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Original Research

Peroxidases Produced by New Ligninolytic *Bacillus* strains Isolated from Marsh and Grassland Decolourized Anthraquinone and Azo Dyes

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Abstract

The biotechnological relevance of ligninolytic organisms remains topical and may remain so in the foreseeable future. The enzyme battery produced by ligninolytic bacteria, including Bacillus species, has shown immense industrial significance. Consequently, peroxidases produced by newly isolated ligninolytic Bacillus strains from the marsh and grassland in Hogsback forest reserve of the Eastern Cape Province of South Africa were evaluated for decolourization of anthraquinone (Remazol Brilliant Blue R-RBBR) and azo (Congo Red-CR) dyes. Maximum dye decolourization was observed with the peroxidase from *Bacillus* sp. NWODO-3: CR (69.89±2.64 %) and RBBR (72.12±0.38 %). Dye decolourization readings for peroxidases from the other Bacillus strains were CR: 55.06±5.48 %, RBBR: 70.45±0.0 % (*Bacillus* sp. MABINYA-1), 42.62±5.55 % and 42.42±4.82 % against CR for *Bacillus* sp. MABINYA-2 and *Bacillus* sp. FALADE-1, respectively. RBBR was less susceptible to the attack by crude peroxidase produced by *Bacillus* sp. MABINYA-2 and *Bacillus* sp. FALADE-1 as the dye decolourization activities observed were 4.91±0.36 % and 1.19±0.0 %, respectively. These results suggest the industrial relevance of peroxidases from the new ligninolytic Bacillus strains in bioremediation.

Keywords: bioremediation, dye decolourization, ligninolytic bacteria, lignin-modifying enzymes

Introduction

Lignin constitutes a major hassle in the conversion of lignocellulosic-carbonoclastic materials to value-added products. An example of such a valorization process is the utilization of lignocellulose as feedstock for biofuel production. Hence, efficient delignification of lignocellulosic biomass is imperative. The biological approach to delignification involves the use of microbes or microbial products such as enzymes [1], perhaps due to the mild reaction conditions, increased yield and low energy requirements [2].

The lignin degradation potentials of several fungal species have been studied often and their effectiveness vastly documented [3-6]. However, the emergence of some classes of bacteria as ligninolytic microbes is quite nascent and requires an adept combination of biotechnological tools to unravel the immense potentials

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possessed by the microbes. Some classes of bacteria whose emerging role in lignin degradation has been reported include actinomycetes, α-proteobacteria and γ-proteobactria [7]. Similarly, members of the Bacillus genus have been reported to possess ligninolytic abilities [8, 9], and some of these members include *Bacillus* sp. LD003 [8], *Bacillus* sp. CS-1 and *Bacillus* sp. CS-2 [9], and *Bacillus ligniniphilus* L1 [10]. The lignin-degradation activities shown by these microbes have been partly attributed to the production of oxidative enzymes, predominantly peroxidases [11].

Besides the delignification activity of peroxidases, the unique oxidative properties of the enzymes have seen other applications, including dye decolourization and xenobiotic degradation emerge [1]. The high redox potentials of microbial peroxidases and the ability to oxidize recalcitrant phenolic compounds underscore the relevance of these microbes to humanity. Synthetic dyes are recalcitrant to degradation and thus constitute an environmental nuisance upon discharge as industrial effluent. Consequently, the effective removal from the environment through partial or complete degradation remains a challenge.

A physico-chemical treatment approach has been applied for removal of the noxious substances (dye) from the environment. However, these approaches, including adsorption and flocculation, have high limitations and are inefficient [12]. The other downside of these techniques includes high operational cost and the creation of secondary pollution [13]. The biological approach, which includes the use of microorganisms and sub-molecules such as enzymes in the degradation of dye in effluents, has been effective and is saddled with fewer limitations [1]. Hence, the exploration of microbial diversity, besides the already known species, for dye decolourization potentials becomes imperative. Consequently, the ligninolytic bacteria species isolated from marsh and grassland of Hogsback forest reserve were evaluated for peroxidase production and the decolourization of dyes with varied arene substituents.

Material and Methods

Sampling Site and Sample Collection

Samples of decaying wood, soil, sediment, moist rock scrapings and water were collected from marsh and grassland in Hogsback forest reserve of the Raymond Mhlaba Municipality, Eastern Cape, South Africa and transported on ice to the Applied and Environmental Microbiology Research Group (AEMREG) Laboratory, University of Fort Hare, Alice, South Africa for analysis.

