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Decolourization of synthetic dyes by laccase produced from *Bacillus* sp. NU2

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ABSTRACT

Advanced industrialization has caused an increase in the continuous discharge of hazardous effluents in the environment. This study evaluated the potential of the laccase synthesized by *Bacillus* sp. NU2 to degrade five synthetic dyes. Sawdust, wheat bran and peels of banana and tangerine were utilized as carbon sources for bacterial growth and laccase production. The produced crude enzyme was purified to homogeneity to determine its molecular weight. The kinetic activity of the purified laccase was determined using 2,2′-azinobis-(3-ethylbenzothiaz oline-6-sulfonic acid) (ABTS). The toxicity of the laccase-treated dye solution was assessed on *Bacillus* sp. NU2 growth. The result showed optimum laccase yield from the tangerine peel medium. The purified laccase gave a specific activity of 349.94 U mg^{-1} and a molecular weight of 55 kDa, respectively. The purified laccase displayed a strong affinity for ABTS substrate with an enzyme activity of 31.21 U mg^{-1}. It was optimum at 60 °C and pH 8, with catalytic efficiency (K_{cat}/K_m) of 23.93 mmol L^{-1}. The decolourization effects on Congo Red, Methyl Orange, Remazol Brilliant Blue R, Reactive Blue 4 and Malachite Green were 87%, 70%, 65%, 63% and 51%, respectively. The toxicity assay of laccase degraded dyes on *Bacillus* sp. NU2 showed a growth reduction of 36.75% (Malachite Green), 12.57% (Congo Red), 17.19% (Methyl Orange), 38.41% (Remazol Brilliant Blue R) and 28.14% (Reactive Blue 4). The laccase produced by *Bacillus* sp. NU2 holds a high catalytic potential for the detoxification of dye effluents in an environmental system.

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Introduction

Advanced industrialization and novel technologies present opposing challenges which include toxic compounds discharged into the environment. The agricultural, textile and paper mill sectors produce varieties of noxious compounds in significant proportions. The azo, triphenylmethane and anthraquinone dyes are components of synthetic material that are important in the textile sector [1, 2], and about 10–15% of these industrial dyes are discharged into the environment through wastewater after each mechanical process. These dyes end up in freshwater basins through seepage and leachate processes leading to water pollution and ecosystem contamination [3]. Dye polluted environment causes mutagenicity and toxicity to flora and fauna, leading to complete habitat degradation and destruction [4, 5]. contextualizing the magnitude of health challenges posed by the continuous discharge of industrial dye effluents in the environment, the World Bank estimated that about 20% of freshwater contamination arises from the textile industries alone [6, 7]. The demand for durable textile has driven innovation towards the synthesis of dyes with high resistance to degradation upon exposure to the environment. Consequently, novel dyes formed with intricate aromaticity and light resistance have displayed increased accumulation and persistence characteristics in the environment [7].

In a safe environment, efficient, low-cost waste disposal systems and an adequate treatment process is required before effluents discharge into receiving watersheds. The physicochemical approaches for textile dye effluent removal, such as the adsorption, illumination, precipitation, ozonation, activated carbon, coagulation and switched assimilation, is insufficient,

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as only a few fractions of the colourants are removed during the treatment process [8]. However, the visibility and continuous application of the physico-chemical dye decolourization techniques lingers due to the high costs associated with the techniques and the maintenance cost [9, 10]; hence, the need for a viable alternative method is on the rise.

Currently, there is a global trend towards environmentally friendly approaches like biological remediation of contaminated environment. Thus, exploration of microbial biomolecules for dye removal is topical [11], and there is an accumulating body of recent accounts for the microbial debasement of dyes [8, 12, 13]. In most cases, insufficiencies in the microbial system present negative measures that inhibit the dye removal system. In addition, azo dye toxicity hampers microbial growth, thereby constituting a factor in the insufficiencies articulated in the dye decolourization process.

