Isolation and Characterization of Potential Lignin Peroxidase-Producing Bacteria from Compost Samples at Richards Bay (South Africa)

SINDISWA L. DUBE[1], FOLUSO O. OSUNSANMI[1]* , BONGEKILE P. NGCOBO[1], LONDIWE B. MKHWANAZI[1],
ZANELE Z. JOBE[1], RAPHAEL T. ARULEBA[2], REBAMANG A. MOSA[3] and ANDREW R. OPOKU[1]

[1] Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa, South Africa
[2] Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, Cape Town, South Africa
[3] Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Hatfield, South Africa

A b s t r a c t

Lignin recalcitrance is a key issue in producing value-added products from lignocellulose biomass. *In situ* biodegradable lignin-modifying enzymes-producing bacteria are considered a suitable solution to lignin biodegradation problems, but exploitation of ligninolytic bacteria is still limited to date. Hence, this study aimed to isolate and characterize potential lignin peroxidase ligninolytic bacteria from decomposing soil, sawdust, and cow dung at Richard Bay, South Africa. The samples were collected and cultured in the lignin-enriched medium. Pure isolated colonies were characterized through 16S rRNA gene sequencing. The ability of the isolates to grow and utilize aromatic monomers (veratryl and guaiacol alcohol) and decolorize lignin-like dyes (Azure B, Congo Red, Remazol Brilliant Blue R) was evaluated. Of the twenty-six (26) bacteria isolates 10 isolates, including *Pseudomonas* spp. (88%), *Enterobacter* spp. (8%), and *Escherichia coli* (4%) were identified as true lignin peroxidase producers. *Pseudomonas aeruginosa (*CP031449.2) and *E. coli (*LR025096.1) exhibited the highest ligninolytic activities. These isolates could potentially be exploited in the industry and wastewater treatment as effective lignin degrading agents.

K e y w o r d s: lignin, bacteria, ligninolytic activity, lignin peroxidase, 16S rDNA

Introduction

Lignocellulose is a significant plant component and varies among plant species (de Gonzalo et al. 2016). The demand for value-added products such as organic acids, biofuel, and microbial polysaccharides from lignocellulose biomass is increasing worldwide (Chaturvedi and Verma 2013). It is due to the renewable and abundant nature of the lignocellulose biomass. However, the recalcitrant nature of lignin remains a major bottleneck to unleashing useful products during various industrial operations. Due to inadequate treatment and waste management plans, lignin waste biomass from industrial and agricultural sectors continue to pose an environmental threat (Falade et al. 2017). Hence, valorizing lignocellulose waste is a welcome call for a proper waste management strategy.

Despite the commonly used chemical pre-treatment strategies to delignify lignocellulose biomass, there is a growing interest in biological delignification. In addition to the high capability of microorganisms to effectively degrade lignin, biological hydrolysis of lignin is also saddled with limited drawbacks (Datta et al. 2017). Unlike the chemical approach associated with high energy requirements and environmental threats, the biological hydrolysis of lignin is cost-effective, eco-friendly, and reduces environmental pollutants (Kumar and Chandra 2020).

The high capability of microorganisms to effectively degrade lignin is due to their ability to produce lignin-modifying enzymes (LMEs). Among the LMEs (laccase, manganese peroxidase, versatile peroxidase, etc.), lignin peroxidase is the most effective (Al-Dhabaan 2019). Lignin peroxidase (LiP), a heme peroxidase, utilizes hydrogen peroxide to oxidize a wide variety of aromatic model compounds (of a phenolic and non-phenolic nature) of lignin into mono-aromatic structures (Janusz et al. 2017). LiP thus could play a pivotal role in treating

* Corresponding author: F.O. Osunsanmi, Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa, South Africa; **e-mail: alafin21@unizulu.ac.za**

industrial waste and degradation of xenobiotics such as dyes, organophosphorus, and polycyclic aromatic hydrocarbons (Falade et al. 2017). Such versatile industrial applications of LiP increase the need to search for LiP-producing microorganisms.

Although white rot basidiomycetes have intensively been studied as a source of potent ligninolytic extracellular oxidative enzymes, the ligninolytic use of fungus is limited (Su et al. 2018). Bacteria candidature as ligninolytic agents is currently gaining attention in research. It is due to lesser ethical issues regarding bacteria use, environmental adaptation, spontaneous growth, and the ease of genetic manipulation of bacteria (Tian 2016). Hence, there is a growing interest in exploring bacteria as potential ligninolytic agents. In searching for bacteria that can produce lignin-modifying enzymes (LMEs), bacterial species classified into actinomycetes, α-Proteobacteria, and γ-Proteobacteria have been identified (Wang et al. 2016). Such bacteria include *Streptomyces viridosporus*, and strains of *Nocardia* and *Pseudomonas* (Zimmermann 1990; Bugg et al. 2011).

The Richards Bay environment, a central industrial hub in the Kwa-Zulu Natal province of South Africa, is characterized by loops of hemp of lignin-based wastes and untreated industrial wastewater, posing environmental hazards (Okello et al. 2022). Such an environment might serve as a reservoir for bacterial genome pool to exploit novel bacteria strains with excellent lignin-modifying enzymes. Therefore, this study intended to isolate and characterize potential ligninolytic bacterial strains with lignin peroxidase production capacity from compost samples: sawdust, decaying soil, and cow dung from the Richards Bay environment.