Hogsback lies on the Amathole Mountains of the Eastern Cape Province of South Africa, with geographical coordinates S32°.598' E26°.938'. The pristine forest reserve has waterfalls, and trout fishing is common. The Hogsback forest reserve has been described as the second-largest per unit area in South Africa comprising indigenous forests, with pockets of Afromontane rain forests covering a large area. Additionally, marsh and grassland are other features of Hogsback.

Isolation of Ligninolytic Bacteria

Lignin degrading bacteria were isolated using standard techniques [14] with slight modification as previously described by Falade et al. [11].

Lignin Degradation Assay

The isolates were further evaluated for ligninolytic potential using the modified method of Taylor et al. [15] as described by Falade et al. [11] in a previous study.

Bacteria Identification

Isolates with promising ligninolytic activity were characterized using 16S rRNA gene sequence analysis as previously described elsewhere [11]. Phylogenetic analysis was conducted by the neighbour-joining method using MEGA 7.0.21 [16].

Peroxidase Activity Screening

Qualitative peroxidase activity was determined as described by López et al [17]. Briefly, isolates were inoculated in nutrient agar and incubated at 30°C for 48 h. Thereafter, 30 µL of equal parts of 0.4% (v/v) hydrogen peroxide (H_2O_2) and 1% pyrogallol in water was added to the colony. Colonies with yellow-brown colour were recorded as positive.

Peroxidase Production and Enzyme Preparation

Peroxidase was produced in a submerged fermentation system using the method described by Falade et al. [11]. Fermented broth was centrifuged (15000 rpm) for 10 min at 4ºC using benchtop cold centrifuge (SIGMA 1-14K). The supernatant was subsequently utilized for peroxidase assay.

Peroxidase Activity Quantitation

Peroxidase activity was quantified through the measurement of the rate of hydrogen peroxide-dependent oxidation of pyrogallol to purpurogallin in line with standard methods [18, 19], but with slight modification as reported elsewhere [11].

Dye Decolourization Assay

Lignin-mimicking dyes Remazol Brilliant Blue R (RBBR) and Congo Red (CR) were assessed for decolourization [8]. About 5 µL of an 18 h culture was aseptically inoculated in dye-agar with the following composition: K_2HPO_4 (4.55 g/L), KH_2PO_4

(0.53 g/L), $MgSO_4$ (0.5 g/L), NH_4NO_3 (5 g/L), yeast extract (0.1 g/L), glycerol (40 mM), dye (100 mg/L RBBR; 50 mg/L CR), and agar (15 g/L). The cultures were incubated at 30ºC and examined daily for growth and development of decolourization zones. The total incubation time was 168 h.

Furthermore, the rate of decolourization of the dyes was similarly evaluated in line with the modified method of Kalyani et al. [20]. The reaction mixture (400 µL) contained dye (100 mg/L), potassium phosphate buffer (0.1 M, pH 6) and culture supernatant (crude enzyme). The reaction was initiated via the addition of 0.5% hydrogen peroxide (30% w/w) and subsequently incubated at 25±2ºC for 30 min. Absorbance was read at 490 nm and 590 nm being the maximum wavelength for CR and RBBR, respectively [21], using SynergyMx 96-well microtitre plate reader (BioTeK Instruments). A reaction mixture without the crude enzyme served as the control. Dye decolourization was measured by monitoring the decrease in absorbance of each dye and expressed as percentage decolourization:

$$\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100\%$$

Data Analysis

All data, including absorbance readings and halo zones, were subjected to analysis of variance (ANOVA) using GraphPad Prism 7 at a 5% (*P≤0.05*) confidence interval. Where applicable, results were presented as mean values±standard deviation (STD).

Results and Discussion

Isolation of Ligninolytic Bacteria

A total of 49 ligninolytic bacteria were isolated from marsh and grassland in Hogsback located in Raymond Mhlaba Municipality, Eastern Cape, South Africa (Table 1). The isolates were presumed to be ligninolytic due to their ability to utilize alkali lignin as the sole carbon source in an enrichment medium. Isolation of bacteria with ligninolytic potential from different samples has previously been reported [8, 9, 15, 22, 23]. Hemati et al. [24] reported the isolation of lignocellulolytic bacteria from composts and soils in Iran. Naz [25] isolated and characterized ligninolytic bacteria from Kuthrel Agro Field in India. Also, Lai et al. [26] reported the isolation of thermophilic ligninolytic bacteria from oil palm empty-fruit bunch compost in Malaysia. However, this is the first report of isolation of ligninolytic bacteria from Hogsback forest reserve in South Africa.