Nevertheless, many cases have outlined the toxicity level of metabolites generated after the degradation of various synthetic dyes. Biodegradation of textile dyes leads to the development of toxic products and amines in most cases [14]. Hence, it is important to evaluate the degraded product. Biotoxicity to microbial cells was reported to possibly occur by an intermediate metabolite after Reactive Black B degradation [15]. Mansour et al. [16] reported that the metabolites of the degradation of azo-dyes were more lethal than the initial dye in the presence of araclor-induced rat liver microsome preparations. In addition, Iark et al. [4] stated that the biodegradation of Congo Red (CR) by laccase resulted in an open benzene ring intermediate product with a reduced toxicity level. Forootanfar et al. [17] reported a similar decrease in toxicity after degradation of Acid Orange 67, Disperse Yellow 79, Basic Yellow 28, Basic Red 18, Direct Yellow 107 and Direct Black 166 synthetic dyes. This implies that synthetic dyes can be degraded and detoxified by ligninolytic enzymes.

The microbial oxidoreductases, laccase, peroxidase, etc. have shown a propensity for removal of various azo dyes [18–22], but laccase has been widely utilized and has outrun other enzymes due to its catalytic activation energy which does not require a cofactor but available molecular oxygen as an electron acceptor to carry out its oxidation reaction [2, 21, 22]. Nonetheless, agro-residues are excellent substrates utilized in fermentation processes to induce laccase production in amounts of interest [10, 23, 24]. In addition, hemicellulose, cellulose and lignin are the component of agro-waste, and they serve as a rich carbon substrate inducing laccase synthesis [25].

The aptitudes of agro-waste as a promoter for laccase synthesis with potent oxidoreductive activity motivated this study. The aim of this study was to explore a bacterial laccase producer that could efficiently utilize various lignocellulosic wastes as a nutrient source for extracellular laccase production. Further, we purified laccase and applied it to degrade and detoxify various synthetic dyes.

Materials and methods

Bacterial culture

Axenic bacterial culture was from the BioCat collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare. The bacteria species was isolated from hospital wastewater and was identified molecularly using 16S rRNA sequence analysis. The partial bacterial 16S rRNA sequence was deposited in GeneBank and phylogenetic analysis was conducted using the MEGA X as described by Kumar et al. (2018).

Agrowaste pre-treatment

Banana peel (BP) and tangerine peel (TP) were chopped into small particle sizes, washed and air-dried under a steady current at room temperature for about 14 days. Wheat bran (WB) and sawdust (SD) were washed and air-dried over a steady air current for about 14 days. The air-dried agro-residues were pulverized using a mill blender (Hamilton Breach HBF 500S, Virginia, United States) and stored at room temperature until further use.

Agrowaste as carbon source for laccase production

The lignocellulosic wastes (BP, TP, SD and WB) were used as carbon sources to induce laccase production by applying the standard method [25]. Briefly, the medium was minimal salt medium (MSM) of the following composition (in g/L): 1.1 – KH_2PO_4; 0.72 – K_2HPO_4; 0.5 – KNO_3; 0.003 – $MnSO_4{\cdot}7H_2O$; 0.572 – $MgSO_4.7H_2O$; 4 – fructose; 0.009 – $FeSO_4$; 0.004 Vanillin; 0.03 – $CuSO_4$, 0.2 – Yeast; 0.1 – $ZnSO_4$; 1 – $NaNO_3$. The medium also contained 0.03% (*w*/*v*) of 2,6-dimethoxyphenol (2,6-DMP) as a laccase inducer. Additionally, the medium was supplemented separately with 2 g of the listed carbon sources and autoclaved at 121 °C for 15 psi. The *Bacillus* sp. NU2 overnight broth was standardized with normal saline to 0.5 McFarland at 600 nm. Then, 3% of the standardized culture was inoculated into 50 mL MSM and incubated at 140 rpm under 30 °C. Then, the medium was

harvested and centrifuged at 10,500×*g* for 13 min at 4 °C using the SIGMA-1-14K bench-top centrifuge. The cell-free supernatant was considered our crude enzyme and was screened for laccase activity.