Experimental

Materials and Methods

Chemicals. Unless otherwise stated, the chemicals and reagents used in this study were of analytical grades. Nutrient both, nutrient agar, and salts were purchased from Merck (Germany). The kraft lignin, Congo Red, ferric chloride, hexahydrate, sodium azide, pyrogallol, veratryl alcohol, guaiacol alcohol, Malachite green, Remazol Brilliant Blue, Luria broth base, pyrogallol, hydrogen peroxide, disodium salts, and glycerol were supplied by Sigma Aldrich (USA). Bacteriological agar was bought from Lasec (South Africa).

Sample collection. The compost samples (sawdust, soil, and cow dung) were collected at locations within Richards Bay, South Africa, during summer in March 2021. The soil samples were collected along the uMlalazi river and eSkhaleni forest with coordinates of LA: 28.936164 and LO: 31.763771, and LA: −29.993591

and LO: 30.877430, respectively. Decomposed sawdust sample was collected from Mtunizini's Furniture Company (LA: −28.958938 and LO: 31.758050). Cow dung was collected at Farmhouse, University of Zululand (LA: 28.7576 and LO: 32.0497). The samples were kept in sterilized plastic containers and transported to the laboratory, where they were used within 24 hr. The samples (10 g) were mixed with sterile water (90 ml) and centrifuged (30 minutes; 170 rpm). Then, the suspension was diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively. Afterward, the different dilutions (0.1 ml) were added to the prepared medium and daub uniformly. The ethical clearance (UZREC 171110–030 PGM 2018/575) was obtained from the University of Zululand Research Ethics Committee (UZREC) for the use of bacteria for research purposes.

Isolation of ligninolytic bacteria strain. Ligninolytic bacteria were isolated using the enriched media (Minimal Salts Medium Containing Lignin; MSM-L) and the method described by Sasikumar et al. (2014). Briefly, 1 g/l kraft lignin as the sole carbon source was added to the minimal salts medium (MSM), composed of 4.55 g/l K_2HPO_4, 0.53 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 5 g/l NH_4NO_3, and 0.1 g/l yeast extract. The reagents were vigorously mixed and autoclaved at 121°C for 15 minutes. Enrichment culture was prepared by adding each sample (5 g) to a 250 ml Erlenmeyer flask containing MSM-L broth (95 ml). The culture was then incubated (30°C; 140 rpm) in an orbital shaker (Infors HT Ecotron; Infors HT, Switzerland) for 168 hr. The enriched culture (1 ml) was transferred and mixed with 9 ml of sterilized NaCl (0.9%) solution at room temperature. Serial dilutions were made using 1 ml of the liquid solution, and 100 μl of the serially diluted solutions were poured and spread on MSM-L agar plates containing nystatin (50 mg/l) to prevent fungal development. The plates were then incubated at 30°C for 168 hours, until ligninolytic bacteria became visible. The cultures were then purified using the streaking technique to obtain single colonies, and these were kept in glycerol (20%) at −80°C for later use.

Screening of isolated bacteria for ligninolytic activities. *Evaluation of the ligninolytic potential of bacterial isolates using lignin model compounds.* The method described by Taylor et al. (2012) was adopted to evaluate the bacterial ligninolytic potential using lignin model compounds (guaiacol and veratryl alcohols). The isolated bacterial suspension (5 μl; $OD_{620} \approx 1.0$) was inoculated into different agar plates consisting of KH_2PO_4 (0.53 g/l), NH_4NO_3 (5 g/l), $MgSO_4$ (0.5 g/l), K_2HPO_4 (4.55 g/l), 0.1 g/l of yeast extract, veratryl or guaiacol alcohol (0.1% v/v), and 15 g/l agar. The plates were then incubated for 168 hours at 30°C, after which the plates were flooded with Gram iodine solution to determine degradation zones.

Dye decolorization assay. The isolates with ligninolytic activity were evaluated for their ability to decolorize certain dyes, viz. Remazol Brilliant Blue R (RBBR), Congo Red (CR), Methylene Blue (MB), and Azure B (AZ), using the method of Bandounas et al. (2011). Briefly, 5 μl of the overnight culture (18 hours) was aseptically inoculated onto dye agar plates made up of KH_2PO_4 (0.53 g/l), NH_4NO_3 (5 g/l), $MgSO_4$ (0.5 g/l), K_2HPO_4 (4.55 g/l), 0.1 g/l yeast extract, 40 mM of glycerol, dye (100 mg/l AZ, 100 mg/l MB, 50 mg/l CR or 100 mg/l RBBR), and 15 g/l of agar. The plates were then incubated at 30°C for 168 hours and observed regularly for growth and formation of zones of decolorization.

Qualitative lignin peroxidase activity of the isolated bacteria. All positive isolates from the dye decolorization assay were evaluated qualitatively for peroxidase activity (Falade et al. 2017). The isolates were separately grown on nutrient agar plates and incubated at 30°C for 48 hours. Subsequently, hydrogen peroxide (0.4%; 30 μl) and pyrogallol (1%) were mixed with the grown colonies on the plates. The colonies with the yellow-brown color indicated a positive result.

Qualitative laccase activity of the isolated bacteria. Ligninolytic bacteria were also tested for laccase activity using the method described by Kameshwar and Qin (2017). The isolated ligninolytic bacteria strains were activated overnight and inoculated on MSL agar plates supplemented with 0.1% w/v ABTS, 1.6% w/v Agar, and 20% w/v glucose. The plates were incubated in the dark for 10 days at 30°C. The development of green color on colonies indicated positive results.

Biochemical test. The bacterial isolates with ligninolytic activity were subjected to standard biochemical tests, including catalase, oxidase, starch hydrolysis, urease, indole, and Simmon's citrate test (Badr 2018; Al-Dhabaan 2019).

The 16S rRNA gene sequencing of the isolates. The 16S rRNA genes of the bacterial isolates with ligninolytic activities were analyzed. The 16S rDNA was amplified, and sequence analyzed through BLAST (NCBI). The bacteria genomic DNA was extracted from the colonies using *Quick*-DNA™ Fugal/Bacterial Miniprep Kit (Zymo Research, USA). The targeted region of 16S rDNA was tagged with One*Taq*® Quick-Load® 2X Master Mix (New England Biolabs® Inc., USA) along with 16S–1492R (5'-CGGTTACCTTGTTAC-GACTT-3') and 16S-27F (5'-AGAGTTTGATCMTG-GCTCAG-3') primers, respectively. The products were then run through a gel and extracted with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). The DNA fragments extracted were further sequenced with Nimagen (The Netherlands), BrilliantDye™ Terminator v3.1 Cycle Sequencing Kit (BRD3-1000) and purified with Zymo Research (USA), ZR-96 DNA Sequencing Clean-up Kit™. The pure fragments were analyzed using the ABI 3500xL Genetic Analyzer (Applied Biosystems™, ThermoFisher Scientific Inc., USA). The file generated by the ABI analyzer was further analyzed with GLC Bio Main Workbench v7.6 with the aid of BLAST search (NCBI). The phylogenetic tree was constructed by neighbor joining methods, and 99% similarity with existing isolates in the database was considered (Xiong et al. 2020).

Lignin degradation capability study of the pure cultures. The bacterial isolates with ligninolytic enzyme activity were evaluated for lignin biodegradation capability following the method described by Dicko et al. (2020), with slight modification. The experiment was carried out on a medium containing kraft lignin (3 g/l) as a carbon source. The purified colonies were inoculated on nutrient broth and incubated for 48 hours at 30°C to activate the colonies. Afterward, bacterial suspensions (2%) in saline were cultured in conical flasks (250 ml) containing MSM-L broth fortified with glucose (1%) until the OD_{620} was 1 nm. It was incubated for 6 days at 30°C in a shaker (120 rpm). Aliquots (2 ml) were taken daily from each flask, bacterial growth (A_{620}), and pH were measured accordingly. The collected aliquots were centrifuged (15,000 rpm; 5 minutes), and their supernatants (250 μl) were mixed with phosphate buffer (750 μl). The absorbance of color reduction and lignin degradation was read at A_{465} and A_{280}, respectively, using a spectrophotometer. Graphs of bacterial growth, lignin degradation, pH vs. time, and bacterial growth were plotted.

Quantitative assays for the lignin peroxidase activity. The lignin peroxidase activity of the isolates was determined by monitoring the rate of H_2O_2-dependent oxidation of pyrogallol to purpurogallin (Falade et al. 2017). Pyrogallol (310 μl of 5% w/v) was pipetted into an Eppendorf tube with potassium phosphate buffer (100 mM; pH 6) and mixed. Afterward, the cultured supernatant (25 μl) was added to the solution and mixed by the vortex. The mixture without the enzymes served as blank. The reaction was initiated by the addition of 15 μl of 0.5% v/v of H_2O_2. The direct increase in absorbance was read at 420 nm every 30 seconds for 2–3 minutes at room temperature, using BioTeK Synergy™ HT 96-well Microtitre Plate Reader (BioTeK® Instruments Inc., USA). The enzymatic activity was subsequently calculated using the following equation:

$$\frac{Units}{ml}\,enzyme = \frac{(\Delta A420/30\,sec\,Test\,Sample - \Delta A420/30\,sec\,Blank)\,(3)\,(df)}{(12)\,(0.1)}$$

where: 3 = volume (ml) of the assay, df = dilution factor, 12 = extinction coefficient of 1 mg/ml of purpurogallin at 420 nm (determined internally), and 0.1 = volume (ml) of enzyme used.

Data Analysis. Unless otherwise stated, all the experiments were triplicated, and the results were

expressed as mean ± standard deviation (SD). The data for decolorization assay was analyzed by one-way ANOVA and comparison analysis by Dunn's post hoc using GraphPad Prism software (version 6).

Results

Isolation of ligninolytic bacteria. Table SI depicts the potential ligninolytic isolates from the compost samples. Twenty-six (26) bacteria strains were successfully isolated from cow dung, soil, and sawdust samples.

Evaluation of ligninolytic activity of the isolates. The results indicating the ability of the isolates to degrade and utilize guaiacol and veratryl alcohols are presented in Table I. After over 168 hours, the isolated microbes could grow on lignin monomers – veratryl and guaiacol alcohol – as a carbon source. While fifteen (58%) of the total isolates were able to degrade both

Table I
Degradation of lignin model compounds; guaiacol and veratryl alcohols by the bacterial isolates from the compost samples.

S/N	Bacterial code	Isolation source	Guaiacol alcohol	Veratryl alcohol
1	Old A	Old cow dung	++	++
2	*Old A	Old cow dung	++	++
3	OOld A	Old cow dung	++	+++
4	Old A1	Old cow dung	+	+
5	*Old A2	Old cow dung	+	++
6	Old A2	Old cow dung	+++	+++
7	/*New A	Fresh cow dung	–	++
8	New A	Fresh cow dung	+++	+++
9	*New A	Fresh cow dung	–	++
10	New B	Fresh cow dung	++	++
11	ONew B	Fresh cow dung	–	–
12	*New B	Fresh cow dung	–	–
13	Saw A	Sawdust	–	–
14	Saw A1	Sawdust	–	+
15	Saw A2	Sawdust	+++	+++
16	Saw B	Sawdust	–	–
17	*Saw B	Sawdust	–	+++
18	Saw B1	Sawdust	–	++
19	Soil A	Soil	–	+
20	*Soil A	Soil	–	+++
21	Soil A1	Soil	+	++
22	*Soil B	Soil	+	+++
23	Soil B	Soil	++	+++
24	Soil B1	Soil	+++	+++
25	Soil 2	Soil	+	++
26	Soil 7	Soil	+	++

– – negative, + – weak, ++ – moderate, +++ – strongly positive

Table II
Decolourization of synthetic lignin-like dyes (halo zone measured in mm) by the selected ligninolytic bacterial isolates.

S/N	Bacterial code	RBBR (mm)	AZ (mm)	CR (mm)
1	Old A	15.0 ± 0.10^c	35.0 ± 0.04^b	0.00 ± 0.00^f
2	*Old A	26.0 ± 0.00^b	40.0 ± 0.01^d	42.0 ± 0.01^a
3	OOld A	24.0 ± 0.06^b	30.0 ± 0.00^c	31.0 ± 0.10^c
4	Old A1	19.0 ± 0.00^d	29.0 ± 0.05^c	0.00 ± 0.00^f
5	*Old A2	0.00 ± 0.00^f	34.0 ± 0.04^e	10.0 ± 0.20^d
6	Old A2	35.0 ± 0.10^a	42.0 ± 0.11^d	40.0 ± 0.08^a
7	New A	8.00 ± 0.50^e	40.0 ± 0.00^d	20.0 ± 0.01^e
8	New B	30.0 ± 0.00^h	50.0 ± 0.01^a	26.0 ± 0.02^b
9	Saw A2	15.0 ± 0.04^c	35.0 ± 0.02^b	12.0 ± 0.50^d
10	Soil A1	0.00 ± 0.00^f	8.00 ± 0.01^g	24.0 ± 0.10^b
11	*Soil B	0.00 ± 0.00^f	25.0 ± 0.02^f	19.0 ± 0.00^e
12	Soil B	34.0 ± 0.00^a	50.0 ± 0.02^a	19.0 ± 0.10^e
13	Soil B1	10.0 ± 0.06^e	37.0 ± 0.01^b	40.0 ± 0.03^a
14	Soil 2	49.0 ± 0.00^h	25.0 ± 0.08^f	49.0 ± 0.01^h
15	Soil 7	35.0 ± 0.02^a	16.0 ± 0.04^h	35.0 ± 0.05^i

The superscripts represented significant different ($p < 0.05$).
RBBR – Remazol Brilliant Blue R, AZ – Azure B, CR – Congo Red

guaiacol and veratryl alcohols, only seven (27%) of the isolates degraded veratryl alcohol only. Four (15%) of the isolates could not grow on any of the alcohols.

Dye decolorization assay. The ligninolytic potential of 15 isolates that degraded both alcohols (Table I) was further confirmed with dye decolorization assay, and the results are shown in Table II. It is apparent that all 15 isolates successfully degraded Azure B, although at various degrees of efficiency. Meanwhile, 12 isolates degraded Remazol Brilliant Blue R only, and 13 degraded Congo Red only. Only 10 isolates effectively degraded all the tested dyes out of which isolate Old A2 (obtained from old cow dung) showed the highest ligninolytic activity when compared to the other isolates.

Qualitative determination of lignin peroxidase and laccase activity. All 10 isolates that displayed positive results on the tested dyes were qualitatively evaluated for lignin peroxidase and laccase activities. The results revealed that all the isolates possess a prolific capability to produce lignin peroxidase (Table III). However, all the isolates showed to be non-laccase producers.

Biochemical characteristics of the bacterial isolates. The results of the biochemical characteristics of the targeted 10 isolates with ligninolytic activity are presented in Table IV. The isolates showed positive catalase, starch hydrolysis, and indole tests. However, they could not utilize urease or citrate as a sole carbon source.

Sequencing the 16S rRNA gene of the isolates. 16S rDNA gene sequencing fingerprinting was per-

Table III
Lignin peroxidase and Laccase producing potential
of the isolated bacteria.

S/N	Bacterial code	Lignin peroxidase activity	Laccase activity
1	*Old A	+++	–
2	OOld A	+++	–
3	Old A2	+++	–
4	New A	+++	–
5	New B	+++	–
6	Saw A2	+++	–
7	Soil B	+++	–
8	Soil B1	+++	–
9	Soil 2	++	–
10	Soil 7	++	–

– – negative,
+ – weak,
++ – moderate,
+++ – strongly positive

formed the 10 isolates identified: seven *P. aeruginosa* isolates, two *Enterobacter* species (*Enterobacter kobei* and *Enterobacter xiangfangensis*) and one *Escherichia coli* isolate (Table V).

Lignin degradation capability study of the pure cultures. The results demonstrating the capability of the bacterial isolates to degrade and utilize kraft lignin are presented in Fig. 1. All the isolates showed appreciable levels of growth; it is noteworthy that the isolates' growth corresponded with their lignin degradation capability and the changes in the pH levels. *E. coli* and *P. aeruginosa* showed the most appreciable lignin degradation ability compared to the other isolates.

Quantitative assays for the lignin peroxidase activity. The qualitative lignin peroxidase activity in the recovered supernatant of the bacteria cultures is presented in Table VI. While all the isolates produced the enzyme, only *P. aeruginosa* (CP031449.2) and *E. coli* (LR0250096.1) could produce an appreciable quantity of lignin peroxidase (1.99×10^{-1} U/l). In addition, the

Table IV
Characteristics of ligninolytic bacteria from cow dung, sawdust, and soil samples.

Bacterial code	Catalase test	Oxidase test	Starch hydrolysis	Urease test	Indole test	Citrate test
*Old A	+	–	+	–	+	–
OOld A	+	+	+	–	+	–
Old A2	+	+	+	–	+	–
New A	+	+	+	–	+	–
New B	+	+	+	–	+	–
Saw A2	+	–	+	–	+	–
Soil B	+	–	+	–	+	–
Soil B1	+	–	+	–	+	–
Soil 2	+	+	+	–	+	–
Soil 7	+	–	+	–	+	–

– – negative, + – positive

Table V
Identification of the isolated bacterial cultures by 16S rDNA.

Bacterial name	Accession number (16S rRNA gene sequence)	Bacterial code
Pseudomonas aeruginosa	KR136350.1	Soil B
Pseudomonas aeruginosa	KT799669.1	*Old A
Pseudomonas aeruginosa	MF144461.1	OOld A
Pseudomonas aeruginosa	KR136350.1	New B
Pseudomonas aeruginosa	KR136350.1	New A
Pseudomonas aeruginosa	CP031449.2	Saw A2
Pseudomonas aeruginosa	KT799669.1	Soil B1
Escherichia coli	LR025096.1	Old A2
Enterobacter kobei	CP032897.1	Soil 7
Enterobacter xiangfangensis	MH304301.1	Soil 2

Table VI
Quantitative analysis for the lignin peroxidase activity
of the bacteria isolates.

S/N	Bacterial strains	Lignin peroxidase activity (U/l)
1	*Pseudomonas aeruginosa* (MF144461.1)	1.37×10^{-2a}
2	*Escherichia coli* (LR025096.1)	1.99×10^{-1b}
3	*Pseudomonas aeruginosa* (KR136350.1)	8.70×10^{-2c}
4	*Pseudomonas aeruginosa* (CP031449.2)	1.99×10^{-1b}
5	*Enterobacter xiangfangensis* (MH304301.1)	1.09×10^{-2d}
6	*Enterobacter kobei* (CP032897.1)	8.55×10^{-2c}

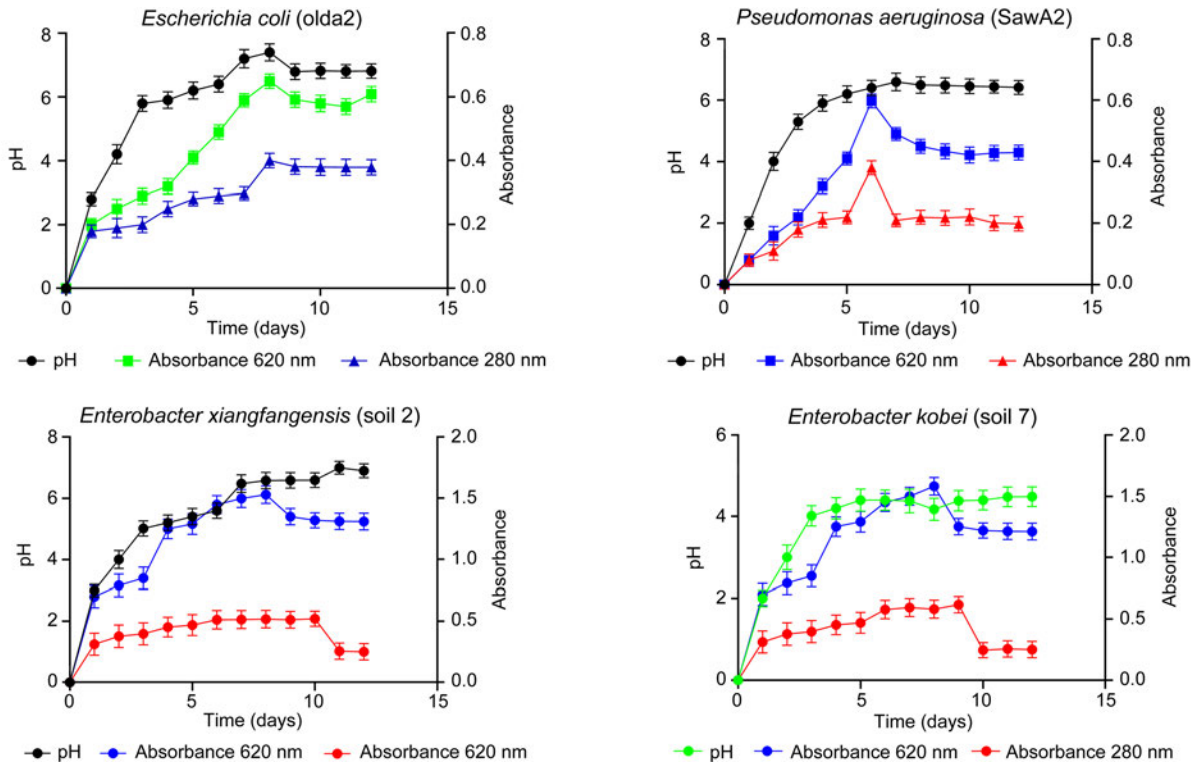The superscripts represented significant different ($p < 0.05$).

Fig. 1. Graphs of lignin degradation capability, pH variation, and growth of *E. coli*, *P. aeruginosa*, *E. kobei*, and *E. xiangfangensis*.
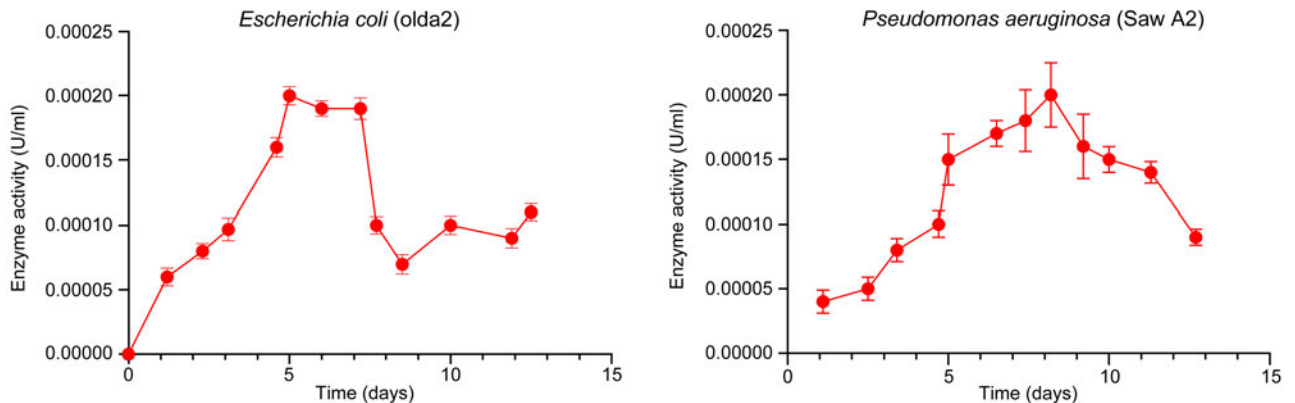

Fig. 2. Enzyme activity (the recovered supernatant) of *E. coli* (LR0250096), and *P. aeruginosa* (CP031449.2) on the lignin enriched medium.

two bacteria also exhibited reasonable exponential enzyme activities, with optimum activity occurring between 5 to 9 days of growth (Fig. 2).

Discussion

This study isolated twenty-six potential ligninolytic bacteria from samples collected in the Richards Bay environment. Most of the isolates were identified (Table SI) as *P. aeruginosa* (88%), *Enterobacter* spp. (8%), and *E. coli* (4%). These ligninolytic bacteria were all found to be γ-Proteobacteria. The phylogenetic tree, showing the relationship between the *P. aeruginosa* and the *Enterobacter hormaechei* in this study, and other

ligninolytic bacteria is presented in Fig. 3. The identified isolates all showed 99% similarity to the previously isolated strains (Xiong et al. 2020).

According to Lopez et al. (2006), using lignin as a carbon source for the selection of ligninolytic microorganisms is regarded as one of the most appropriate methods worldwide. However, the utilization of lignin model compounds such as veratryl and guaiacol alcohols, and the decolorization of industrial dyes as exhibited by the isolates (Table I and II), is the evidence that the colonies have the potential to utilize and degrade lignin (Husain 2006; Bandounas et al. 2011; Falade et al. 2017).

However, growth on polymeric lignin or lignin monomers is not necessarily a good measure of the ligninolytic potential. The ligninolytic potential, independent
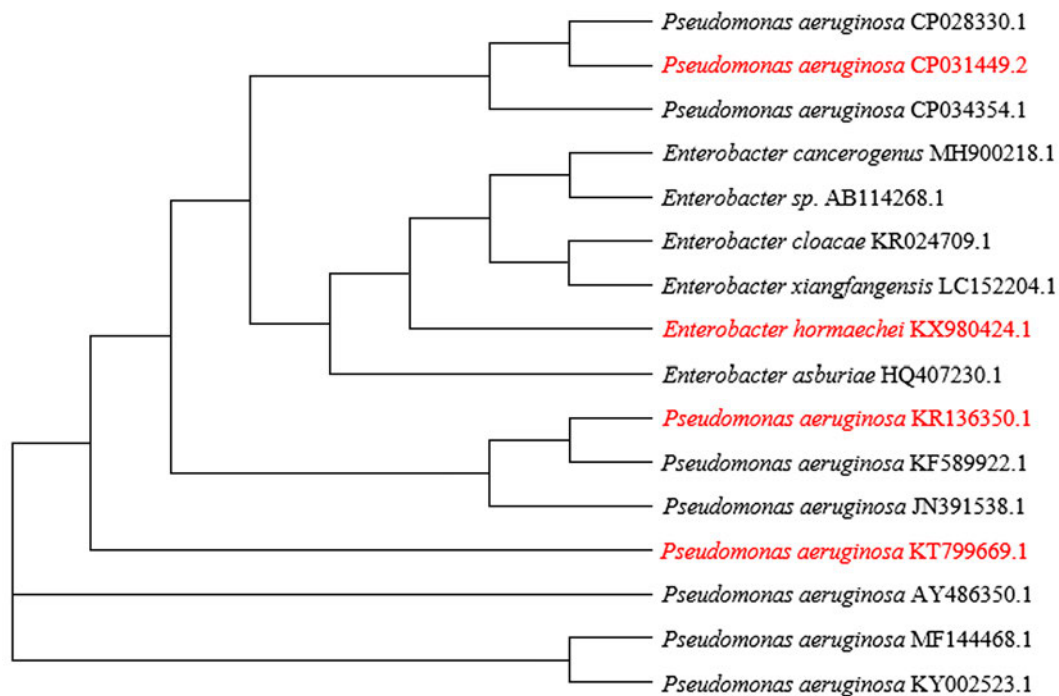
Fig. 3. The phylogenetic tree of the bacteria from this study (red) in relation to some other ligninolytic bacteria in the NCBI database.

from lignin utilization, was studied with the decolorization of synthetic lignin-like dyes (Falade et al. 2017). The selected organisms were able to decolorize Azure B. Although most LMEs can decolorize various dyes, previous studies (Falade et al. 2017) have revealed that Azure B decolorization is associated explicitly with high redox potential agents like lignin peroxidases (LiP). Other LMEs, such as laccase and manganese peroxidase-producing bacteria, cannot decolorize Azure B (AZ). Since fifteen of the isolated ligninolytic bacteria degraded AZ (Table II), it is apparent that these bacterial isolates were potential LiP producers. However, out of the 15 isolates, 10 isolates were able to decolorize Azure B only. These pure colonies gave a positive pyrogallol test for peroxidase, and the supernatant of cultures quantitatively revealed the activity of peroxidase (Arora and Gill 2011). Therefore, they were selected as potential lignin peroxidase producers, and this was confirmed by the production of a crude enzyme complex that contained a peroxidase, most likely, a lignin peroxidase. They were not laccase producers (Table III). All the selected isolates also showed high catalase and oxidase activities and were capable of hydrolyzing starch (Table IV).

The lignin degradation capabilities of these bacterial strains were further examined, and the evidence for ligninolytic activity was based on) the bacterial growth on lignin enriched medium and the lignin degradation. Most of the tested bacterial strains were able to degrade lignin. The isolated organisms were cultured on lignin (with 1% glucose), and after a period of cultivation, phenolic (or derived lignin monomers) appeared and

increased with growth in the culture. We believe that the organisms produced these phenolic compounds in the culture as a result of lignin (a polyphenolic complex) being degraded. We also agree that the lignin degradation should be monitored as directly as possible. However, synthetic dyes may mimic lignin (Sana et al. 2017). In this present study, the lignin degradation was prominent with *E. coli* LR0250096.1 and *P. aeruginosa* CP031449.2, as their growth increased with the degradation of lignin (Fig. 1). These results are consistent with literature reports on the ligninolytic activity of *Pseudomonas* sp. and *E. coli* species (Bandounas et al. 2011; Sana et al. 2017).

Interestingly, of 26 isolates obtained, ten were identified as true lignin peroxidase producers with *E. coli* LR0250096.1, and *P. aeruginosa* CP031449.2 considered the best lignin peroxidase producers, exhibiting activity of about 1.99×10^{-1} U/l (Table VI, Fig. 2). Other bacteria strains such as *Klebsiella pneumonia*, *Bacillus* species, *Citrobacter* species, *Serratia marcescens*, and *Pseudomanos* spp. have also been reported to have LMEs-producing potential (Zainith et al. 2019; Kumar and Chandra 2020).

Conclusion

This study attempted to isolate and characterize lignin-degrading bacteria capable of producing lignin peroxidase. The isolated *Pseudomonas* and *Enterobacter* species and *E. coli* were identified as true lignin peroxidase producers. The highest ligninolytic activity

exhibited by *P. aeruginosa* (CP031449.2) and *E. coli* (LR025096.1) suggests that these isolates could be exploited in the industrial treatment of lignin and detoxification of recalcitrant industrial dyes that invariably end up in the environment. For further studies, enzyme purification, enzyme's efficiency, and the mechanism of its action will be ascertained.

ORCID
Folusо O Osunsanmi https://orcid.org/0000-0002-1710-6966

Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Al-Dhabaan FA. Morphological, biochemical and molecular identification of petroleum hydrocarbons biodegradation bacteria isolated from oil polluted soil in Dhahran, Saud Arabia. Saudi J Biol Sci. 2019 Sep;26(6):1247–1252. https://doi.org/10.1016/j.sjbs.2018.05.029
Bandounas L, Wierckx NJ, de Winde JH, Ruijssenaars HJ. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. BMC Biotechnol. 2011 Oct;11:94.
https://doi.org/10.1186/1472-6750-11-94
Badr MS. Isolation and characterization of bacteria isolated from ice cream samples in Hyderabad, India. J Pure Appl Microbiol. 2018 Dec;12(4):2275–2282. https://doi.org/10.22207/JPAM.12.4.68
Bugg TD, Ahmad M, Hardiman EM, Singh R. The emerging role for bacteria in lignin degradation and bio-product formation. Curr Opin Biotechnol. 2011 Jun;22(3):394–400.
https://doi.org/10.1016/j.copbio.2010.10.009
Chaturvedi V, Verma P. An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. 3 Biotech. 2013 Oct;3(5):415–431.
https://doi.org/10.1007/s13205-013-0167-8
Datta, R, Kelkar, A, Baraniya D, Molaei A, Moulick A, Meena RS, Formanek P. Enzymatic degradation of lignin in soil: a review. Sustainability. 2017 Jul;9(7):1163. https://doi.org/10.3390/su9071163
de Gonzalo G, Colpa DI, Habib MH, Fraaije MW. Bacterial enzymes involved in lignin degradation. J Biotechnol. 2016 Oct;236:110–119. https://doi.org/10.1016/j.jbiotec.2016.08.011
Dicko M, Ferrari R, Tangthirasunun N, Gautier V, Lalanne C, Lamari F, Silar P. Lignin degradation and its use in signaling development by the coprophilous ascomycete *Podospora anserina*. J Fungi. 2020 Nov;6(4):278. https://doi.org/10.3390/jof6040278
Falade AO, Nwodo UU, Iweriebor BC, Green E, Mabinya LV, Okoh AI. Lignin peroxidase functionalities and prospective applications. MicrobiolgyOpen. 2017 Feb;6(1):e00394.
https://doi.org/10.1002/mbo3.394

Husain Q. Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review. Crit Rev Biotechnol. 2006 Oct–Dec;26(4):201–221. https://doi.org/10.1080/07388550600969936
Janusz G, Pawlik A, Sulej J, Swiderska-Burek U, Jarosz-Wilkolazka A, Paszczynski A. Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol Rev. 2017 Nov;41(6):941–962. https://doi.org/10.1093/femsre/fux049
Kameshwar AKS, Qin W. Qualitative and quantitative methods for isolation and characterization of lignin-modifying enzymes secreted by microorganisms. Bio-Energy Research. 2017 Mar;10(1):248–266.
http://doi.org/10.1007/s12155-016-9784-5
Kumar A, Chandra R. Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. Heliyon. 2020 Feb;6(2):e03170. https://doi.org/10.1016/j.heliyon.2020.e03170
Lopez MJ, Guisado G, Vargas-Garcia MC, Suárez-Estrella F, Moreno J. Decolorization of industrial dyes by ligninolytic microorganisms isolated from composting environment. Enzyme Microb Technol. 2006 Dec;40(1):42–45.
https://doi.org/10.1016/j.enzmictec.2005.10.035
Okello NO, Okello TW, Zunckel M. Public perceptions of air quality status and suggestions for improvement: The case of Richards Bay and its surroundings, uMhlathuze Local Municipality, South Africa. Clean Air J. 2022 Jun;30(1).
https://doi.org/10.17159/caj/2020/30/1.8001
Sana TG, Lugo KA, Monack DM. T6SS: The bacterial "fight club" in the host gut. PLoS Pathog. 2017 Jun 8;13(6):e1006325.
https://doi.org/10.1371/journal.ppat.1006325
Sasikumar V, Priya V, Shankar CS, Sekar SD. Isolation and preliminary screening of lignin degrading microbes. J Acad Ind Res. 2014 Nov;3(6):291–294.
Su Y, Yu X, Sun Y, Wang G, Chen H, Chen G. Evaluation of screened lignin-degrading fungi for the biological pre-treatment of corn stover. Sci Rep. 2018 Mar;8:5385–5396.
https://doi.org/10.1038/s41598-018-23626-6
Taylor CR, Hardiman EM, Ahmad M, Sainsbury PD, Norris PR, Bugg TD. Isolation of bacterial strains able to metabolize lignin from screening of environmental samples. J Appl Microbiol. 2012 Sep;113(3):521–530. https://doi.org/10.1111/j.1365-2672.2012.05352.x
Tian JH, Pourcher AM, Peu P. Isolation of bacterial strains able to metabolize lignin and lignin-related compounds. Lett Appl Microbiol. 2016 Jul;63(1):30–37.
https://doi.org/10.1111/lam.12581
Wang L, Nie Y, Tang YQ, Song XM, Cao K, Sun LZ, Wang ZJ, Wu XL. Diverse bacteria with lignin degrading potentials isolated from two ranks of coal. Front Microbiol. 2016 Sep;7:1428.
https://doi.org/10.3389/fmicb.2016.01428
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 Sep;69(3):339–347.
https://doi.org/10.33073/pjm-2020-037
Zainith S, Purchase D, Saratale GD, Ferreira LFR, Bilal M, Bharagava RN. Isolation and characterization of lignin-degrading bacterium *Bacillus aryabhattai* from pulp and paper mill wastewater and evaluation of its lignin-degrading potential. 3 Biotech. 2019 Mar;9(3):92. https://doi.org/10.1007/s13205-019-1631-x
Zimmermann W. Degradation of lignin by bacteria. J Biotechnol. 1990 Feb;13(2–3): 119–130.
https://doi.org/10.1016/0168-1656(90)90098-V

Supplementary materials are available on the journal's website.