Organisms with ligninolytic potential are promising candidates for delignification of feedstock for bioethanol production [1]. They are as well significant in the valorization of lignocellulose to other value-added products. Besides the imperativeness of ligninolytic organisms in the valorization of lignocellulosic biomass, they also have the potential for the production of ligninolytic enzymes, including peroxidases and laccase [25, 27], with significant prospective industrial applications. Furthermore, ligninolytic bacteria have shown prospect in bioremediation. For instance, Hooda et al. [28] employed ligninolytic bacteria for the treatment of pulp and paper mill effluent. The study established that *Brevibacillus agri*, a lignin-degrading bacteria, was able to reduce the colour and chemical oxygen demand (COD) of the treated wastewater. Similarly, Raj et al. [29] reported the bioremediation and toxicity reduction potential of ligninolytic bacteria. Specifically, treatment of pulp and paper mill effluent by *Paenibacillus* sp. LD-1, a ligninolytic organism, showed that the COD and BOD (biological oxygen demand) of the treated effluent was reduced by 78 and 83%, respectively, after 144 h, while about an 86% reduction in phenol content was recorded after the treatment. It is therefore evident that ligninolytic bacteria are promising candidates for the treatment of wastewater effluent discharged from pulp and paper mills as lignin is a major component of the effluent [29].

Lignin Degradation Potential

Besides the utilization of alkali lignin as the sole carbon source, the ligninolytic potential of the isolates was also assessed using their ability to utilize and degrade some lignin model compounds, including 2-Methoxyphenol (guaiacol) and 3,4-Dimethoxybenzyl alcohol (veratryl alcohol). The use of different lignin model compounds and aromatic monomers such as benzaldehyde, benzoic acid, guaiacol, veratryl alcohol, catechol, syringol, vanillic acid, and vanillin for screening of organisms for ligninolytic potentials has been reported [8, 11, 15, 30]. Table 2 showed that 13 isolates (26.5 %) were able to utilize and degrade both guaiacol and veratryl alcohol. The degree of degradation of the compounds by the isolates was measured by the diameter of the halo zone with isolate HBB1A having the highest zone of degradation for the two compounds (guaiacol: 42.0±0.0 mm, veratryl alcohol: 41.0±1.0 mm), while HBB29C had the lowest zone of degradation (guaiacol: 9.0±1.0 mm, veratryl alcohol: 7.0±1.0 mm). The degraded part was revealed as a clear zone around the bacterial colony on plate while the brown colouration of the un-degraded part resulted from the interaction of hydrogen iodide (generated from dissolving potassium iodide and iodine in water) with the two aromatic alcohols in the presence of oxygen after being flooded with Gram's iodine [11] at 168 h of incubation. The clear zone around the bacterial colony in this study could either be a result of utilization of the compounds or their degradation by extracellular enzymes, including lignin-modifying enzymes. Furthermore, the capability of bacterial strains to utilize

Table 1. Colonial morphology of isolated bacteria on alkali lignin plate.

S/N	Isolate ID	Isolation source	Form	Pigmentation	Elevation	Margin
1.	HBB1A	Decaying wood	Circular	Creamy	Raised	Entire
2.	HBB1B	Decaying wood	Circular	Yellowish	Raised	Entire
3.	HBB2A	Decaying wood	Circular	Transparent	Flat	Undulate
4.	HBB2B	Decaying wood	Circular	Milky	Raised	Entire
5.	HBB4A	Soil	Circular	Creamy	Flat	Entire
6.	HBB4B	Soil	Irregular	Creamy	Flat	Undulate
7.	HBB4C	Soil	Irregular	Transparent	Flat	Undulate
8.	HBB5A	Soil	Irregular	Creamy	Flat	Undulate
9.	HBB5B	Soil	Irregular	Creamy	Flat	Undulate
10.	HBB6A	Sediment	Circular	Transparent	Raised	Entire
11.	HBB7A	Sediment	Irregular	Creamy	Flat	Undulate
12.	HBB8A	Sediment	Irregular	Brownish	Flat	Undulate
13.	HBB8B	Sediment	Circular	Transparent	Raised	Entire
14.	HBB9A	Soil scrapping	Circular	Creamy	Raised	Entire
15.	HBB10A	Soil scrapping	Circular	Creamy	Raised	Entire
16.	HBB11A	Soil scrapping	Circular	Creamy	Raised	Entire
17.	HBB11B	Soil scrapping	Circular	Creamy	Flat	Entire
18.	HBB12A	Decaying wood	Circular	Creamy	Raised	Entire
19.	HBB13A	Soil scrapping	Circular	Creamy	Raised	Entire
20.	HBB13B	Soil scrapping	Circular	Yellowish	Raised	Entire
21.	HBB13C	Soil scrapping	Circular	Whitish	Raised	Entire
22.	HBB14A	Decaying wood	Irregular	Creamy	Flat	Undulate
23.	HBB15A	Moist soil	Irregular	Whitish	Flat	Undulate
24.	HBB16A	Decaying wood	Irregular	Milky	Flat	Undulate
25.	HBB18A	Decayed wood	Circular	Yellowish	Raised	Entire
26.	HBB19A	Water	Circular	Creamy	Raised	Entire
27.	HBB20A	Water	Circular	Creamy	Raised	Entire
28.	HBB21A	Sediment	Irregular	Whitish	Flat	Undulate
29.	HBB21B	Sediment	Circular	Yellowish	Raised	Entire
30.	HBB22A	Water	Circular	Yellowish	Raised	Entire
31.	HBB22B	Water	Circular	Creamy	Raised	Entire
32.	HBB23A	Sediment	Circular	Yellowish	Raised	Entire
33.	HBB23B	Sediment	Circular	Creamy	Raised	Entire
34.	HBB24A	Sediment	Circular	Yellowish	Raised	Entire
35.	HBB25A	Rock scrapping	Circular	Creamy	Raised	Entire
36.	HBB26A	Waterfall sediment	Circular	Yellowish	Flat	Entire
37.	HBB27A	Waterfall sediment	Circular	Yellowish	Raised	Entire
38.	HBB27B	Waterfall sediment	Irregular	Creamy	Flat	Undulate
39.	HBB28A	Rock scrapping	Circular	Yellowish	Raised	Entire

Table 1. Continued.

40.	HBB29A	Rock scrapping	Irregular	Creamy	Flat	Undulate
41.	HBB29B	Rock scrapping	Circular	Yellowish	Raised	Entire
42.	HBB29C	Rock scrapping	Circular	Creamy	Raised	Entire
43.	HBB30A	Decayed wood	Circular	Yellowish	Raised	Entire
44.	HBB31A	Soil particles	Irregular	Transparent	Flat	Undulate
45.	HBB32A	Sediment	Circular	Creamy	Flat	Entire
46.	HBB32B	Sediment	Irregular	Creamy	Flat	Undulate
47.	HBB34A	Decayed wood	Circular	Creamy	Raised	Entire
48.	HBB35A	Moist decayed wood	Irregular	Transparent	Flat	Undulate
49.	HBB35B	Moist decayed wood	Irregular	Creamy	Flat	Undulate

and degrade guaiacol and veratryl alcohol as observed in this study may also be attributed in part to the activity of alcohol dehydrogenase, which is required to oxidize aromatic alcohols. This finding is in agreement with the work of Tian et al. [31], where ligninolytic bacteria belonging to Serratia, Pseudomonas, Stenotrophomonas and Mesorhizobium species were able to mineralize guaiacol, veratryl alcohol and biphenyl. However, our results contradict the findings of Bandounas et al. [8], who reported that certain bacterial strains including *Pandoraea norimbergensis* LD001, *Pseudomonas* sp. LD002 and *Bacillus* sp. LD003 were not able to utilize or degrade the alcoholic forms of the aromatic monomers investigated, including guaiacol and veratryl alcohol. Moreover, Ravi et al. [30] reported the ability of a *Pseudomonas* sp. to utilize ferulate, *p*-coumarate, benzoate and vanillin. However, the organism could not utilize or grow on syringate and guaiacol. Likewise, *Burkholderia* sp. strain CCA53 did not utilize guaiacol and veratryl alcohol [32]. Nevertheless, it was able to utilize some other lignin model aromatic monomers, including benzaldehyde, benzoic acid, catechol, syringol, vanillin, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol. The seeming discrepancies in the utilization of lignin model compounds by the different bacteria might be due to ecological reasons as strains studied by Bandounas et al. [8] were isolated from soil beneath decomposing wood logs in the Netherlands, the bacteria from the study of Ravi et al. [30] was isolated from Baltic sea sediments, and Akita et al. [32] isolated their ligninolytic bacteria from soil samples collected in Japan. The bacterial isolates used in this study were from different environments in South Africa. In order to clearly understand these discrepancies, the metagenomics analysis of the samples is imperative.

Table 2. Ligninolytic potential of bacterial isolates.

S/N	Positive isolates	Diameter of halo zone for GA (mm)	Diameter of halo zone for VA (mm)
1.	HBB1A	42.0 ± 0.0^{a}	41.0 ± 1.0^{a}
2.	HBB1B	40.0 ± 2.0^{a}	39.0 ± 1.0^{a}
3.	HBB4A	24.0 ± 0.0^{b}	23.0 ± 1.0^{b}
4.	HBB5A	26.0 ± 0.0^{c}	29.0 ± 1.0^{c}
5.	HBB5B	20.0 ± 0.0^{d}	25.0 ± 1.0^{d}
6.	HBB7A	22.0 ± 2.0^{d}	32.0 ± 0.0^{c}
7.	HBB10A	12.0 ± 2.0^{e}	13.0 ± 1.0^{e}
8.	HBB11B	18.0 ± 2.0^{f}	20.0 ± 0.0^{f}
9.	HBB22A	31.0 ± 1.0^{g}	21.0 ± 1.0^{f}
10.	HBB29A	31.0 ± 1.0^{g}	34.0 ± 0.0^{g}
11.	HBB29B	30.0 ± 2.0^{g}	34.0 ± 0.0^{g}
12.	HBB29C	9.0 ± 1.0^{h}	7.0 ± 1.0^{h}
13.	HBB30A	17.0 ± 1.0^{i}	10.0 ± 0.0^{i}

GA: Guaiacol; VA: Veratryl Alcohol. Values represent mean±standard deviation, number of replicate, n = 3. Values with the same superscript letter along the same column are not significantly different ($P>0.05$).

Bacterial Identity

Four bacterial isolates with promising ligninolytic potential (HBB5A, HBB5B, HBB7A and HBB29A) were identified using 16S rRNA gene sequence analysis and the results showed that all the isolates belong to Bacillus genus. The results of the BLAST search of the respective nucleotide sequences in the NCBI database revealed that HBB5A, HBB5B and HBB29A had 99% similarity to *Bacillus lentus* strain FJAT-10603 (JN450800), *Bacillus* sp. strain NC62 (KY454505) and *Bacillus* sp. strain FJAT-25753 (KR077842), respectively. However, HBB7A was 100% similar to *Bacillus* sp. strain HP5F2 (KM187486) and [*Brevibacterium*] *frigoritolerans* strain Hb-1 (KC139406), as it also showed 99% similarity to several strains of Bacillu*s* species. The respective 16S rRNA gene sequences of the identified organisms are available in the NCBI database with the following

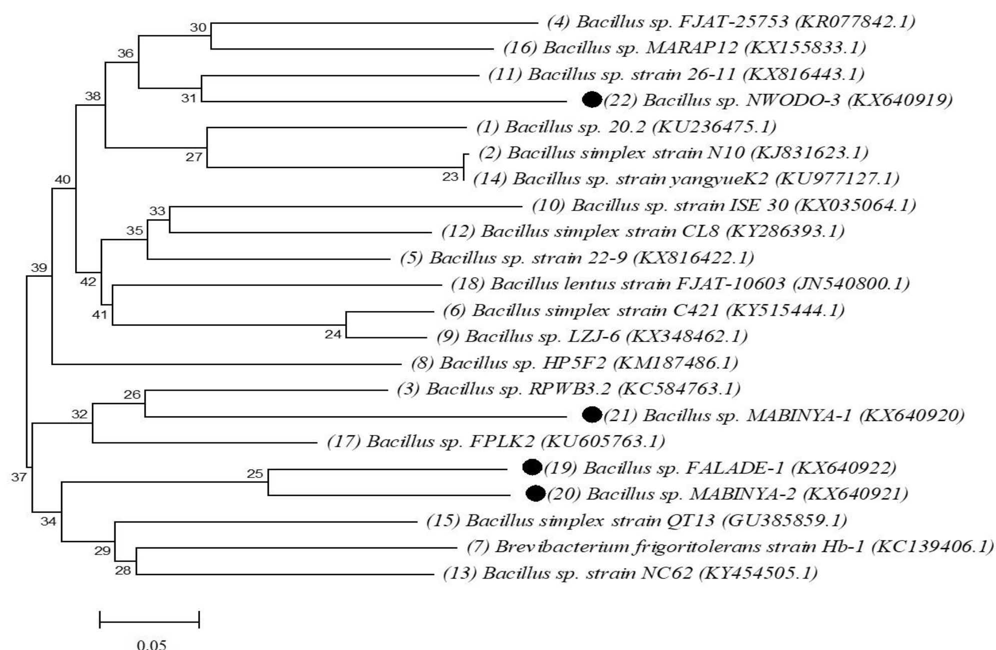

Fig. 1. Phylogenetic tree showing the evolutionary relationships of ligninolytic Bacillus strains in this study and selected Bacillus species in the NCBI database. The ligninolytic bacteria isolated and sequenced in this study are shown in black tips with ID Numbers 19-22. The bootstrap values of 1000 replicates are shown next to the branches while accession numbers are indicated in parentheses.

names and accession numbers: HBB5A: *Bacillus* sp. strain NWODO-3 (KX640919), HBB5B: *Bacillus* sp. strain MABINYA-1 (KX640920), HBB7A: *Bacillus* sp. strain MABINYA-2 (KX640921) and HBB29A: *Bacillus* sp. strain FALADE-1 (KX640922). The unrooted phylogenetic tree indicating the evolutionary relationships of the Bacillus strains in this study with other Bacillus species available in the NCBI database is shown in Fig. 1. The evolutionary analysis showed that strains FALADE-1 and MABINY-2 are more closely related as they cluster in the same clade.

The identity of these organisms as *Bacillus* sp. further confirms the emerging role of Bacillus species in lignin degradation. This finding is consistent with previous studies on the ligninolytic activities of Bacillus species where Bandounas et al. [8] reported the ligninolytic potential of *Bacillus* sp. LD003 isolated from soil in Netherlands. Also, Chang et al. [9] reported the lignin-degrading activity of *Bacillus* sp. CS-1 and *Bacillus* sp. CS-2 from forest soils in Japan. This is also corroborated by Zhu et al. [10], who documented the lignin degradation potential of *Bacillus ligniniphilus* L1 from sea sediment in South China. Furthermore, the results of Naz [25] have also implicated Bacillus species in lignin degradation activity. The emerging ligninolytic activity of Bacillus species confers on this class of bacteria the potential for application in biological delignification, consequently enhancing valorization of lignocellulosic biomass to value-added products of economic importance such as biofuel.

Peroxidase Production by Novel Ligninolytic *Bacillus* species

Bacillus species have been described as one of the most significant industrial producers of enzymes, partly owing to their capacity to produce a large amount of extracellular enzymes [33]. To assess the potential of the newly isolated ligninolytic Bacillus species to produce extracellular peroxidases, the qualitative peroxidase

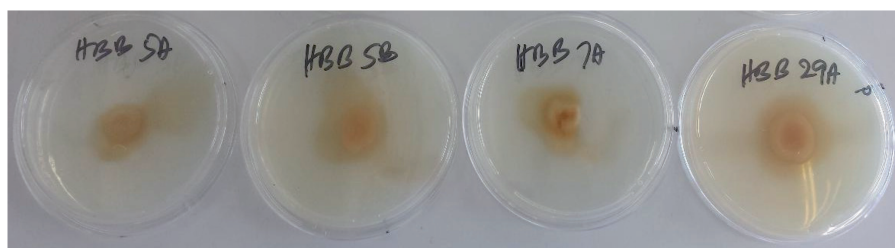

Fig. 2. Qualitative peroxidase activity screening. HBB5A: *Bacillus* sp. NWODO-3, HBB5B: *Bacillus* sp. MABINYA-1, HBB7A: *Bacillus* sp. MABINYA-2, HBB29A: *Bacillus* sp. FALADE-1.

Table 3. Peroxidase production by novel ligninolytic Bacillus species.

S/N	Organisms	Peroxidase activity (U mL^{-1})
1.	*Bacillus* sp. NWODO-3	3.03
2.	*Bacillus* sp. MABINYA-1	1.52
3.	*Bacillus* sp. MABINYA-2	4.08
4.	*Bacillus* sp. FALADE-1	6.53

activity of the organisms was determined. The results revealed that all the ligninolytic Bacillus species assessed showed peroxidase activity on plate (Fig. 2) with the presence of yellowish-brown colouration on the bacterial colony after its reaction with 0.4% (v/v) hydrogen peroxide (H_2O_2) and 1% pyrogallol at 48 h of incubation on nutrient agar [17]. To further evaluate the peroxidase production potentials of these organisms, the level of extracellular peroxidase produced by the ligninolytic Bacillus species was determined using a quantitative assay as described in the method, and the result is presented in Table 3. *Bacillus* sp. FALADE-1 exhibited the highest potential for peroxidase production with 6.53 U mL^{-1} followed by *Bacillus* sp. MABINYA-2 and *Bacillus* sp. NWODO-3 with 4.08 U mL^{-1} and 3.03 U mL^{-1} of peroxidase, respectively, while *Bacillus* sp. MABINYA-1 produced 1.52 U mL^{-1} of peroxidase. However, all the ligninolytic Bacillus species in this study showed promising potential for peroxidase production. The peroxidase yield by these organisms is higher than what was reported by Rao and Kavya [34] and Musengi et al. [35], where *Bacillus subtilis* and *Streptomyces* sp. BSII#1 produced 0.00045 U mL^{-1} and 1.30 U mL^{-1} of peroxidase, respectively. This finding is consistent with previous related studies that have reported the potential production of different lignin-modifying enzymes by various ligninolytic bacteria. Min et al. [36] extracted a dye-decolourizing peroxidase from *Bacillus subtilis* KCTC2023, a ligninolytic bacteria. Moreover, Chantarasiri and Boontanom [27] in their study reported the potential of ligninolytic *Lysinibacillus sphaericus* JD1103 to produce lignin peroxidase. In one of our previous studies, we also reported the peroxidase production potential of two ligninolytic proteobacteria: *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 [11]. Similarly, Xu et al. [37] recorded excellent lignin peroxidase and manganese peroxidase activities in *Pseudomonas putida* NX-1 with kraft lignin degradation activity.

Dye Decolourization

Decolourization of lignin-like dyes such as Azure B, Methylene Blue, Toluidine Blue O, Malachite Green, Indigo Carmine, RBBR, and CR, etc., has been used to determine the ligninolytic potential of bacteria [8]. To further confirm the ligninolytic potential of the isolates, the 13 positive isolates for the degradation of lignin model compounds (guaiacol and veratryl alcohol) were evaluated for their abilities to decolourize two ligninolytic indicator dyes: RBBR and CR, with different arene substituent attachment positions.

RBBR is a recalcitrant anthraquinone dye with its aromatic substituent attached at the *para* position of its structural backbone (anthraquinone), while CR is an azo dye having its two azo groups (-N=N-) that serve as the chromophore attached at the *ortho* position [11]. The position of attachment of the various arene rings may probably contribute to the recalcitrance of synthetic dyes to degradation. Consequently, degradation of dyes with *ortho, meta* and *para* arene substituents would suffice as a novel mechanism of dye degradation.

The results of the qualitative assessment of the bacterial isolates for dye decolourization as revealed in Table 4 showed 5 isolates (38.46 %) decolourized RBBR, while 11 isolates (84.62 %) were positive for decolourization of CR within 72 h. This finding suggests that CR (azo dye) is more susceptible to decolourization than RBBR, an athraquinone dye. However, only *Bacillus* sp. NWODO-3, *Bacillus* sp. MABINYA-1, *Bacillus* sp. MABINYA-2 and *Bacillus* sp. FALADE-1 were able to decolourize both RBBR and CR in this study. The dye decolourization observed in this study could be a result of enzymatic oxidation by extracellular enzymes secreted by the bacteria. Moreover, decolourization of CR and RBBR by the Bacillus species also indicate ligninase activity [24, 38], which further justifies the peroxidase production potential of the studied organisms. Other ligninolytic bacteria species with dye decolourization potential have been reported. In a study by Tian et al. [31], the authors

Table 4. Qualitative assessment of bacteria for dye decolourization

S/N	Isolates	RBBR	CR
1.	HBB1A	-	+
2.	HBB1B	-	+
3.	HBB4A	-	+
4.	HBB5A	+	+
5.	HBB5B	+	+
6.	HBB7A	+	+
7.	HBB10A	+	-
8.	HBB11B	-	+
9.	HBB22A	-	+
10.	HBB29A	+	+
11.	HBB29B	-	-
12.	HBB29C	-	+
13.	HBB30A	-	+

+: positive; -: negative; RBBR: Remazol Brilliant Blue R; CR: Congo Red.

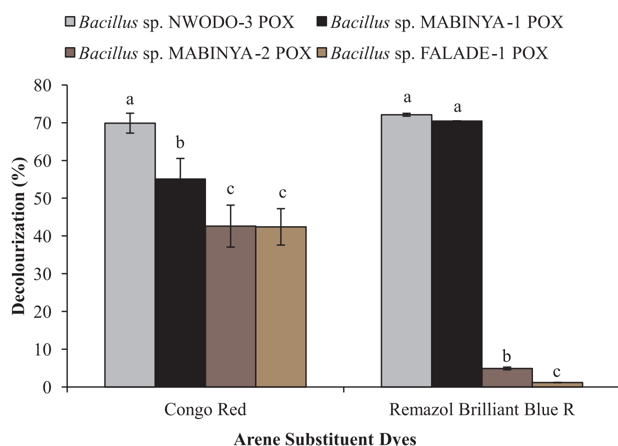

Fig. 3. Decolourization of anthraquinone and azo dyes by peroxidases from new ligninolytic Bacillus strains. Error bars with the same alphabet are not significantly different (*P>0.05*). POX: Peroxidase.

reported the decolourization of methylene blue and RBBR by ligninolytic *Serratia* sp. In another study by Chantarasiri and Boontanom [27], CR and RBBR were also decolourized by *Lysinibacillus sphaericus* JD1103, a ligninolytic bacteria isolated from wetlands in Thailand.

Nevertheless, enzyme-based dye decolourization is of greater interest, partly due to its efficiency, greater specificity and non-dependence on the growth rates of organisms [20, 39]. The involvement of microbial enzymes including peroxidases, laccases and azo reductase in biodegradation of dyes has been suggested [20]. Moreover, crude and purified forms of bacterial peroxidase have been used in dye decolourization [40, 41]. However, considering the cost of enzyme purification, the use of crude enzyme is being encouraged. Therefore, the potential activity of crude peroxidase produced by the new ligninolytic Bacillus species in dye decolourization was evaluated by incubating the culture supernatant from the organisms with the dyes (CR and RBBR) at 25±2ºC for 30 min in a reaction mixture containing phosphate buffer (pH 6) and hydrogen peroxide. The results (Fig. 3) revealed that crude peroxidase from *Bacillus* sp. NWODO-3 showed the highest decolourization activity for both CR (69.89±2.64 %) and RBBR (72.12±0.38 %), followed by peroxidase from *Bacillus* sp. MABINYA-1 (CR: 55.06±5.48 %, RBBR: 70.45±0.0 %). However, there was no significant difference (*P<0.05*) in the decolourization of CR by peroxidases from *Bacillus* sp. MABINYA-2 (42.62±5.55 %) and *Bacillus* sp. FALADE-1 (42.42±4.82 %). Although there was a significant difference (*P<0.05*) in the decolourization of RBBR by peroxidases from *Bacillus* sp. MABINYA-2 (4.91±0.36 %) and *Bacillus* sp. FALADE-1 (1.19±0.0 %), their activities were insignificant. The disparity observed in the decolourization of CR and RBBR by peroxidases from the Bacillus species may probably be due to the structural variation of the dyes. The result indicates that crude peroxidases from *Bacillus* sp. NWODO-3 and *Bacillus* sp. MABINYA-1 showed the best potential activity for arene substituent dye decolourization. This finding is comparable to a previous related study by Kalyani et al. [20], who reported the decolourization of various textile dyes (such as methyl orange, reactive red 2, reactive blue 59 etc.) by peroxidase from *Pseudomonas* sp. SUK 1 with highest decolourization activity of 72% on methyl orange. On the other hand, Rekik et al. [42] reported 5% decolourization of Poly R-478 by peroxidase from *Streptomyces griseosporeus* SN9 after 48 h. In a similar study by Guo et al. [43], crude manganese peroxidase effectively decolourized an azo dye, Acid Red 18 (AR 18), with more than 80% decolourization efficiency recorded after 60 min under optimum conditions.

Conclusions

In conclusion, the four novel ligninolytic Bacillus strains identified in this study have shown promising potential for delignification and production of peroxidases with industrial relevance in bioremediation. The ability of crude peroxidases from *Bacillus* sp. NWODO-3 and *Bacillus* sp. MABINYA-1 to decolourize both anthraquinone and azo dyes confers on them the potential for application in textile effluent treatment and synthetic dye transformation. However, further study on detailed characterization of the enzymes and optimization of the dye decolourization process is imperative for industrial applicability.

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Conflict of Interest

The authors declare no conflict of interest.

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