Process optimization for laccase production

Temperature, pH, agitation speed and time course parameters were imputed for laccase production following the slight modification techniques described in [26]. Concisely, 3% standardized culture was inoculated into 50 mL autoclaved MSM of pH 6 in 250-mL Erlenmeyer flasks. To determine the temperature optimum for laccase production, each flask was incubated under temperatures of 25, 30, 35, 40, 45, 50, 55 and 60 °C (IncoShake Incubator, Labotec, South Africa), while the optimal pH conditions were investigated in the range of 3–11 and the liquid culture was incubated at 45 °C (Jenway Instrument, UK), respectively. Furthermore, the agitation speed parameters selected were 50, 100, 150 and 200 (rpm) (Inco Shake Incubator, Labotec, South Africa). The time course was assayed for 15 consecutive incubation periods. The prepared medium was at pH 5 and the incubation was at 45 °C. The crude extract was aseptically withdrawn every 24 h and tested for laccase activity. All experiments were performed in triplicates. After each process, to determine the optimized parameters, the culture broth was harvested and centrifuged, and the supernatant was assayed for laccase activity.

Quantitation of laccase activity

Laccase activity was measured by monitoring the oxidation of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) spectrometrically using the method of Bourbonnais and Paice [27] with slight modification: 50 µL of the crude supernatant was allowed to react with 50 µL of 2 mmol L^{-1} ABTS solution in 100 mmol L^{-1} potassium phosphate buffer (pH 6). The mixture was incubated at 30 °C for 10 min, and the reaction was stopped with 40 µL of 15% trichloroacetic acid (TCA). Absorbance for laccase activity was measured at 420 nm using a UV-spec microtiter reader (SynergyMX, BioTek, USA). The catalytic action on ABTS was interpreted in international units (IU), in which 1 IU of enzyme is required to oxidize 1 µmol of ABTS per minute.

Protein concentration was determined following the method of Bradford [28], and bovine serum albumin (BSA) was prepared as standard. The protein concentration was monitored at absorbance of 595 nm.

Enzyme purification

The crude laccase was purified using a standard technique [10]. The harvested medium was centrifuged at 10,500×*g* under 4 °C for 10 min, and the supernatant was withdrawn for the salting-out process. About 80% ammonium sulphate was added into the 200 mL harvested crude extract. It was kept overnight at 4 °C under stirring conditions and was centrifuged at 10,500×*g* for 10 min at 4 °C. The resulting precipitate was withdrawn and dissolved in 10 mL of 100 mmol L^{-1} potassium phosphate buffer PPB (pH6) for the dialysis process. The dissolved precipitate was dialyzed against the same buffer (PPB, pH 6; 100 mmol L^{-1}), and the buffer was changed every six hours over four consecutive cycles. The dialysate was centrifuged at 10,500×*g* for 10 min, and protein concentration and laccase activity were determined. The DEAE-Sepharose ion exchange resin (Sigma-Aldrich, South Africa) Econo-column (BioRad, South Africa) was set up at 2.5 × 10 cm, and the dialysate was eluted through 100 mmol L^{-1} PPB (pH 6). Then NaCl gradient (0, 0.2–1.2 mol L^{-1}) was used to elute resin-bound protein, and the protein concentrations were determined at 280 nm wavelength, while laccase activity was read at 420 nm using 2 mmol L^{-1} ABTS for each eluted fraction. The fractions with laccase activity were collected and concentrated with 4 mol L^{-1} sucrose solution following gel filtration assay. The concentrated protein was subjected to DEAE-Sephacryl S-200 gel (Sigma-Alrich, South Africa) filtration and the column (Econo, BioRad, South Africa) was equilibrated using 100 mmol L^{-1} PPB (pH 6). The protein was eluted using the same buffer and 4 mL fractions were collected separately at a slow flow rate of 0.4 mL/min. The protein concentration and laccase activity were determined after each purification step. The fractions with laccase activity were pooled and further characterized. Absorbance was read using a microplate reader (SYNERGY Mx BioTek Instrument, USA).

Enzyme characterization

Molecular weight determination

The molecular weight of the purified laccase was determined following a standard procedure. The mini protein two gel electrophoresis (Bio-Rad, South Africa) was utilized. In brief, 10% polyacrylamide gel containing 5 µL protein standard (BioLabsP7717S) and 10 µL denatured purified laccase was loaded into the gel and ran for 35 min, at 200 V. Then the gel was stained in a container containing 50% methanol and 7% acetic acid and the procedure was repeated twice under

gentle stirring condition. The protein band was visualized by staining with SyproRuby (Sigma-Aldrich, South Africa) at room temperature at 50 rpm overnight. Then the gel was viewed using the UV-transilluminator, and the protein band size was analyzed alongside the protein standard.

Optimum temperature and pH for laccase activity

The optimal range of temperature and pH for laccase activity was assayed by subjecting the purified laccase at a range of temperatures at 30, 40, 50, 60, 70, 80, 90 and 100 °C and pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 11.0) following the standard method Edoamodu and Nwodo [24]. The optimal temperature for laccase activity was determined by adding the purified crude laccase at different temperature regimes. Also, the optimal pH condition for laccase activity was determined by measuring the enzyme activity at the range of pH values which was prepared using 100 mmol L^{-1} of the given buffer solutions. The pH values were adjusted using sodium citrate (pH 3.0–5.0), potassium phosphate (pH 6.0–8.0) or sodium carbonate buffer (pH 9.0–11.0). Laccase activity was determined using a standard procedure [29].

Laccase substrate specificity and kinetic activity

The substrate specificity of the purified laccase was assayed on the following substrates: ABTS, 2,6-DMP, potassium ferrocyanoferate (PFC) and guaiacol as previously described [24]. Furthermore, laccase activity kinetic parameters, Michaelis–Menten constant K_m, V_{max} and K_{cat}/K_M, were determined at a range of ABTS concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 mmol L^{-1}) in PPB (100 mmol L^{-1} pH 6). Lineweaver–Burk plot was used for enzyme activity determination.

Dye decolourization assay

The oxidative potential of the purified laccase for dye removal was evaluated on five synthetic dyes following a standard technique [24]. The dyes were Malachite Green (MG), Congo Red (CR), Methyl Orange (MO), Remazol Brilliant Blue R (RBBR) and Reactive Blue 4 (RB4). About 50 mg/L of each dye was dissolved in sterile tap water. Purified laccase and each dye solution were allowed to react at a 1:1 ratio. The 3 mL reaction volume contained 1 mL acetate buffer (pH 5.5), 1 mL dye solution, and 1 mL purified laccase; the mixture was incubated at 30 °C for 1 h. The procedure was repeated for all five dyes, and the absorbance was read, respectively, at 579, 497, 507, 595 and 595 nm using a microplate reader (SYNERGY Mx BioTek Instrument, USA) instrument. Triplicate experimentation was performed, and the percentage decolourization was calculated using the following equation:

$$\%\text{decolourization} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 is the initial absorbance of the mixture immediately after the addition of the enzyme and A_t is the absorbance at a given time.

Dye detoxification assay

The toxic potential of laccase-treated dyes (MG, CR, MO, RBBR and RB4) was tested on *Bacillus* sp. NU2 growth as previously described [24]. About 3 mL of laccase treated dye was added into sterilized Muller-Hinton broth and about 50 µL overnight standardized (1.5×10^8 CFU mL^{-1}) *Bacillus* sp. NU2 broth was inoculated in the broth and incubated at 37 °C for 18 h. Bacterial growth was measured through optical density at 600 nm. A similar procedure was carried out with each untreated dye, while the control was Muller-Hinton broth only. The per cent growth inhibition (% GI) was calculated as follows:

$$\%\text{Growth inhibition}(\%\text{GI}) = \left(1 - \text{OD}_{600a} / \text{OD}_{600c}\right) \times 100$$

where OD_{600a} represents the absorbance of the sample, and OD_{600c} represents the control absorbance; the control absorbance was further used to determine the per cent growth inhibition (%GI).

Data analysis

All experimental procedures were performed in triplicates. Data were analyzed with the statistical tools in Microsoft 365 Excel v 6.1.

Results and discussion

Molecular identification and phylogenetic relatedness

The 16S rRNA molecular analysis of the extracted DNA from the isolated microbial strain shows it belongs to the Bacillaceae family and the Bacilli class of microbial species. The nucleotide sequence of the bacterial strain was deposited in GenBank as *Bacillus* sp. strain NU2 under accession number MN686607. The phylogenetic analysis of the *Bacillus* sp., NU2 partial sequence showed a close relatedness with already deposited bacterial sequences of *Bacillus cereus* and *Lysibacter segetis* extracted from the NCBI database, with a

well-defined distance clustering around our choice bacterial species (Figure 1). In a similar finding, *Enterobacter asburiae* strain ESI and *Enterobacter* sp. strain Kamsi extracted from marine sediment showed a close relatedness with other bacterial sequences extracted from NCBI [24]. Likewise, a gamma-proteobacterium, *Enterobacter* sp. AI1 isolated from cow dung showed 99% similarity to other bacterial sequences compared from the NCBI database.

Agrowaste as a carbon source for laccase production

Bacillus sp. NU2 efficiently utilized sawdust (SD), banana peel (BP), wheat bran (WB) and tangerine peel (TP) and gave a laccase activity of 19.6, 19.9, 21 and 22.1 (U mg^{-1}), respectively (Figure 2). Our isolate was able to utilize extensively the agro-wastes added in the medium. However, TP appeared to be the best nutrient source, with an optimal laccase yield of 22.1 U mg^{-1}. In a similar study that applied sawdust, banana peel, rice bran and wheat bran for *Bacillus subtilis* MTCC 2414 laccase production, wheat and rice bran were efficient in supporting laccase production [30]. Furthermore, Kumar et al. [31] reported rice bran as a substantial agro-waste utilized by *Bacillus* sp. strain AKRC01, with a low laccase titre value produced (1.625 U mg^{-1}). In comparison to these reports, our isolate of choice, *Bacillus* sp. NU2, actively utilized all four types of agro-waste showing no significant difference among the activity of crude laccase produced on each of them. This suggests that our laccase producer could be suitably applied for laccase production on a commercial scale, and this also highlights its wide substrate specificity to various agro-waste. In another study, *Bacillus* sp. PK4 significantly utilized wheat bran, rice husk, groundnut shell and sugarcane bagasse, producing substantial laccase activity [32]. In addition, *Pseudomonas* sp. S2 optimally utilized potato peel for laccase production [33]. The remarkable laccase activity obtained by utilization of all used lignocellulosic waste might be due to the vitamins, proteins, and also to adequate hydrolysis of other components contained in the agro-waste material. Most importantly,

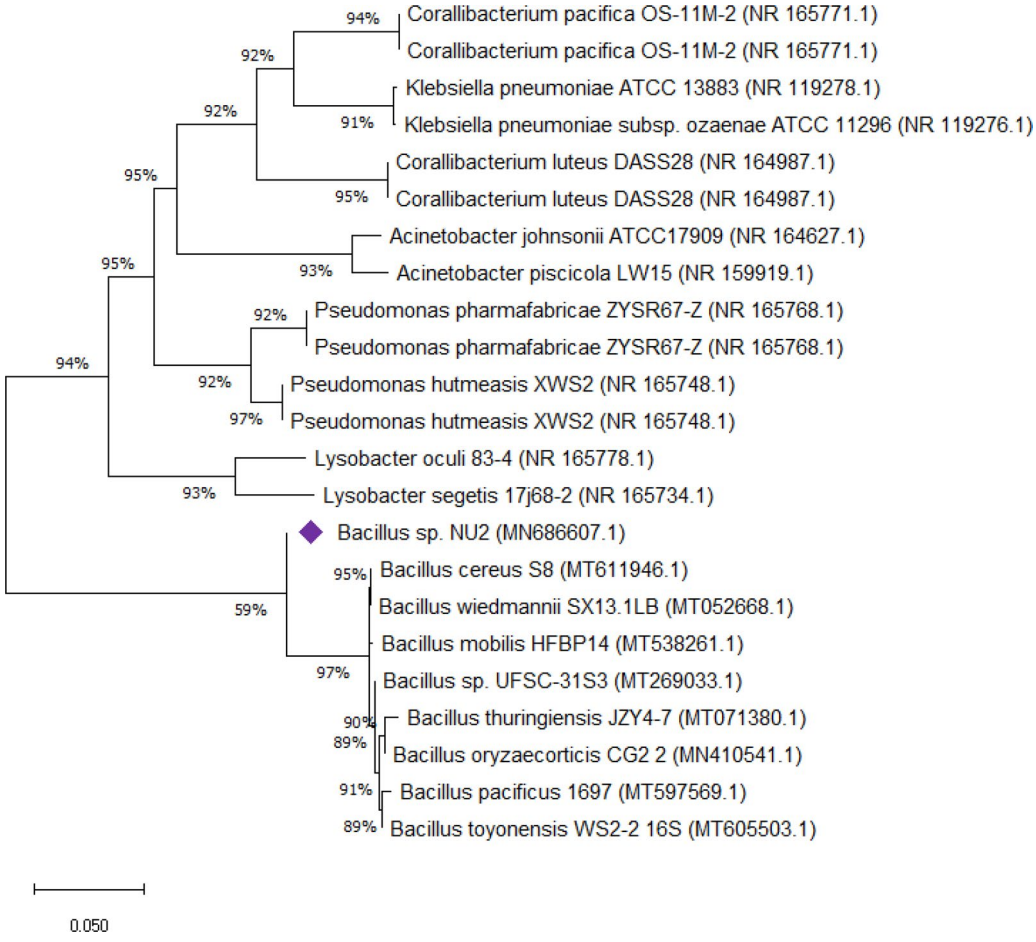

Figure 1. Phylogenetic tree of *Bacillus* sp. NU2 partial sequence relatedness to other bacterial sequences retrieved from the NCBI database. The evolutionary history was deduced utilizing the neighbour-joining method using MEGA X.

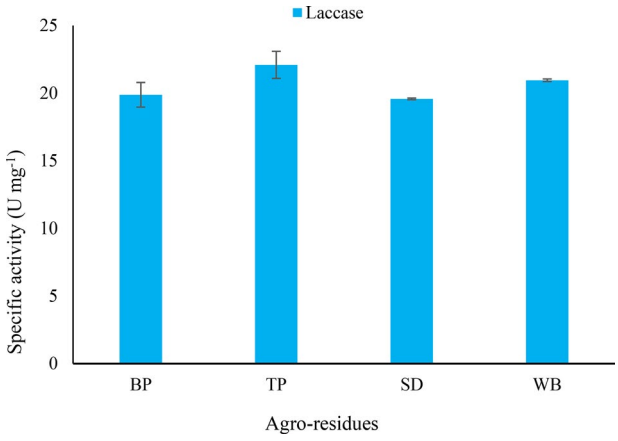

Figure 2. Effect of standardized *Bacillus* sp. NU2 in a sterile minimal salt medium of pH 6 containing banana peel (BP), tangerine peel (TP), sawdust (SD) and wheat bran (WB) incubated at 30 °C for laccase production. The crude laccase activity was measured after 6 days of incubation.

agro-waste was reported as the most promising feedstock for laccase production from various organisms [23]. In addition, the optimal laccase activity in the TP medium might be due to a water-soluble aromatic compound which might have the potential to induce or stimulate laccase synthesis within the medium. The choice of carbon source is important, since selecting an appropriate agro-residue in an enzyme production medium can significantly influence the potential of an organism to secrete the secondary metabolites of interest in a large quantity [34], and this was achieved by the rich lignin content of the selected agro-waste used.

Optimized conditions for laccase production

Remarkable enzyme activity of the crude laccase produced by *Bacillus* sp. NU2 was obtained at the temperature range of 25–45 °C but the maximum activity was recorded at 45 °C with a specific activity of 29.38 U mg^{-1}, while on further incubation at 50–60 °C the laccase activity decreased (Figure 3(a)). Bacteria grow at a certain temperature range that could be suitable for secreting enzyme at a remarkable titre within that temperature environment [35]. The reduction in enzyme activity observed from 50 to 60 °C might have resulted from lower metabolic activity of the microorganism within the medium during continuous heating, thereby leading to low substrate to product conversion. *Bacillus* sp. