BMC Microbiology

RESEARCH Open Access

Enterobacter spp. isolates from an underground coal mine reveal ligninolytic activity

Bame J. Rammala[1*], Santosh Ramchuran[2], Viren Chunilall[3,4] and Nerve Zhou[1*]

Abstract

Lignin, the second most abundant renewable carbon source on earth, holds significant potential for producing biobased specialty chemicals. However, its complex, highly branched structure, consisting of phenylpropanoic units and strong carbon-carbon and ether bonds, makes it highly resistant to depolymerisation. This recalcitrancy highlights the need to search for robust lignin-degrading microorganisms with potential for use as industrial strains. Bioprospecting for microorganisms from lignin-rich niches is an attractive approach among others. Here, we explored the ligninolytic potential of bacteria isolated from a lignin-rich underground coalmine, the Morupule Coal Mine, in Botswana. Using a culture-dependent approach, we screened for the presence of bacteria that could grow on 2.5% kraft lignin-supplemented media and identified them using 16 S rRNA sequencing. The potential ligninolytic isolates were evaluated for their ability to tolerate industry-associated stressors. We report the isolation of twelve isolates with ligninolytic abilities. Of these, 25% (3) isolates exhibited varying robust ligninolytic ability and tolerance to various industrial stressors. The molecular identification revealed that the isolates belonged to the *Enterobacter* genus. Two of three isolates had a 16 S rRNA sequence lower than the identity threshold indicating potentially novel species pending further taxonomic review. ATR-FTIR analysis revealed the ligninolytic properties of the isolates by demonstrating structural alterations in lignin, indicating potential KL degradation, while Py-GC/MS identified the resulting biochemicals. These isolates produced chemicals of diverse functional groups and monomers as revealed by both methods. The use of coalmine-associated ligninolytic bacteria in biorefineries has potential.

Keywords Bioprospection, Ligninolytic bacteria, Biorefinery, Biochemicals

*Correspondence:
Bame J. Rammala
bame.rammala@studentmail.biust.ac.bw
Nerve Zhou
zhoun@biust.ac.bw
[1]Department of Biological Sciences and Biotechnology, Botswana International University of Science and Technology, Private Bag 16, Palapye, Botswana, South Africa
[2]Council for Science and Industrial Research, Chemicals Cluster, Pretoria, South Africa
[3]Council for Science and Industrial Research, Biorefinery Industry Development Facility, Durban, South Africa
[4]School of Life Sciences, School of Engineering, University of KwaZulu Natal, Durban, South Africa

Introduction

Lignin, the second most abundant biopolymer on earth [1], is often underutilized despite being renewable. Generated in large quantities as a byproduct from industries like pulp and paper mills, agro-, and bioethanol production, its recalcitrant nature necessitates various pretreatment strategies for effective bioconversion [2–5]. One challenge is its strong covalent binding to hemicellulose, forming lignin-carbohydrate complexes, which complicates efficient extraction and use [6]. In bioethanol production, lignin inhibits saccharification and can deactivate enzymes [2].

Typically considered a 'waste' product, lignin is often disposed of by burning as low-grade fuel or dumping into wastewater, which is environmentally unfriendly [7, 8]. The global annual supply of lignin ranges from 67 to 81 million tons [9]. Valorizing lignin through physical, chemical, and thermochemical approaches has several drawbacks, such as high energy demands and complex product synthesis [10, 11]. However, biological valorization using microorganisms in biorefineries has gained momentum [12–16], offering a dual solution by managing waste streams and addressing nonrenewable fuel challenges.

In a bio-circular economy, lignin helps reduce waste generation and greenhouse gas emissions. It serves as a valuable resource for producing specialty biochemicals through microbial metabolic pathways, aligning with circular economy principles [17–19]. The use of lignin as a feedstock for biorefineries enables the production of biofuels, biochemicals, and biomaterials, thus promoting sustainable practices [20–22]. Additionally, lignin can be integrated into biodegradable materials such as bioplastics [23], contributing to waste reduction and environmental conservation. Moreover, its carbon-rich composition makes it a valuable tool for carbon sequestration [24], aiding in the fight against climate change.

One of the major drawbacks of bio-valorization is finding robust lignin-degrading microorganisms to overcome the recalcitrant nature of lignin. The most studied ligninolytic microorganisms are fungi [25–27]. However, the use of fungi poses many challenges due to their slow growth rate, requirement for co-substrates and difficulty in genetic modifications, making their use less scalable [28]. In contrast, ligninolytic bacteria are emerging as promising candidates due to their shorter culture period, genetic amenability, phenotypic plasticity, and ability to degrade a broader spectrum of recalcitrant pollutants than fungi [29–32]. Recent advancements in the study of lignin-degrading bacteria focused on genomic and plasmid-based approaches offer new avenues for lignin valorization. For example, Nawaz et al. identified and sequenced a novel *Pseudomonas putida* strain Hu 109 A revealing key enzymes and pathways involved in lignin breakdown [33]. Additionally, Spence et al. genetically engineered *Rhodococcus jostii* RHA1 to enhance its ligninolytic ability showing potential for improving lignin degradation efficiency [34]. Moreover, ligninolytic bacteria generate fewer inhibitors and have a selective action that prevents loss of the cellulose fraction [35]. Hence, employing ligninolytic bacteria might increase the probability of finding a cost-effective and optimal route for lignin disposal. Although some bacteria with ligninolytic properties have been described, they reportedly remain poorly established due to their inefficient lignin valorization abilities [36].

The search for efficient lignin-depolymerizing bacteria from lignin-rich environments in natural habitats is promising [37–39]. For example, Wang et al. isolated strain TH-21, identified as *Raoultella ornithinolytica* from the soil of tropical rainforest, which demonstrated significant lignocellulose-degrading ability and efficiently utilized xylose and other sugars for the production of 2,3-butanediol (2,3-BDO) [40]. Alternatively, ligninolytic bacteria isolated from coal mine environments may exhibit robust lignin-degrading capabilities and high tolerance to industrial stressors, making them promising candidates for biotechnological applications in lignin valorization. In this study, we sought to isolate potential ligninolytic and robust bacteria from an underground coal mine owned by the Morupule Coal Mine in Palapye, Botswana. Coal possesses a molecular structure similar to that of lignin, increasing the potential to find ligninolytic microorganisms [41]. Moreover, the extreme conditions of coal mines could select for bacteria with unique and robust ligninolytic capabilities, potentially yielding novel enzymes or pathways for lignin degradation. Here, we described the isolation and identification of potential ligninolytic bacteria using Kraft Lignin-enriched media and demonstrated their ligninolytic capabilities and tolerance to stressors, which are important attributes of industrial strains. We demonstrated the ligninolytic potential of the isolates by analyzing the degradation of Kraft lignin (KL) using ATR-FTIR. Additionally, we identified the degradation products using Py-GC/MS. The findings were used to suggest a more cost-effective and environmentally benign lignin valorization route.

Materials and methods

Study area and sample collection

Coal samples were collected from the Morupule Coal Mine, located 14 km northwest of Palapye (22. 5071 °S and 27. 0264 °E) along the Serowe-Palapye Road in Central Botswana (Fig. 1). Coal mining commenced in 1973. This underground mine employs a room and pillar method to extract its semi bituminous thermal coal [42]. The mine contains a total of four seams, and the coal seam at our sample site has a thickness of 7.89 m.

Fig. 1 Map of Botswana showing the location of the Morupule Coal Mine. This map was adapted from Makoba et al. [44]

The coal-bearing strata have a total thickness of 60 m [43]. Our coal sample was obtained at a mining depth of 112.84 m. A 20×20 cm block mined using an automaker was randomly selected from among other blocks within the coalbed. To maintain the integrity of the samples, we employed aseptic techniques during collection, utilizing sterile Ziplock bags. The bags were transported to the laboratory at room temperature (25 °C) and stored at 4 °C for further investigation.

Screening for ligninolytic activity using Kraft lignin (KL) media

The inner part of the coal block was cut under sterile conditions, and 1 g of the coal sample was crushed using a sterile pestle and mortar and placed in a 15 mL centrifuge tube. A concentration of 2.5% (KL) [25] (Sigma Aldrich, CAS No. 8068-05-01, USA) was prepared, and its pH was adjusted to 7 [45] and supplemented with 100 µL/L trace metals [46] and 100 µL/L vitamins [47], as well as 10 mg/L cycloheximide to select against fungi. A total of 10 mL of KL was pipetted into 15 mL centrifuge tubes. The centrifuge tubes were tightly closed and incubated for 7 days at 37 °C and shaken at 150 rpm in an incubating shaker (VWR® Incubated Large Capacity Shaker 230 V, SN:120501001, USA). Assuming that all bacteria that grew in this media had lignin-degrading or lignin-utilizing abilities, the incubated media were then examined under a microscope (Olympus CX22, Tokyo, Japan) for the presence of bacteria. After ascertaining the presence of cells, the samples were serially diluted tenfold ($10^0 - 10^{-3}$) and plated onto nutrient agar (NA) plates. To obtain pure cultures, distinct colonies were selected and re-streaked on NA. Gram staining was subsequently conducted to distinguish between gram-negative and gram-positive bacteria.

Characterization of ligninolytic bacteria

Lignin assimilation assay

Bacterial isolates were evaluated for their ability to assimilate lignin using minimal salt media enriched with KL (KL-MSM) [48]. The growth and assimilation of the isolates were monitored by measuring biomass accumulation over a predetermined time of 7 days. For each isolate, overnight cell cultures were grown in nutrient broth (NB) and harvested by centrifugation at 8 000 × g for 5 min using a microcentrifuge (Heraeus Pico 17, Am Kalkberg Osterode, Germany), and the resulting cell pellets were resuspended in 100 µL of 0.01 M phosphate buffer solution (PBS) with an adjusted pH of 7.4. The cell concentration was estimated by measuring the absorbance at OD_{600nm} using a spectrophotometer (Spectronic™ 200E, Madison, USA). The assimilation assay was performed in centrifuge tubes (2 mL), each containing 1 mL of KL-MSM, and the cells were inoculated to an initial OD_{600nm} of 0.1. The tubes were tightly closed, incubated at 37 °C and shaken at 150 rpm for 7 days. The samples were harvested at 0 h (as a control) and after 168 h (7 days), after which their optical densities were measured and recorded. This experiment was performed in triplicates and repeated three times.

Lignin assimilation capacity

After confirmation of assimilation, we sought to determine the capacity (rate) at which the isolates assimilated KL, measured as the rate of biomass increase per given time (growth rate), as an index of KL degradation rate. A subset of 6 bacterial isolates that had accumulated the highest biomass (OD_{600nm} of ≥1), was selected. The overnight cultures were prepared, harvested, quantified, and inoculated as described above, except that the optical density readings were recorded at 4-h intervals for 24 h. Isolates with high growth rates of ≥0.3 h^{-1} were selected for fermentation assays. These experiments were performed in triplicates and repeated three times.

Lignin fermentation assay

Fermentative ability, which is crucial for industrial processing, was assessed in isolates with the specified growth rate threshold. Four isolates with an average growth rate of ≥0.3 h^{-1} were selected. The fermentative capability test utilized glucose as the carbon source instead of KL. This adjustment was necessary because the use of dark KL hindered the observation of color changes. Overnight cultures were grown on NA as described above. The cultures were inoculated into Durham test tubes containing

4 mL of phenol red differential media supplemented with 1 mL of 1% glucose. The tubes were incubated at 37 °C for 24 h at 150 rpm in an incubating shaker. Fermentation capability was determined by the change in media color from red to yellow and the formation of gas bubbles [49]. A negative control inoculated with the nonfermenting *Pseudomonas aeruginosa* strain ATCC 10,145 was used [50], while a fermenting *Bacillus cereus* strain, ATCC 10,987, was used as a positive control [51]. Cultures exhibiting gas formation and/or a change in color from red to yellow were selected for further investigation. This experiment was performed in triplicates and repeated three times.

Molecular identification of potential ligninolytic bacteria

DNA extraction and PCR

A total of three isolates demonstrated fermentative ability. These were identified using molecular techniques, amplifying the 1500 bp highly conserved 16 S rRNA region [52]. Overnight cultures grown in NB were harvested and resuspended in sterile distilled water (dH_2O). DNA extraction was performed using the boiling method as described elsewhere [53]. Briefly, the pure cultures were repeatedly boiled (5 times) at 100 °C for 10 min, followed by cooling on ice for 5 min. The resulting DNA samples served as templates for PCR amplification. A total of 2 µL of the template, 1.5 µL of primers (16 S F-GGAGGCAGCAGCAGCAGTAGGGAATA and 16 S R-TGACGGGCGGTGAGTACCAG) [54] at a concentration of 1.0 µM, and 23 µL of Phusion® High-Fidelity DNA Polymerase PCR master mix (Biolabs, Ipswich, Massachusetts, USA) were used. The Thermo Scientific Proflex™ PCR System was used following parameters adapted and modified from [55]. This involved initial denaturation at 98 ℃, primer annealing at 57 ℃ for 20 s, extension at 72 ℃ for 1 min 30 s and a final elongation cycle at 72 ℃ for 5 min at a holding temperature of 4 ℃. Post-PCR amplification fragments were visualized by agarose gel electrophoresis in a 1% (w/v) gel prepared using 1× Tris-Borate-EDTA (TBE), stained with 20 µL ethidium bromide and observed using a UVP GelDoc-It®TS3 Imager (P/N 97-0783-02, Cambridge, UK).

Amplicon sequencing and bioinformatics analysis

Successful PCR amplicons were sent to Inqaba Biotec™ for Sanger sequencing. The resulting sequences were manually edited and modified using BioEdit Sequence Alignment Editor Version 7.0.5.3 [56]. This approach was used to clean and prepare sequence data for more accurate and reliable analysis, leading to better analysis of the sequences. The putative species were searched against the National Centre for Biotechnology Information nucleotide database using the Basic Local Alignment Search Tool (BLAST) (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud (https://www.ezbiocloud.net/). A statistically validated sequence homology threshold of 98.65% by Kim et al. [57] was adopted to differentiate between species. Notably, factors such as sequence quality and consensus could affect the percentage identity of the isolates. Visualizing the sequences with BioEdit to identify and eliminate irregularities and aligning them to the reference strain with multiple sequence alignment (MUSCLE) ensured that the sequences used for phylogenetic analysis were of sufficient quality. All species with a sequence identity lower than this threshold (98.65%) was denoted as "aff." (Latin: affinis, meaning related to or neighboring in taxonomy). This means an uncertain identity which requires further taxonomic investigation for accurate validation of their identity.

Phylogenetic analyses

To elucidate the phylogenetic relationships among the isolates, Molecular Evolutionary Software version 10. 2. 6 (MegaX) [58] was used to align the sequences. Sequence alignment was performed using MUSCLE, and a maximum likelihood phylogenetic tree was subsequently generated [59]. The phylogenetic tree was generated using MegaX. To classify the isolates into specific species, we utilized type strain of the *Enterobacter* genus, NR118568.1 *Enterobacter cloacae*. The selection of the type strain began with a thorough literature review to identify the type strain for the target species. The type strain was further verified using the American Type Culture Collection (https://www.atcc.org/). Finally, NCBI was used to find further taxonomic information on the target species and for cross-referencing the type strain with the original species description to ensure accuracy.

Industry-associated stress tolerance assays

The fermentative capabilities of the three isolates were additionally evaluated under simulated industrial stressors to gauge their performance in potential industrial applications. The industrial traits of interest investigated included thermotolerance, osmotolerance, alkaline pH tolerance and tolerance to growth inhibitors. Cell cultures were prepared as previously mentioned and diluted sixfold starting from OD_{600nm} 0.1 to 0.003125 in a 96-well plate. The cells were then stamped onto solid media using an 8×6 replicator stamp and incubated at 37 ℃ as described by [60]. The experiments evaluated osmotolerance on plates containing 0.5 M, 1 M, and 1.5 M NaCl; growth in inhibitors at 0.1 M NA-$FeSO_4$, 0.1 M NA-$CuSO_4$, and 2.5% KL-MSM agar; pH stress tolerance at pH values of 8, 10 and 12; and thermotolerance at 40 ℃, 45 ℃ and 50 ℃. The plates were then incubated for 24 h under aerobic conditions. On the other hand, some plates were subjected to strict anaerobic incubation by placing the plates in a Thermo Scientific™ Oxoid™ Anaero Jar

2.5 L [61]. Due to limited resources, thermotolerance was investigated under aerobic conditions only. These experiments were all performed in triplicate and repeated three times. The representative plates were scanned using a scanner (Epson Perfection V600 photo).

GC–MS and ATR-FTIR assessment of the degradation products of lignin

Growth of isolates for analyses

Overnight cultures were prepared as described in the above sections and diluted to an initial OD_{600nm} of 0.1. The appropriate volumes of the cells were then aliquoted into sterile luer-lock Fisherbrand™ syringes (containing 3 mL of KL-MSM) [60], ensuring that no air was trapped in the syringes. The syringes were capped and then incubated at 37 ℃ and shaken at 150 rpm in a rotary shaker for three weeks to allow fermentation to proceed to completion.

Metabolite extraction

Metabolite extraction methods for attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) and pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) were adapted from [62, 63]. Briefly, bacterial-treated and untreated samples of KL were first sonicated for 5 min at a high frequency and 25 °C using a ScienTech Ultrasonic cleaner (Johannesburg, South Africa). The samples were then centrifuged at 8000 x g for 30 min at room temperature to separate the cell biomass from particulate matter. The supernatants were transferred to sterile 15 mL centrifuge tubes, and the pH was adjusted to approximately 1 using 1 M hydrochloric acid, confirmed with litmus pH test strips. Metabolites were extracted by mixing the samples with an equal volume (3 mL) of ethyl acetate three times, allowing the mixture to settle each time. The resulting mixture separated into residual, aqueous, and ethyl acetate layers. The organic layer was then transferred into 2 mL sterile microcentrifuge tubes for further analysis.

Attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectral analysis was performed at room temperature to monitor structural and functional group changes in KL after degradation by various bacteria. The spectra were measured using a Bruker Vertex 70v connected to a PMA50 module and a Hyperion series IR microscope. Ten microliters of each degraded and undegraded extract sample was analyzed as a liquid in a vacuum to eliminate any extraneous interactions [64]. The spectra ranged from 4 000 cm^{-1} to 400 cm^{-1} with the resolution set at 4 $cm^{-1,}$ and 64 scans were performed per sample. Baseline corrections were performed using OPUS 7.8 software (Ettlingen, Germany). Peak reports were also generated using OPUS spectroscopy software, and a Merck IR spectrum table and chart (Sigma Aldrich: https://www.sigmaaldrich.com/BW/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ir-spectrum-table) was used to determine the bands, bonds and functional groups.

Pyrolysis-gas chromatography/mass spectroscopy (Py-GC/MS)

Py-GC/MS analysis was performed to identify products of lignin degradation by the isolates. The extracts were analyzed as solids using a multishot pyrolyzer, EGA/PY-3030 D (Frontier Lab, Japan), connected to a Shimadzu gas chromatograph/mass spectrometer (QP2010 SE). Approximately 100 to 150 µg of the sample was pyrolyzed at 550 °C for 20 s, and the interface temperature of the analytical column was 350 °C. The chromatographic separation of the pyrolysis products was performed using an ultra-alloy capillary column (Frontier Lab, Japan) (30 m × 0.25 mm, 0.25 µm). The injection temperature was set at 280 °C, and the column flow rate was set at 1.0 mL/min with helium used as the carrier gas. The GC temperature program used was as follows: (i) hold at 50 °C for 2 min; (ii) ramp from 50 °C to 200 °C at a rate of 3 °C/min; and (iii) hold for another 4 min. The ion source and interface temperatures in the mass spectrometer were set to 200 °C and 300 °C, respectively. The scan range used for the mass-selective detector was from m/z 40–650. Pyrolysis products were identified by comparing their mass spectra to mass spectra available in the National Institute of Standards and Technology (NIST) and Wiley libraries [65].

Statistical analysis

Statistical analysis was conducted using STATISTICA version 13.2 (StatSoft Inc., Tulsa, Oklahoma). Statistical differences were determined using one-way ANOVA. Subsequently, a post hoc Tukey's honest significant difference test (HSD) was used to establish significant differences between the isolates. A 95% confidence level ($p \leq 0.05$) was used.

Results and discussion

Coal mine-associated isolates reveal varying potential ligninolytic activity

A total of twelve isolates screened using KL containing media were obtained from the Morupule Coal Mine. The presence of ligninolytic bacteria in such environments has been attributed to the molecular similarity between coal and lignin [41]. The findings highlight the diverse biomass accumulation capabilities among the isolated bacterial strains. A total of 6 isolates (C01, C04, C10, C08, C06, and C09) accumulated biomass above $OD_{600nm} \geq 1.00$, whereas C05, C02, C12, C07, C11 and

C03 accumulated biomass less than $OD_{600nm} \geq 1.00$ and were therefore regarded as less efficient (Fig. 2, see also Supplementary materials S1). Among the 6 isolates that exhibited biomass accumulation above $OD_{600\ nm} \geq 1.00$, three isolates, namely, C01, C04, and C10, demonstrated notably high biomass accumulation rates. Interestingly, these isolates displayed similar levels of biomass accumulation, indicating comparable performance within this subgroup. Consequently, they were categorized into group A due to their consistent and robust biomass accumulation abilities. Isolates C08, C06 and C09 were categorized into groups B, C and D, respectively. Furthermore, statistical analysis revealed significant differences in biomass accumulation among the labeled groups (see also Supplementary materials S2 and S3). This may emphasize the heterogeneity in biomass accumulation capacities in potential ligninolytic bacterial strains. Bacterial strains with efficient biomass accumulation capabilities can serve as promising candidates for biorefineries because they potentially possess highly efficient metabolic pathways. The isolates' capacity to accumulate substantial biomass in the presence of KL suggests that they are resource-efficient and capable of high-yield production. This trait is vital for biorefineries, where economic success hinges on achieving high throughput.

Ligninolytic isolates displayed varying rates of growth

In addition to the ability to assimilate lignin, the rate of assimilation is an essential factor in selecting potential industrial strains. A subset of four isolates (C01, C04, C10 and C08) exhibited notably high growth rates ranging from $0.431 \pm 0.00231\ h^{-1}$ to $0.303 \pm 0.0167\ h^{-1}$ (Fig. 3; see also Supplementary Materials S4). This subset demonstrated a robust growth rate, suggesting its unique capacity for rapid proliferation under the experimental conditions. Isolate C08 demonstrated the highest growth rate, followed by isolate C10, which also exhibited commendable growth, albeit slightly lower than that of C08, suggesting efficient proliferation. Despite a considerably lower growth rate, C01 still showed commendable kinetics above the set threshold. Conversely, while exhibiting high growth, isolate C04 appeared to have the lowest growth rate among the subsets. A statistical analysis revealed significant differences in growth rates among the isolates (see also Supplementary materials S5 and S6). A superior performance, coupled with an elevated rate of growth, is a crucial trait in an efficient biorefinery process because it may enhance enzyme production [66], reduce processing time and costs and increase the overall productivity of the biorefinery [67].

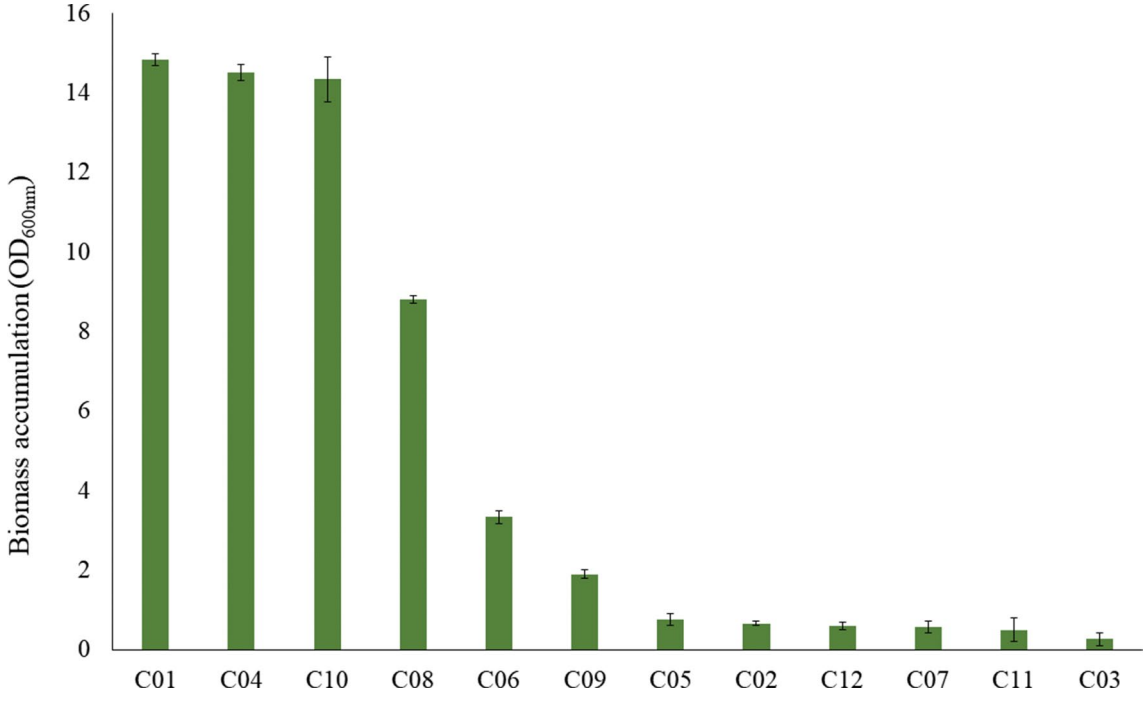

Fig. 2 Biomass accumulation of the twelve ligninolytic bacteria. Ligninolytic activity of the coal isolates, emphasizing six isolates with notable biomass. The assay measured biomass accumulation (OD_{600nm}) after seven days in KL-MSM broth, which was subsequently grown anaerobically at 37 ℃ and shaken at 150 rpm. Biomass accumulation for the six isolates ranged from $14.8\ h^{-1} \pm 0.153$ to $1.90^{-1} \pm 0.100$. The significance level was set at $p \leq 0.05$

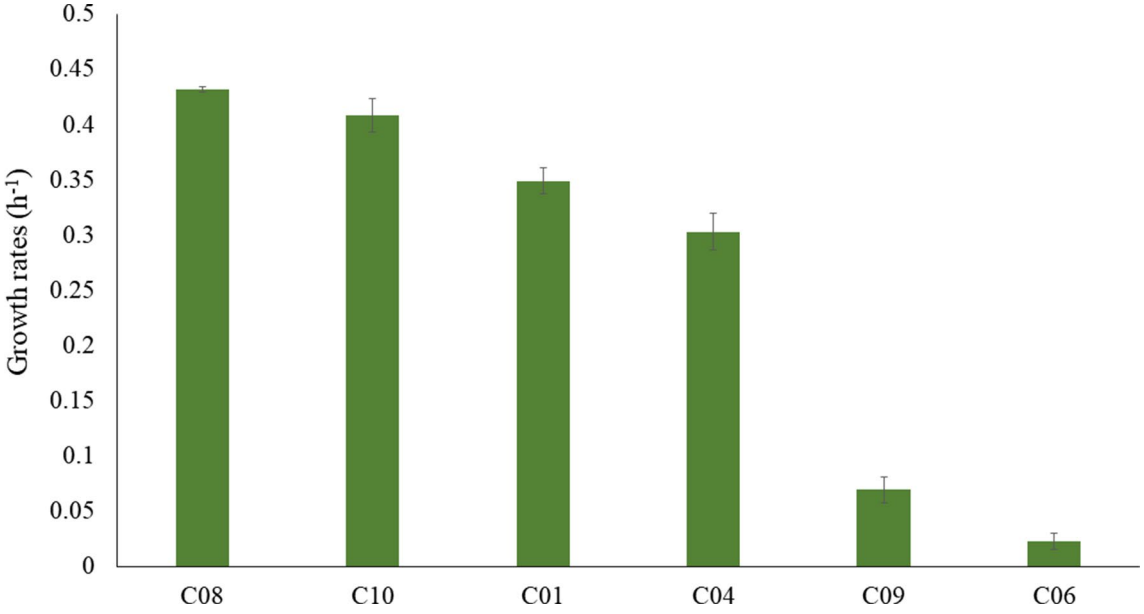

Fig. 3 Growth rates of the six ligninolytic bacteria grown in KL-MSMs. Growth rates of six ligninolytic bacteria cultivated in KL-MSM as monitored at OD600, measured every four hours for 24 h, and plotted using a logarithmic function exponential trendline. The plates were incubated anaerobically with continuous shaking at 150 rpm at 37 °C. The growth rates varied from 0.432 h^{-1} ± 0.00231 to 0.0222 h^{-1} ± 0.00739. The significance level was set at $p \leq 0.05$

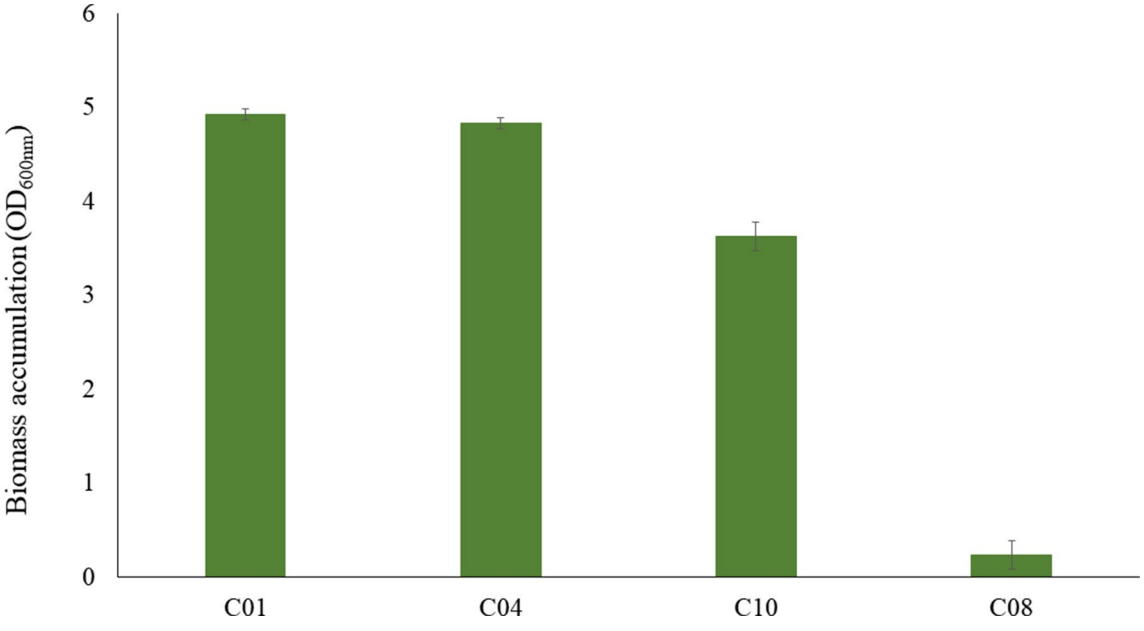

Fig. 4 Biomass accumulation of the four isolates within a short period of time (24 h). The assay measured the accumulated biomass (OD_{600nm}) during growth in KL-MSM (broth) under anaerobic conditions at 37 ℃ with shaking at 150 rpm. Biomass assimilation varied from 4.93 h^{-1} ± 0.0577 to 0.233 h^{-1} ± 0.153. Statistical significance was set at $p \leq 0.05$

Diverse biomass accumulation abilities within short periods of time

Rapid assimilation in a relatively short period of time is an important attribute of a production strain. This can lead to shorter fermentation times, which impacts the efficiency of a biorefinery. Of the four isolates described above, three rapidly utilized KL within 24 h. These are C01, C04 and C10. Their growth rates were 4.93±0.0577 h^{-1}, 4.83±0.0577 h^{-1} and 3.63±0.153 h^{-1}, respectively (Fig. 4, see also Supplementary materials S3). Despite their similar growth rates, isolates C01 and C04 accumulated more biomass than isolate C10. Statistical analysis revealed no significant difference in biomass accumulation between isolates C01 and C04, while isolate C10 exhibited a lower biomass and showed statistical significance from the other two isolates (see supplementary

Table 1 Carbohydrate fermentation results of the three isolates. Fermentative potential was assessed in all three isolates. Isolates that induced a color change from red to yellow in the media and generated a bubble in the Durham tube were recorded as having fermentation ability. Controls included a negative control (*P. Aeruginosa*), a sterile negative control, and a positive control (*B. cereus*)

	Color change		
Sample ID	**Before fermentation**	**After fermentation**	**Gas formation**
C01	Red	Yellow	No gas formation
C04	Red	Yellow	No gas formation
C10	Red	Yellow	No gas formation
B. cereus (ATCC 10145)	Red	Yellow	No gas formation
Sterile control	Red	Red	No gas formation
P. aeruginosa (ATCC 10987)	Red	Red	No gas formation

materials S8 and S9). These findings may highlight potential variations in the increased metabolic activities of the isolates, further indicating their potential versatility and stability for use in biorefineries.

Fermentative potential of the isolates

The fermentative capability of an industrial strain provides a strategic advantage for lignin valorization, eliminating the necessity for oxygenation in feedstocks, reducing operational costs and being sustainable [68]. Assessment of fermentative capacity, conducted through a carbohydrate fermentation test, indicated that the three isolates exhibited promising fermentation ability (Table 1, Supplementary Materials S10). The isolates induced a color change in the media from red to yellow, signifying a degree of fermentation without notable gas generation. This finding aligns with the findings of other researchers, who reported that ligninolytic bacteria can ferment simple sugars [69]. The observed fermentation potential strengthens the prospect that these isolates can be used to effectively ferment lignocellulosic biomass for the production of target biochemicals. Studies indicate that reducing sugars are produced from the degradation of lignocellulosic biomass [70]. Furthermore, glucose has been reported to enhance the production of laccase, an important enzyme in lignin degradation [71]. The ability of these isolates to ferment glucose suggests that they could be valuable in biorefineries, where reducing sugars are possible products of degradation. These isolates could utilize sugars to enhance the production of ligninolytic enzymes.

Coal mine-associated isolates belong to the Genus *Enterobacter* spp

We investigated the identity of the three isolates due to their observed ligninolytic potential. Molecular identification revealed that all the isolates belonged to the genus *Enterobacter* (Table 2). Unsurprisingly, this genus belongs to the γ-Proteobacteria class which has been reported for its ligninolytic capability [72]. Based on the species homology threshold of 98.65% which is used to determine if a bacterial species belongs to an existing or new species as described by Kim et al. [57], it can be concluded that isolate C04 is not identical to *Enterobacter sichuanensis.* This is because it displays a lower homology percentage than the threshold (98.58%). These results suggest that isolate C04 could be a new species pending further studies and was named as *Enterobacter* aff. *sichuanensis*. On the other hand, isolates C10 and C01 exhibit high homologies of 99.88% and 98.50% respectively to the type strain *Enterobacter cloacae* (NR 118568.1) suggestive of their affiliation to this species. These results agree with other studies as they reveal that members of the *Enterobacter* genus comprise of facultative anaerobes capable of lignin degradation under both aerobic and anaerobic conditions. Examples such as *Enterobacter soli*, *Enterobacter aerogenes*, and *Enterobacter lignolyticus* have demonstrated lignin depolymerization capabilities [73, 74].

Notably given the similarities between coal and lignin, it was anticipated that our coal derived isolates would exhibit ligninolytic potential [75]. Considering the harsh conditions in underground coalmines, it is reasonable to anticipate the isolation of robust ligninolytic bacteria from this environment.

A phylogenetic tree (Fig. 5) was constructed to infer the relationships among the identified *Enterobacter* spp isolates. The results revealed distinct clustering, suggesting a close relationship among *Enterobacter cloacae*

Table 2 Bacteria isolates exhibiting robust ligninolytic ability and tolerance to various industrial stressors

Isolate ID	Isolate Accession number	Nearest species match	Nearest species match accession number	Number of nucleotides	% Identity	Number of mismatches	% Coverage
C10	PP715422.1	*Enterobacter cloacae*	NR 118568.1	842	99.88	7/1003	100
C01	PP715420.1	*Enterobacter cloacae*	NR 118568.1	999	99. 50	15/1003	100
C04	PP715421.1	*Enterobacter* aff. *sichuanensis*	NR 179946.1	655	98.58*	15/1003	96

*The identities are below the internal transcribed spacer barcoding threshold of yeasts, which is 98.65% [57]

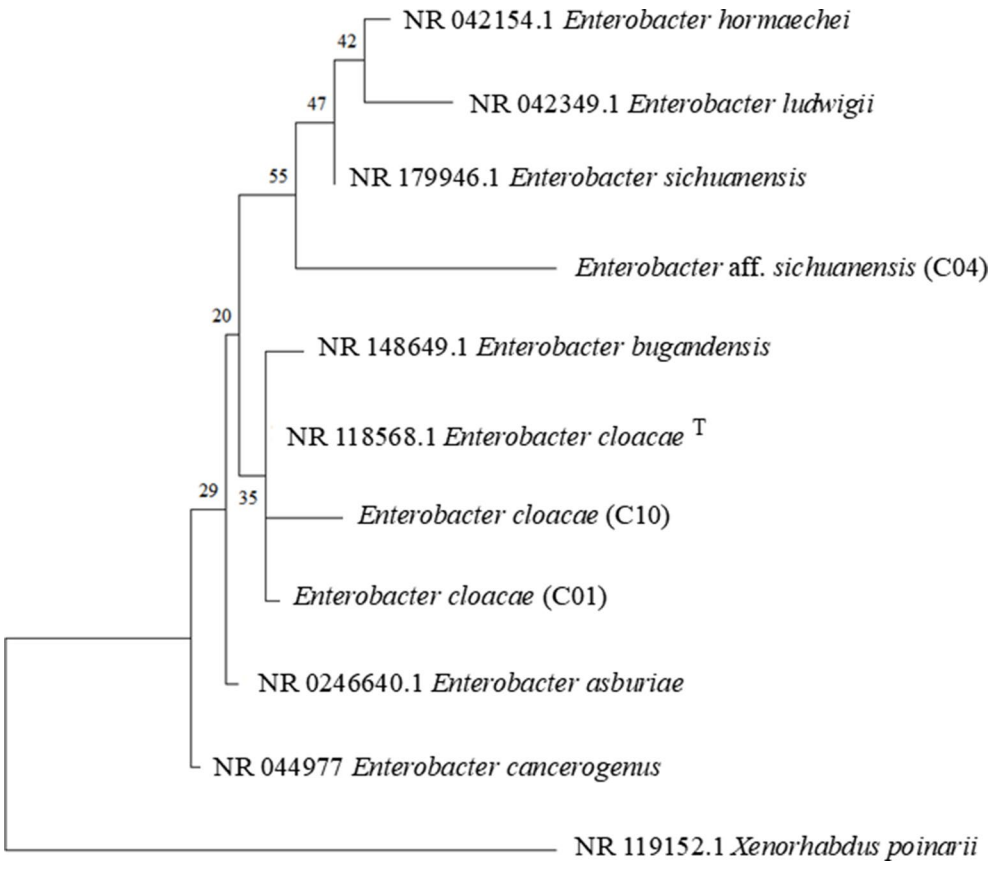

Fig. 5 A phylogenetic tree highlighting the position of the isolated ligninolytic bacteria relative to other strains within *Enterobacteriaceae*. The tree was rooted using NR 119152.1 *Xenorhabduc poinarii* based on 16 S rRNA sequences edited using BioEdit, aligned using MUSCLE and generated in MegaX software. The phylogenetic tree was constructed using maximum likelihood analysis, and the Kimura 2-parameter was set at 1000 bootstrap replicates

(C01), *Enterobacter cloacae* (C10) and type strain NR 118568.1 *Enterobacter cloacae*. Notably, *Enterobacter aff. sichuanensis* (C04) shows no close relationship to the type strain as predicted from the blast results. Literature suggests that *E. sichuanensis* is a newly identified strain [76], hence our strain showing affinity to the species warrants further investigation. The phylogenetic tree further emphasized the genetic diversity within the *Enterobacter* genus. Challenges persist in accurately identifying *Enterobacter* species due to ongoing updates and revisions within the genus [77]. This emphasizes the need for taxonomic research to confirm if C04 is indeed a new species.

Ligninolytic bacteria exhibit desirable industrial traits

The process of lignin valorization through fermentation involves subjecting industrial strains to harsh and stringent pretreatment conditions that can inhibit microbial activity [78]. Employing stress-tolerant industrial strains is appealing for achieving an efficient and cost-effective bioprocess [79]. However, a limitation of these assays is that they may not completely replicate the intricate conditions found in biorefinery processes. Moreover, these assays might not encompass the entire spectrum of stressors that bacteria might face in biorefinery settings, leading to potential limitations in identifying industry-relevant isolates. In the preceding sections, the three identified promising ligninolytic industrial strains were evaluated for their stress tolerance under industrial conditions, including thermal stress, osmotolerance, alkaline pH stress, and metal inhibitor tolerance.

Thermotolerance

Lignin valorization typically involves high-temperature pretreatment processes, with the kraft cooking process utilizing temperatures above 150 ℃ [80]. After pretreatment, the media is cooled to lower temperatures. Although mesophilic fermentation temperatures for experimental microbial depolymerization of KL are preferred [81], the use of a strain that ferments at higher temperatures could reduce cooling costs. Moreover, higher temperatures have been shown to enhance the efficiency of lignin degradation [28]. The growth patterns of the three isolates were observed at different temperatures, revealing colony formation at 40 °C and 45 °C and no observable colony formation at 50 °C (Fig. 6).

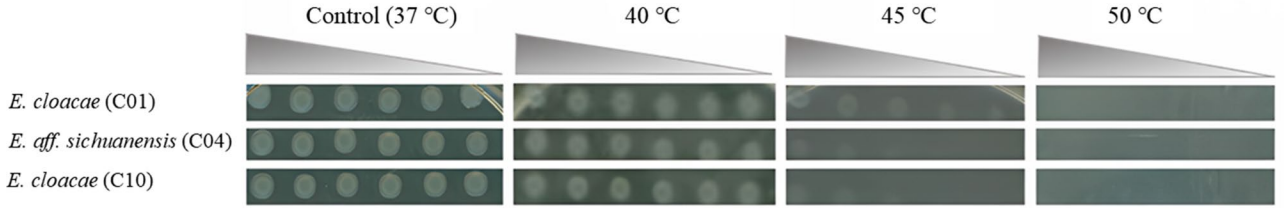

Fig. 6 Thermotolerance of the three selected ligninolytic isolates at three different temperatures. Serial dilutions were prepared, and cultures were inoculated using a 96-well plate and a replicator stamp. Growth was observed for all isolates at the control temperature (37 °C) and test temperatures of 40 °C and 45 °C. No growth was observed at 50 °C

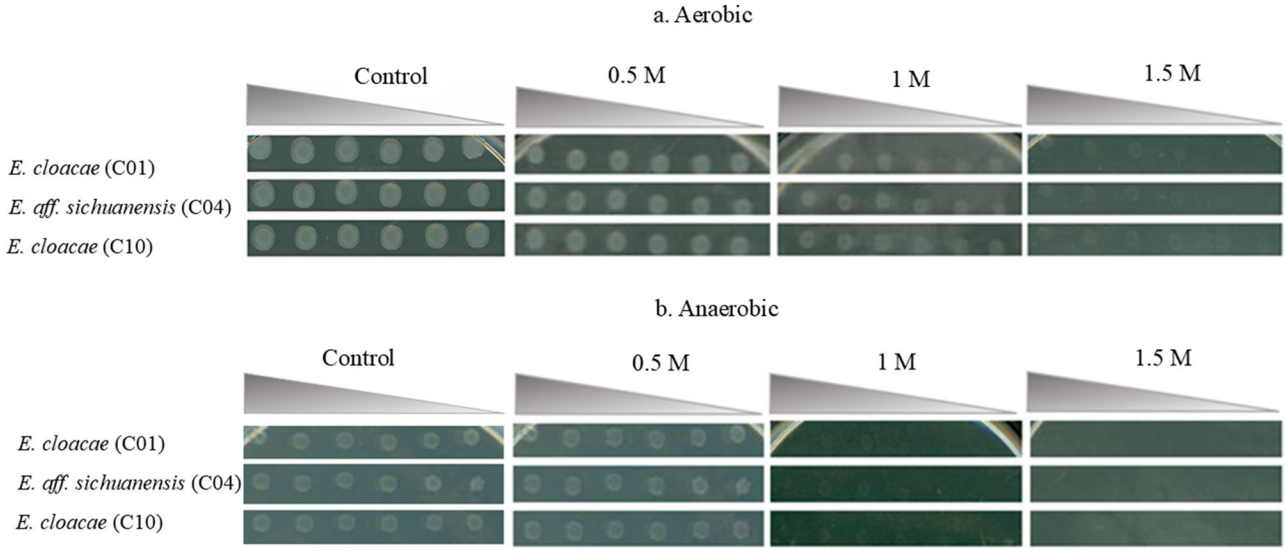

Fig. 7 Osmotolerance of the three selected isolates to three different salt concentrations. The isolates' osmotolerance was assessed using nutrient agar with increasing NaCl concentrations (0.5 M, 1 M, and 1.5 M). Serial dilutions of the cultures were performed using a 96-well plate, and a replicator stamp was used to inoculate the media. The experiment included both aerobic and anaerobic conditions, with nutrient agar without NaCl serving as the control

While these results suggest mesophilic behavior [82], the observed growth at 45 ℃ indicates potential thermophilic characteristics, as described elsewhere [83]. The ability to thrive at elevated temperatures suggests a thermostable nature, which is advantageous for producing thermostable enzymes, enhancing competitiveness in biorefinery processes [84]. Thus, these isolates exhibit promising thermostable potential, making them attractive for biorefinery applications.

Osmotolerance

Lignocellulosic biomass pretreatment methods, including the use of ionic liquids (ILs), often lead to elevated salt levels, creating unfavorable osmotic stress environments [85]. In the biorefinery context, halotolerant or osmotolerant isolates are valuable because they can thrive and efficiently valorize lignin under such conditions. We therefore investigated osmotolerance under both aerobic and anaerobic environments. All isolates demonstrated distinct colony formation under aerobic conditions at salt concentrations of 0.5 M and 1 M. However, only small colonies were observed at 1.5 M (Fig. 7). Under anaerobic conditions, colony formation was observed at all salt concentrations except 1.5 M. These findings indicate that the isolates exhibit a certain degree of tolerance to high sat concentrations or osmotic stress, which establishes a foundation for further investigation. As the use of ionic liquids for lignin extraction has increased in popularity, these isolates are highly promising. Literature suggests that bacteria, particularly in saline environments, may play a crucial role in lignin valorization [86]. In agreement, extensively researched species such as the moderately halotolerant Pseudomonas putida have demonstrated the ability to depolymerize lignin [87].

Alkaline pH stress tolerance

Alkaline stress tolerance is crucial for ligninolytic bacteria, particularly in the valorization of KL, a byproduct of the Kraft pulping process used in paper production. This is because KL is often treated under alkaline conditions during processing [88] highlighting the necessity for bacteria to withstand and thrive in such alkaline environments. The performance of the isolates was evaluated across different pH conditions (pH 8, 10, and

12). Notably, colony formation (from all three isolates) was observed under aerobic conditions at pH 8 and 10, mirroring the alkaline nature of KL, while no colony formation was observed at pH 12 (Fig. 8). Furthermore, colonies were larger under aerobic conditions than under anaerobic conditions, suggesting potential alkaliphilic behavior. The presence of alkaliphilic ligninolytic bacteria at pH 12 under anaerobic conditions in this study holds promise for enhanced biorefinery efficiency, as it may minimize the need for costly pH adjustments, potentially reducing overall production costs. The presence of colonies at pH 12 further enhances their appeal for biorefinery applications.

Metal inhibitor stress tolerance

The presence of metal inhibitors has a negative impact on lignin valorization, as they can induce oxidative stress and enzyme damage [89]. It is therefore crucial to utilize isolates that tolerate such potential inhibitors. This ability was evaluated in the presence of inhibitors such as iron sulphate ($FeSO_4$), copper sulphate ($CuSO_4$), and sulfur in lignin (Fig. 9). In the case of $FeSO_4$ (Fig. 9A), isolates demonstrated colony formation under both aerobic and anaerobic conditions, although colony size was reduced under anaerobic conditions. Under aerobic conditions, all the isolates exhibited some colony formation in the presence of $CuSO_4$ (Fig. 9B), as indicated by the presence of minute colonies. This suggests the presence of natural extremophilic traits that warrant further investigation. Kraft lignin, commonly used in biorefinery processes, has a high sulfur content [90], and the isolates formed colonies under both aerobic and anaerobic conditions on KL-MSM agar (Fig. 9C). The ability of these isolates to form colonies under diverse conditions underscores their natural extremophilic ability. The survival of these isolates in the presence of inhibitors holds promise for their potential application in lignin valorization.

Ability to withstand anoxic conditions

In nature, the complete degradation of lignin is predominantly attributed to fungi and bacteria in the presence of oxygen, owing to the oxygen demand in breaking aromatic rings [91]. Notably, bacteria such as *Tolumonas lignolytica* have demonstrated lignin utilization capabilities under anaerobic conditions [92]. Our findings affirm that all the isolates under study exhibit anaerobic depolymerization potential. Leveraging anaerobic microorganisms in industrial bioprocessing is a cost-effective strategy that circumvents the expenses associated with the oxygen supply [93]. Moreover, the isolates exhibited growth under anaerobic conditions in the presence of additional stressors, showing their extremophilic adaptability.

Isolates produced monomers as specialty chemicals

Structural alterations of KL revealed by ATR-FTIR

A comprehensive ATR-FTIR analysis confirmed lignin degradation by the three isolates, validating their ligninolytic properties observed in preceding studies. The disappearance of peaks in undegraded KL spectra and emergence of new peaks in depolymerized lignin spectra was used an indicator of potential KL degradation (Fig. 10, see also Supplementary materials S12 and S13).

The spectrum of the undegraded control KL exhibited distinctive vibrations characteristic of the lignin

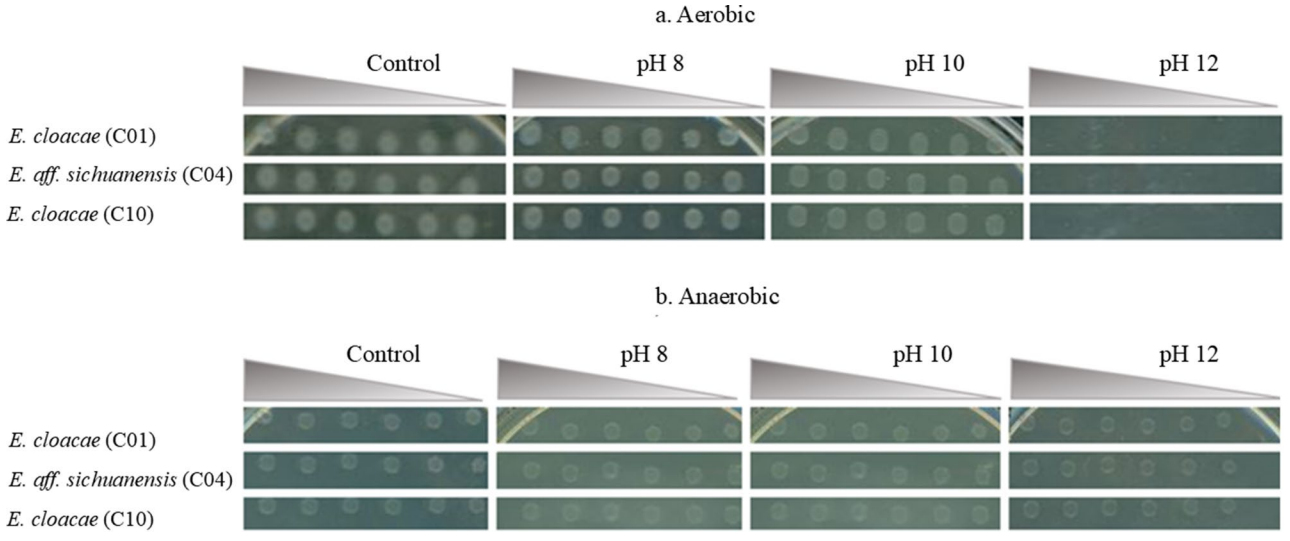

Fig. 8 Alkalinity-pH stress tolerance of selected ligninolytic bacteria. Nutrient agar with varying pH levels (8, 10, and 12) was prepared, with NA at standard pH serving as the control. Serial dilutions of the cultures were performed using a 96-well plate, and a replicator stamp was utilized to inoculate the cultures on the media. All isolates exhibited growth at pH 8 and pH 10 under both aerobic and anaerobic conditions. However, no growth was observed at pH 12 under aerobic conditions, while colony formation occurred under anaerobic conditions

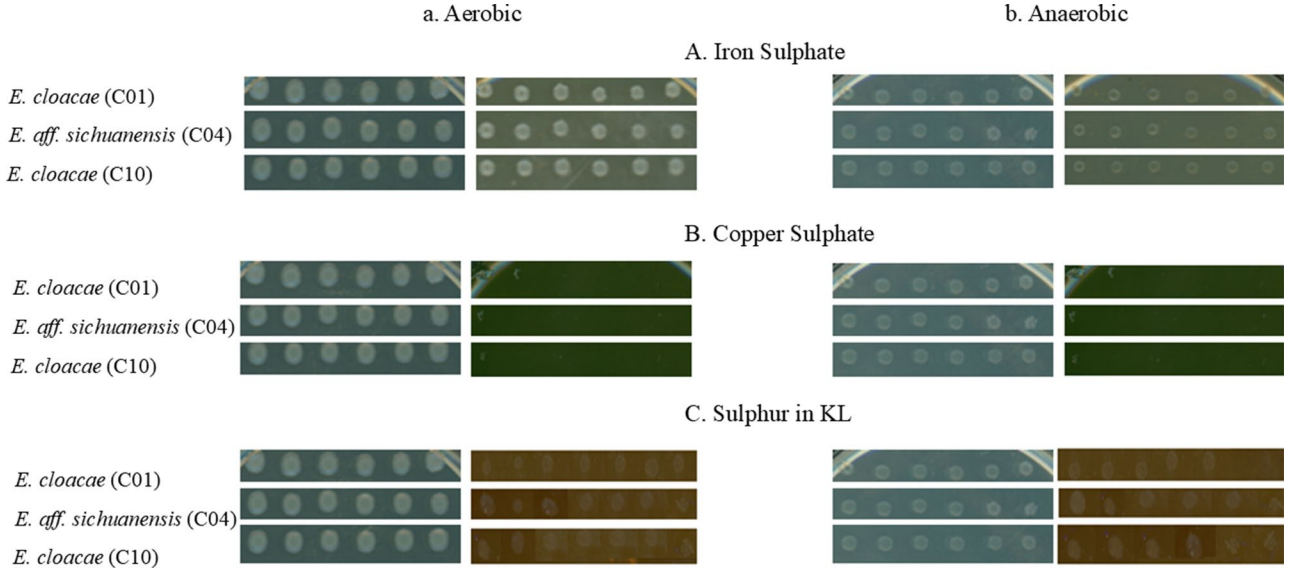

Fig. 9 Metal inhibitor stress tolerance of selected ligninolytic bacteria. Nutrient agar supplemented with 2% $FeSO_4$ (**A**), $CuSO_4$ (**B**), and 2.5% Kraft Lignin (**C**) was prepared. Serial dilutions of the cultures were performed using a 96-well plate, and a replicator stamp was used to inoculate the cultures onto the media. In (**A**), isolates displayed more prominent growth under aerobic conditions than under anaerobic conditions. In (**B**), minute colony formation was observed under aerobic conditions for all isolates, while under anaerobic conditions, only the isolate *E. cloacae* (C01) exhibited potential colony formation. In (**C**), all isolates, under both aerobic and anaerobic conditions, demonstrated colony formation. Nutrient agar without inhibitors served as a control

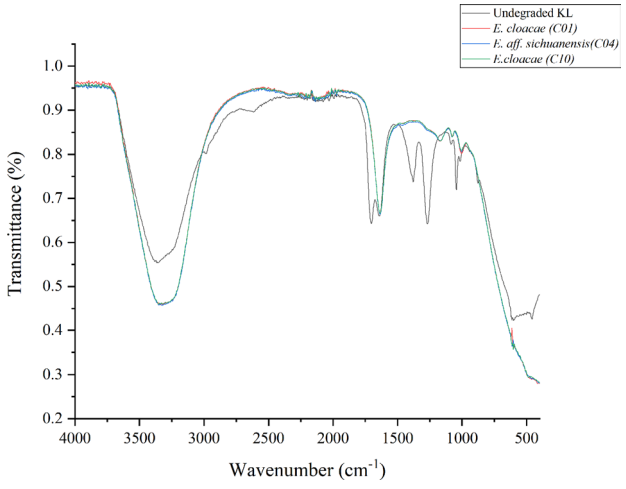

Fig. 10 FTIR spectra of degraded and undegraded Kraft Lignin. ATR-FTIR spectroscopy was employed to unveil potential structural disparities between undegraded and degraded KL. The observed changes are discerned through alterations in the depicted peaks, suggesting the formation and/or elimination of specific functional groups. The undegraded KL strain served as the control for this comparative analysis

structure. Notably, there were strong and intense O–H stretching vibrations at 3357 cm^{-1}, moderate O–H bending vibrations at 1378 cm^{-1} associated with hydroxyl and phenol groups, and C=O stretching vibrations at 1703 cm^{-1} linked to ether and carbonyl linkages within aromatic bonds were observed. Additionally, the spectrum revealed C–O stretching vibrations of the guaiacyl ring at 1268 cm^{-1}, corresponding to alkyl aryl ethers, and moderate C=C stretching vibrations at 1639 cm^{-1}, indicating the presence of aromatic regions. These findings are consistent with the well-documented lignin structure reported elsewhere [94–96].

In contrast, our FTIR analysis of degraded KL demonstrated a clear and identifiable alterations in structure, confirming the ligninolytic capability of the isolates. Notably, the O–H stretching vibrations, which were initially detected between 3319 cm^{-1} and 3352 cm^{-1} indicating the presence of hydroxyl groups in alcohols, became stronger after lignin degradation by each isolate (Fig. 10). This enhancement indicates increased production of hydroxyl functional groups, which suggests that the degradation of KL was successful. Furthermore, upon degradation, the peaks that were previously detected at 1703 cm^{-1}, 1378 cm^{-1}, 1268 cm^{-1}, and 1044 cm^{-1} were no longer present (Fig. 10, Supplementary materials S12 and S13). The presence of these peaks suggests that the isolates specifically targeted the guaiacyl and syringyl vibrations, effectively affecting these bonds. The results of this research are consistent with previous studies [97–99], underscoring the isolates' ability to break down the heterogeneous polymer of lignin into monomers.

The diversity of monomers revealed by Py-GC/MS is a desirable industrial trait of the isolates

The changes in functional groups observed using FTIR suggested the degradation potential of the selected isolates. A high-throughput technique, Py-GC/MS, was used to reveal the respective compounds associated with degradation. Py-GC/MS analysis revealed the production

of several monomers (Fig. 11, Supplementary Materials, S14) with economic and industrial significance, such as styrene, ethyl benzene, 2-methoxyphenol, methyl benzene, and 2-methoxy-4-vinylphenol (Fig. 11). These materials have extensive applications, such as their use in resins, food flavoring, and polymers [100, 101]. Currently, a majority of chemicals on the market are derived from petroleum fuels, rendering their costs to be vulnerable to the volatility of oil prices. However, utilizing lignin in chemical production presents the potential for price stability, given that lignin is derived from renewable feedstocks [102].

The data revealed potentially shared and distinct metabolic profiles among the isolates, with some chemicals produced by all 3 isolates and others by individual isolates. For instance, styrene, 2-methoxyphenol, and 1-octene were consistently produced by all the isolates. Additionally, unique compounds such as nonyl cyclopropane from *E. cloacae* (C01), ethylene monothiocarbonate, 2-methoxy-4-vinylphenol, and 4-ethenyl cyclohexene

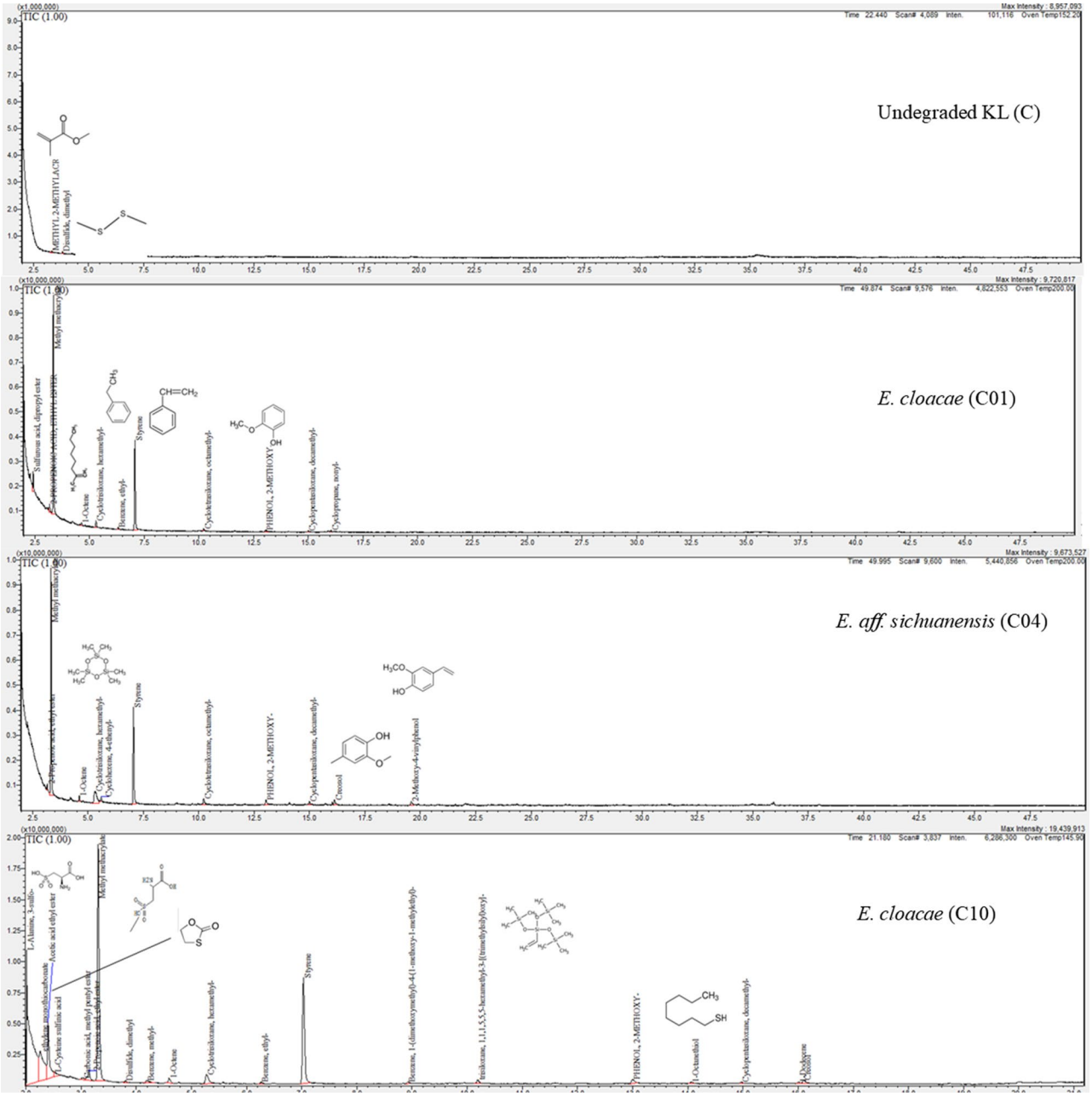

Fig. 11 Py-GC/MS analysis of degraded and undegraded KL and the compounds identified. Kraft lignin degradation was conducted anaerobically in syringes over a 3-week period, followed by compound extraction using ethyl acetate. The chemical structures of the identified compounds were randomly selected and illustrated using ChemDraw. The undegraded Kraft lignin served as the control in this study

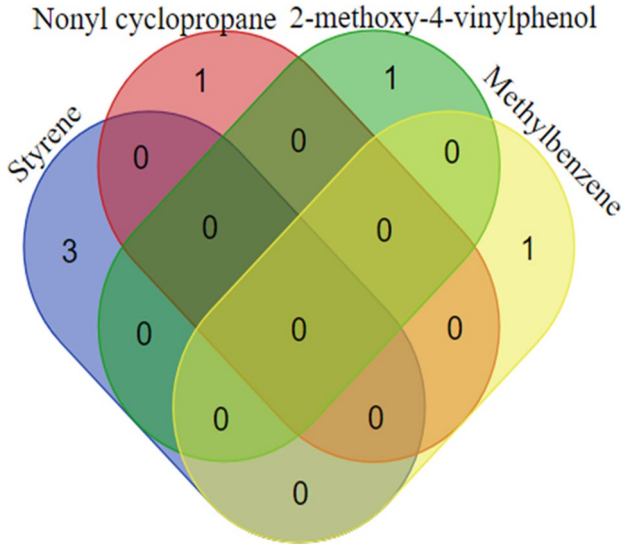

Fig. 12 Venn diagram indicating randomly chosen common and unique products identified via Py–GC–MS. Styrene was commonly produced by all three isolates, while nonyl cyclopropane, 2-methoxy-4-vinylphenol and methylbenzene were exclusively produced by *E. cloacae* (C01), *E.* aff. *sichuanensis* (C04) and *E. cloacae* (C10), respectively

from *E.* aff. *sichuanensis* (C04), as well as L-cysteine sulfinic acid, carbonic acid, methylbenzene, 1-(dimethoxy methyl)-4-(1-methoxy-1-methylethyl)-benzene, 1-dodecene, thiirane, and methyl isobutyrate from *E. cloacae* (C10), were exclusively detected in the individual isolates. Figure 12 displays a Venn diagram that represents the chemicals detected in the Py-GC/MS study, including those that are often recognized and those that are exclusively identified.

Varying amounts of compounds produced

A comprehensive presentation of the performances of the isolates is depicted using a heatmap (Fig. 13). The visual representation provides a means to compare the quantities of products produced by one isolate in proportion to others, so revealing insights into the overall performance of each isolate, for instance, when compared with *E. cloacae* (C10), *E. cloacae* (C01) and *E.* aff. *sichuanensis* (C04) produced considerably higher levels of styrene and 2-methoxyphenol. The specific percentages of area of all products are provided in the Supplementary materials, (Table S14). Although the present levels of degradation products may not have immediate practical applications, establishing this proof of concept is indispensable. This study provides essential insights into the fundamental

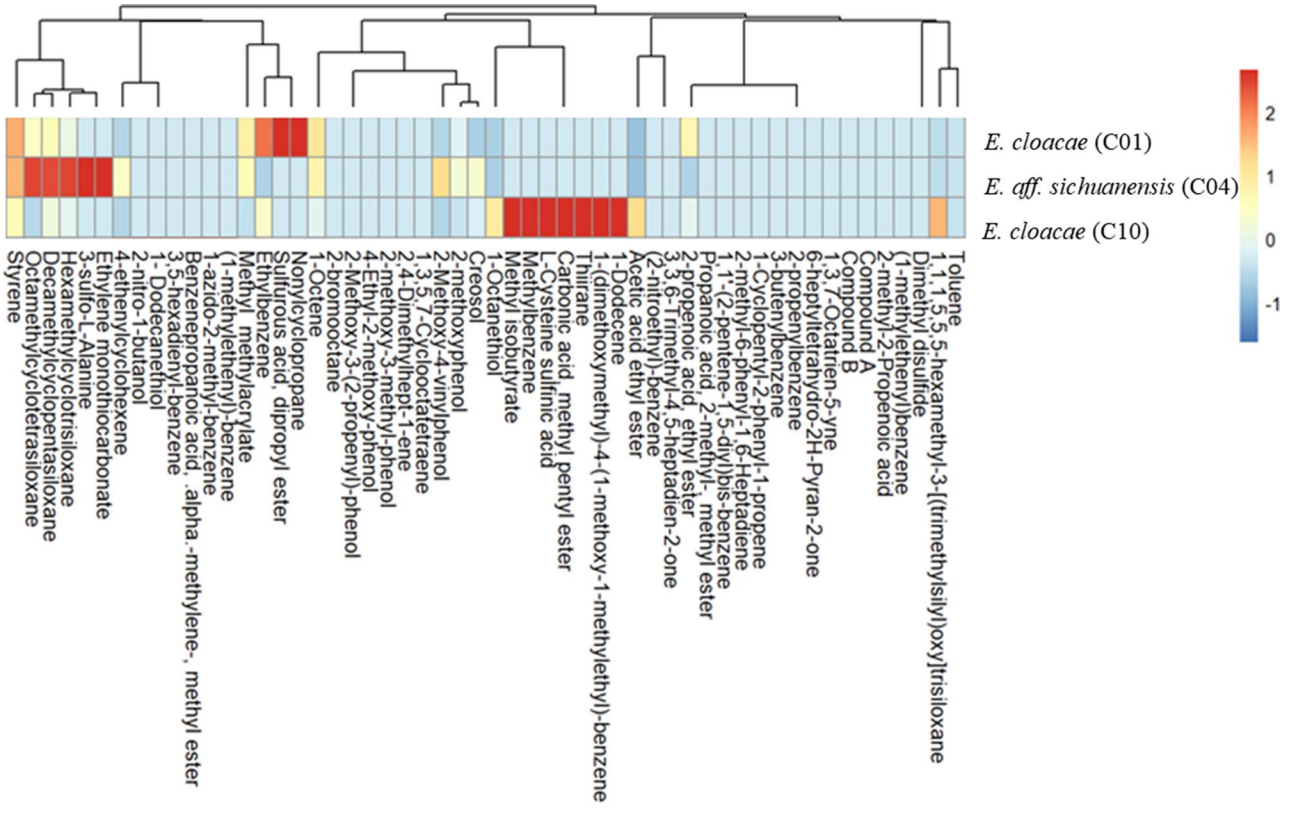

Fig. 13 A heatmap showing the chemicals identified from Py-GC/MS. The three isolated ligninolytic bacteria (rows) correspond to the identified compounds (columns). The score ranged from low (blue) to high (red), indicating the amount of product produced relative to that produced by other isolates and indicating an isolate's overall performance

pathways, guiding the design and optimization of ligninolytic bioprocesses for future practical applications.

Considering the performance of the studied strains, certain chemicals produced from lignin degradation could be extracted for biorefinery use. Techniques such as selective precipitation and fractional distillation can be used for this purpose. Ethylbenzene, produced by the studied isolates, is a key raw material for synthesizing styrene—a precursor for polystyrene [103], which is used in packaging, construction materials, and other applications. Alternatively, leveraging the robust lignin-degrading abilities of the studied isolates alongside the metabolic efficiency of engineered strains offers a promising approach. Implementing integrated bioprocesses, where lignin degradation and chemical production occur simultaneously, can streamline operations and reduce costs. Literature suggests that co-culturing ligninolytic microorganisms potential to improve efficiency [104, 105]. Utilizing the studied isolates to break down lignin into simpler compounds, which can then be utilized by engineered strains, could enable the production of high-value chemicals such as ethylbenzene and others.

Conclusion

Bioprospection for robust ligninolytic microorganisms is vital in advancing carbon-neutral bioprocesses that efficiently convert renewable, inexpensive and abundant but recalcitrant lignin. This study hypothesises that underground coal mine environments, which have comparable structures to lignin, could be a valuable reservoir for discovering industrially relevant ligninolytic strains. Our findings highlight that potentially novel species belonging to the *Enterobacter* genus exhibit significant ligninolytic activity. The isolates produced valuable monomers that can promote the sustainable and effective utilization of lignin. Further research should focus on taxonomic review and characterizing the metabolic pathways and mechanisms employed by these microorganisms to further understand their ligninolytic capabilities. Additionally, exploring genetic and enzymatic factors will be essential for optimizing their application in the industry. Such advancements will contribute to the development of environmentally friendly approaches for lignin waste stream management, aligning with global sustainability goals and fostering the expansion of green technologies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03537-5.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

The authors would like to extend their gratitude to their respective teams at BIUST and CSIR for their contributions. A special thank you to Mr. Tawanda P. Makopa for his invaluable contributions.

Author contributions

BJR: Conducted the experiments; analyzed and interpreted the data; and drafted, wrote, revised and approved the final manuscript.NZ: Data analysis and interpretation: conceptualized, revised and approved the final manuscript. SR: Conducted the Py-GCMS experiments, wrote its methodology. VC: Conducted the Py-GCMS experiments, wrote its methodology.

Funding

This work was funded by the Botswana International University of Science and Technology office of the DVC Research, Development and Innovation, postgraduate research grant (S00368).

Data availability

The datasets generated and/or analyzed during the current study have been deposited in the NCBI repository, https://www.ncbi.nlm.nih.gov/nuccore/PP715420.1?report=GenBank, https://www.ncbi.nlm.nih.gov/nuccore/PP715421.1?report=GenBank, https://www.ncbi.nlm.nih.gov/nuccore/PP715422.1?report=GenBank. Supplementary data are also available as separate files.

Declarations

Ethics approval and consent

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 6 April 2024 / Accepted: 20 September 2024
Published online: 01 October 2024

References

1. Agrawal R, Kumar A, Singh S, Sharma K. Recent advances and future perspectives of lignin biopolymers. J Polym Res. 2022;29(6):222.
2. Agrawal R, Satlewal A, Kapoor M, Mondal S, Basu B. Investigating the enzyme-lignin binding with surfactants for improved saccharification of pilot scale pretreated wheat straw. Bioresour Technol. 2017;224:411–8.
3. Atta-Obeng E, Dawson-Andoh B, Seehra MS, Geddam U, Poston J, Leisen J. Physico-chemical characterization of carbons produced from technical lignin by sub-critical hydrothermal carbonization. Biomass Bioenergy. 2017;107:172–81.
4. Shalma S, Shabbirahmed AM, Haldar D, Patel AK, Singhania RR. Influence of reaction conditions on synthesis and applications of lignin nanoparticles derived from agricultural wastes. Environ Technol Innov. 2023;31:103163.
5. Shao Y, Sun K, Li Q, Zhang L, Wei T, Gao G, Zhang S, Wang Y, Liu Q, Hu X. Dealkaline lignin–the waste from the pulp and paper industry as acid catalyst in biorefinery. Bioresource Technol Rep. 2019;7:100218.
6. Kaur P, Bohidar HB, Pfeffer FM, Williams R, Agrawal R. A comparative assessment of biomass pretreatment methods for the sustainable industrial upscaling of rice straw into cellulose. Cellulose. 2023;30(7):4247–61.
7. Bajwa D, Pourhashem G, Ullah A, Bajwa S. A concise review of current lignin production, applications, products and their environmental impact. Ind Crops Prod. 2019;139:111526.
8. Singh AK, Yadav P, Bharagava RN, Saratale GD, Raj A. Biotransformation and cytotoxicity evaluation of kraft lignin degraded by ligninolytic *Serratia liquefaciens*. Front Microbiol. 2019;10:2364.
9. Nadányi R, Ház A, Lisý A, Jablonský M, Šurina I, Majová V, Baco A. Lignin modifications, applications, and possible market prices. Energies. 2022;15(18):6520.
10. Cao L, Iris K, Liu Y, Ruan X, Tsang DC, Hunt AJ, Ok YS, Song H, Zhang S. Lignin valorization for the production of renewable chemicals: state-of-the-art review and future prospects. Bioresour Technol. 2018;269:465–75.

11. Rashid GM, Bugg TD. Enhanced biocatalytic degradation of lignin using combinations of lignin-degrading enzymes and accessory enzymes. Catal Sci Technol. 2021;11(10):3568–77.
12. Ayşegül Ö, Rakici E, Bektas KI, Çanaçki S, Belduz AO. Isolation of lignin-degrading bacteria from different sources and testing of their ligninolytic activities. Apiterapi ve Doğa Dergisi. 2019;2(2):30–45.
13. Bandounas L, Wierckx NJ, de Winde JH, Ruijssenaars HJ. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. BMC Biotechnol. 2011;11(1):1–11.
14. Duan J, Huo X, Du W, Liang J, Wang D, Yang S. Biodegradation of kraft lignin by a newly isolated anaerobic bacterial strain, *Acetoanaerobium* sp. WJDL-Y2. Lett Appl Microbiol. 2016;62(1):55–62.
15. Xiong X, Liao H, Ma J, Liu X, Zhang L, Shi X, Yang X, Lu X, Zhu Y. Isolation of a rice endophytic bacterium, Pantoea sp. Sd-1, with ligninolytic activity and characterization of its rice straw degradation ability. Lett Appl Microbiol. 2014;58(2):123–9.
16. Zhu D, Zhang P, Xie C, Zhang W, Sun J, Qian W-J, Yang B. Biodegradation of alkaline lignin by *Bacillus ligniniphilus* L1. Biotechnol Biofuels. 2017;10(1):1–14.
17. Devi A, Bajar S, Kour H, Kothari R, Pant D, Singh A. Lignocellulosic Biomass Valorization for Bioethanol Production: a circular Bioeconomy Approach. Bioenergy Res 2022:1–22.
18. Xu Z, Lei P, Zhai R, Wen Z, Jin M. Recent advances in lignin valorization with bacterial cultures: microorganisms, metabolic pathways, and bio-products. Biotechnol Biofuels. 2019;12(1):1–19.
19. Zhao C, Xie S, Pu Y, Zhang R, Huang F, Ragauskas AJ, Yuan JS. Synergistic enzymatic and microbial lignin conversion. Green Chem. 2016;18(5):1306–12.
20. Kaur P, Singh S, Sharma N, Agrawal R. Filling in the gaps in second-generation biorefineries: evaluating rice straw and its bioethanol residue for the production of biogenic silica nanoparticles. Nanatechnol Environ Eng. 2024;9(1):67–76.
21. Sharma N, Allardyce BJ, Rajkhowa R, Agrawal R. Controlled release fertilizer delivery system derived from rice straw cellulose nanofibres: a circular economy based solution for sustainable development. Bioengineered. 2023;14(1):2242124.
22. Sharma N, Kochar M, Allardyce BJ, Rajkhowa R, Agrawal R. Biodegradation of N-fertilizer loaded on cellulose nanofibres to assess their potential use as a controlled-release fertilizer. J Mater Sci. 2023;58(47):17859–72.
23. Zong Q-J, Xu T, Liu H, Xu L, Zhang R-K, Li B-Z, Liu Z-H, Yuan Y-J. Microbial valorization of lignin to bioplastic by genome-reduced Pseudomonas putida. Front Microbiol. 2022;13:923664.
24. Raj T, Verma S, Kumar N, Agrawal R. Role of woody biomass in carbon capture, circular bioeconomy, and biomanufacturing. In: Deepak kumar SK, Karthik Rajendran, Ramesh C. RAY, (ed.) Sustainable biorefining of Woody Biomass to Biofuels and Biochemicals. Elsevier; 2024. pp. 291–318.
25. Asina F, Brzonova I, Voeller K, Kozliak E, Kubátová A, Yao B, Ji Y. Biodegradation of lignin by fungi, bacteria and laccases. Bioresour Technol. 2016;220:414–24.
26. Del Cerro C, Erickson E, Dong T, Wong AR, Eder EK, Purvine SO, Mitchell HD, Weitz KK, Markillie LM, Burnet MC. Intracellular pathways for lignin catabolism in white-rot fungi. Proc Natl Acad Sci. 2021;118(9):e2017381118.
27. Sigoillot J-C, Berrin J-G, Bey M, Lesage-Meessen L, Levasseur A, Lomascolo A, Record E, Uzan-Boukhris E. Fungal strategies for lignin degradation. Adv Bot Res. 2012;61:263–308.
28. Niu J, Li X, Qi X, Ren Y. Pathway analysis of the biodegradation of lignin by Brevibacillus thermoruber. Bioresour Technol. 2021;341:125875.
29. Bugg TD, Ahmad M, Hardiman EM, Singh R. The emerging role for bacteria in lignin degradation and bio-product formation. Curr Opin Biotechnol. 2011;22(3):394–400.
30. Chandra R, Raj A, Purohit H, Kapley A. Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. Chemosphere. 2007;67(4):839–46.
31. Chen Y, Chai L, Tang C, Yang Z, Zheng Y, Shi Y, Zhang H. Kraft lignin biodegradation by *Novosphingobium* sp. B-7 and analysis of the degradation process. Bioresour Technol. 2012;123:682–5.
32. Tian JH, Pourcher AM, Peu P. Isolation of bacterial strains able to metabolize lignin and lignin-related compounds. Lett Appl Microbiol. 2016;63(1):30–7.
33. Nawaz MZ, Shang H, Sun J, Geng A, Ali SS, Zhu D. Genomic insights into the metabolic potential of a novel lignin-degrading and polyhydroxyalkanoates producing bacterium Pseudomonas sp. Hu109A. Chemosphere. 2023;310:136754.
34. Spence EM, Calvo-Bado L, Mines P, Bugg TD. Metabolic engineering of Rhodococcus jostii RHA1 for production of pyridine-dicarboxylic acids from lignin. Microb Cell Fact. 2021;20:1–12.
35. Priyadarshinee R, Kumar A, Mandal T, Dasguptamandal D. Unleashing the potential of ligninolytic bacterial contributions towards pulp and paper industry: key challenges and new insights. Environ Sci Pollut Res. 2016;23(23):23349–68.
36. Akita H, Kimura Z-i, Yusoff MZM, Nakashima N, Hoshino T. Isolation and characterization of *Burkholderia* sp. strain CCA53 exhibiting ligninolytic potential. SpringerPlus. 2016;5(1):1–5.
37. Grgas D, Rukavina M, Bešlo D, Štefanac T, Crnek V, Šikić T, Habuda-Stanić M. Landeka Dragičević T: the bacterial degradation of Lignin—A review. Water. 2023;15(7):1272.
38. Jiang C, Cheng Y, Zang H, Chen X, Wang Y, Zhang Y, Wang J, Shen X, Li C. Biodegradation of lignin and the associated degradation pathway by psychrotrophic *Arthrobacter* sp. C2 from the cold region of China. Cellulose. 2020;27(3):1423–40.
39. Li J, Wang P, Salam N, Li X, Ahmad M, Tian Y, Duan L, Huang L, Xiao M, Mou X. Unraveling bacteria-mediated degradation of lignin-derived aromatic compounds in a freshwater environment. Sci Total Environ. 2020;749:141236.
40. Li F, Pan S, Yang L, Liao S, Yang D, Wang Q, Huang S. Efficient production 2, 3-butanediol from biomass-derived sugars by Raoultella ornithinolytica TH-21, a newly isolated lignocellulose-degrading bacterium. Ind Crops Prod. 2024;217:118841.
41. Wang L, Nie Y, Tang Y-Q, Song X-M, Cao K, Sun L-Z, Wang Z-J, Wu X-L. Diverse bacteria with lignin degrading potentials isolated from two ranks of coal. Front Microbiol. 2016;7:1428.
42. Saubi O, Suglo RS, Konka B. Impacts of fines at Morupule Coal Mine, Botswana. Momona Ethiop J Sci. 2023;15(1):1–16.
43. Hancox P. The coalfields of South-Central Africa: a current perspective. Episodes J Int Geoscience. 2016;39(2):407–28.
44. Makoba M, Moalosi T, Agachi PS, Muzenda E, Mamvura TA. Characterization of Botswana coal from two coal fields: Mabesekwa and Mmamabula to determine its coal rank. Stud Univ Babes-Bolyai Chem 2020, 65(2).
45. Ravi K, García-Hidalgo J, Gorwa-Grauslund MF, Lidén G. Conversion of lignin model compounds by Pseudomonas putida KT2440 and isolates from compost. Appl Microbiol Biotechnol. 2017;101(12):5059–70.
46. Zhang W, Ren X, Lei Q, Wang L. Screening and Comparison of Lignin Degradation Microbial Consortia from Wooden Antiques. Molecules. 2021;26(10):2862.
47. Dhakar K, Jain R, Tamta S, Pandey A. Prolonged laccase production by a cold and pH tolerant strain of Penicillium pinophilum (MCC 1049) isolated from a low temperature environment. *Enzyme research* 2014, 2014.
48. Ravi K, García-Hidalgo J, Nöbel M, Gorwa-Grauslund MF, Lidén G. Biological conversion of aromatic monolignol compounds by a Pseudomonas isolate from sediments of the Baltic Sea. AMB Express. 2018;8(1):32.
49. Reiner K. Carbohydrate fermentation protocol. Am Soc Microbiol. 2012;11:12.
50. Souza R, Gomes F, Brito E, Costa Lelis R. A review on lignin sources and uses. Jornal Appl Biotechnol Bioeng. 2020;7(3):100.
51. Kumar R, Singh A, Maurya A, Yadav P, Yadav A, Chowdhary P, Raj A. Effective bioremediation of pulp and paper mill wastewater using *Bacillus cereus* as a possible kraft lignin-degrading bacterium. Bioresour Technol. 2022;352:127076.
52. Raj A, Chandra R, Reddy MMK, Purohit HJ, Kapley A. Biodegradation of kraft lignin by a newly isolated bacterial strain, *Aneurinibacillus aneurinilyticus* from the sludge of a pulp paper mill. World J Microbiol Biotechnol. 2007;23(6):793–9.
53. Silva GAd, Bernardi TL, Schaker PDC, Menegotto M, Valente P. Rapid yeast DNA extraction by boiling and freeze-thawing without using chemical reagents and DNA purification. Brazilian Archives Biology Technol. 2012;55:319–27.
54. Nazish A, Khattak B, Ali Khan T, Ahmad I, Ullah R, Bari A, Asmari MM, Mahmood HM, Sohaib M, El Askary A. Antinematode Activity of Abomasum Bacterial Culture Filtrates against Haemonchus contortus in small ruminants. Animals. 2021;11(6):1843.
55. Lorenz TC. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. JoVE (Journal Visualized Experiments) 2012(63):e3998. https://doi.org/10.3791/3998
56. Hall T, Biosciences I, Carlsbad C. BioEdit: an important software for molecular biology. GERF Bull Biosci. 2011;2(1):60–1.
57. Kim M, Oh H-S, Park S-C, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol MicroBiol. 2014;64(Pt2):346–51.

58. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35(6):1547.
59. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792–7.
60. Zhou N, Schifferdecker AJ, Gamero A, Compagno C, Boekhout T, Piškur J, Knecht W. Kazachstania Gamospora and Wickerhamomyces subpelliculosus: two alternative baker's yeasts in the modern bakery. Int J Food Microbiol. 2017;250:45–58.
61. Christfort JF, Polhaus CJM, Bondegaard PW, Chang T-J, Te Hwu E, Nielsen LH, Zór K, Boisen A. Open source anaerobic and temperature-controlled in vitro model enabling real-time release studies with live bacteria. HardwareX. 2022;11:e00275.
62. Chandra R, Bharagava RN. Bacterial degradation of synthetic and kraft lignin by axenic and mixed culture and their metabolic products. J Environ Biol. 2013;34(6):991.
63. Chandra R, Singh R. Decolourisation and detoxification of rayon grade pulp paper mill effluent by mixed bacterial culture isolated from pulp paper mill effluent polluted site. Biochem Eng J. 2012;61:49–58.
64. Delmas GH, Benjelloun-Mlayah B, Bigot YL, Delmas M. Functionality of wheat straw lignin extracted in organic acid media. J Appl Polym Sci. 2011;121(1):491–501.
65. Stein S, Mirokhin D, Tchekhovskoi D, Mallard G, Mikaia A, Zaikin V, Sparkmanm D. The NIST mass spectral search program for the NIST/EPA/NIH mass spectra library. *Gaithersburg, MD: Standard Reference Data Program of the National Institute of Standards and Technology* 2002.
66. Xiong Y, Zhao Y, Ni K, Shi Y, Xu Q. Characterization of ligninolytic bacteria and analysis of alkali-lignin biodegradation products. Pol J Microbiol. 2020;69(3):339–47.
67. Zhuo S, Yan X, Liu D, Si M, Zhang K, Liu M, Peng B, Shi Y. Use of bacteria for improving the lignocellulose biorefinery process: importance of pre-erosion. Biotechnol Biofuels. 2018;11:1–13.
68. Khan MU, Ahring BK. Lignin degradation under anaerobic digestion: influence of lignin modifications-A review. Biomass Bioenergy. 2019;128:105325.
69. Naz S. Study of ligninolytic bacteria isolation and characterization from Kuthrel Agro Field of Bhilai-Durg Region. Int J Curr Microbiol App Sci. 2016;5(12):141–50.
70. Ding C, Wang X, Li M. Evaluation of six white-rot fungal pretreatments on corn stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. Appl Microbiol Biotechnol. 2019;103:5641–52.
71. Dong S-J, Zhang B-X, Wang F-L, Xin L, Gao Y-F, Ding W, He X-M, Liu D, Hu X-M. Efficient lignin degradation of corn stalk by trametes with high laccase activity and enzymatic stability in salt and ionic liquid. BioResources. 2019;14(3):5339–54.
72. Ali NS, Huang F, Qin W, Yang TC. A high throughput screening process and quick isolation of novel lignin-degrading microbes from large number of natural biomasses. Biotechnol Rep. 2023;39:e00809.
73. Manter DK, Hunter WJ, Vivanco JM. *Enterobacter soli* sp. aov.: A lignin-degrading γ-Proteobacteria isolated from soil. Curr Microbiol. 2011;62(3):1044–9.
74. Orellana R, Chaput G, Markillie LM, Mitchell H, Gaffrey M, Orr G, DeAngelis KM. Multi-time series RNA-seq analysis of *Enterobacter Lignolyticus* SCF1 during growth in lignin-amended medium. PLoS ONE. 2017;12(10):e0186440.
75. Roy S, Roy M, Shaw A, Soni P. Identification and Characterization of Naphthalene Degrading Bacteria Isolated from Acid Mine Drainage Sample of Barjora Coal Mine, West Bengal, India. 2016.
76. Wu W, Feng Y, Zong Z. Characterization of a strain representing a new Enterobacter species, Enterobacter chengduensis sp. nov. Antonie Van Leeuwenhoek. 2019;112:491–500.
77. Davin-Regli A, Lavigne J-P, Pagès J-M. *Enterobacter* spp.: update on taxonomy, clinical aspects, and emerging antimicrobial resistance. Clin Microbiol Rev. 2019;32(4):e00002–00019.
78. Xu R, Zhang K, Liu P, Han H, Zhao S, Kakade A, Khan A, Du D, Li X. Lignin depolymerization and utilization by bacteria. Bioresour Technol. 2018;269:557–66.
79. Nwaefuna AE, Garcia-Aloy M, Loeto D, Ncube T, Gombert AK, Boekhout T, Alwasel S, Zhou N. Dung beetle-associated yeasts display multiple stress tolerance: a desirable trait of potential industrial strains. BMC Microbiol. 2023;23(1):309.
80. Abdelaziz OY, Brink DP, Prothmann J, Ravi K, Sun M, Garcia-Hidalgo J, Sandahl M, Hulteberg CP, Turner C, Lidén G. Biological valorization of low molecular weight lignin. Biotechnol Adv. 2016;34(8):1318–46.
81. Zhang W, Liu Z. Isolation and performance study of a Novel lignin-degrading strain. Chem Biochem Eng Q. 2021;35(4):431–8.
82. Suresh A, Ramgopal DS, Gopinath KP, Arun J, SundarRajan P, Bhatnagar A. Recent advancements in the synthesis of novel thermostable biocatalysts and their applications in commercially important chemoenzymatic conversion processes. Bioresour Technol. 2021;323:124558.
83. Gomes E, de Souza AR, Orjuela GL, Da Silva R, de Oliveira TB, Rodrigues A. Applications and benefits of thermophilic microorganisms and their enzymes for industrial biotechnology. Gene Expression Syst Fungi: Advancements Appl. 2016:459–92.
84. Zhu D, Adebisi WA, Ahmad F, Sethupathy S, Danso B, Sun J. Recent Development of Extremophilic Bacteria and their application in Biorefinery. Front Bioeng Biotechnol. 2020;8:483.
85. Szalaty TJ, Klapiszewski Ł, Jesionowski T. Recent developments in modification of lignin using ionic liquids for the fabrication of advanced materials–A review. J Mol Liq. 2020;301:112417.
86. Cortes-Tolalpa L, Norder J, van Elsas JD, Falcao Salles J. Halotolerant microbial consortia able to degrade highly recalcitrant plant biomass substrate. Appl Microbiol Biotechnol. 2018;102(6):2913–27.
87. Salvachúa D, Karp EM, Nimlos CT, Vardon DR, Beckham GT. Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria. Green Chem. 2015;17(11):4951–67.
88. Ahmad Z, Mahmood N, Yuan Z, Paleologou M, Xu C. Effects of process parameters on hydrolytic treatment of black liquor for the production of low-molecular-weight depolymerized kraft lignin. Molecules. 2018;23(10):2464.
89. Rekik H, Jaouadi NZ, Bouacem K, Zenati B, Kourdali S, Badis A, Annane R, Bouanane-Darenfed A, Bejar S, Jaouadi B. Physical and enzymatic properties of a new manganese peroxidase from the white-rot fungus trametes pubescens strain i8 for lignin biodegradation and textile-dyes biodecolorization. Int J Biol Macromol. 2019;125:514–25.
90. Evdokimov AN, Kurzin AV, Fedorova OV, Lukanin PV, Kazakov VG, Trifonova AD. Desulfurization of kraft lignin. Wood Sci Technol. 2018;52(4):1165–74.
91. Dashtban M, Schraft H, Syed TA, Qin W. Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol. 2010;1(1):36.
92. Billings AF, Fortney JL, Hazen TC, Simmons B, Davenport KW, Goodwin L, Ivanova N, Kyrpides NC, Mavromatis K, Woyke T. Genome sequence and description of the anaerobic lignin-degrading bacterium *Tolumonas Lignolytica* sp. nov. Stand Genomic Sci. 2015;10(1):1–11.
93. Huang W-C, Tang I-C. Bacterial and yeast cultures–process characteristics, products, and applications. Bioprocess Value-Added Prod Renew Resour 2007:185–223.
94. Nada A-AM, El-Sakhawy M, Kamel SM. Infra-red spectroscopic study of lignins. Polym Degrad Stab. 1998;60(2–3):247–51.
95. Sathawong S, Sridach W, Techato K-a. Lignin: isolation and preparing the lignin based hydrogel. J Environ Chem Eng. 2018;6(5):5879–88.
96. Yang C, Yue F, Cui Y, Xu Y, Shan Y, Liu B, Zhou Y, Lü X. Biodegradation of lignin by *Pseudomonas* sp. Q18 and the characterization of a novel bacterial DyP-type peroxidase. J Ind Microbiol Biotechnol. 2018;45(10):913–27.
97. Hainal AR, Capraru AM, Volf I, Popa VI. Lignin as a carbon source for the cultivation of some *Rhodotorula* species. Cellul Chem Technol. 2012;46:87–96.
98. Khan SI, Zarin A, Ahmed S, Hasan F, Belduz AO, Çanakçi S, Khan S, Badshah M, Farman M, Shah AA. Degradation of lignin by Bacillus altitudinis SL7 isolated from pulp and paper mill effluent. Water Sci Technol. 2022;85(1):420–32.
99. Sharma RK, Mukhopadhyay D, Gupta P. Microbial fuel cell-mediated lignin depolymerization: a sustainable approach. J Chem Technol Biotechnol. 2019;94(3):927–32.
100. Jeong JB, Hong SC, Jeong HJ, Koo JS. Anti-inflammatory effect of 2-methoxy-4-vinylphenol via the suppression of NF-κB and MAPK activation, and acetylation of histone H3. Arch Pharm Res. 2011;34(12):2109–16.
101. Smolarski N. High-value opportunities for lignin: unlocking its potential. Frost Sullivan. 2012;1:1–15.
102. Luo H, Abu-Omar MM. Chemicals from lignin. Encyclopedia sustainable technologies; Elsevier: Amsterdam Neth. 2017;573–85.
103. Luo Z, Qin S, Chen S, Hui Y, Zhao C. Selective conversion of lignin to ethylbenzene. Green Chem. 2020;22(6):1842–50.
104. Ahmed AAQ, Babalola OO, McKay T. Lignin Degradation by Two Isolated Bacillus Sp. Strains and their co-culture potential in the production of platform chemicals. Proceedings Book. 2016.

105. Peláez RDR, Wischral D, Mendes TD, Pacheco TF, Urben AF, Helm CV, Mendonça S, Balan V, de Siqueira FG. Co-culturing of micro-and macro-fungi for producing highly active enzyme cocktail for producing biofuels. Bioresource Technol Rep. 2021;16:100833.

Publisher's note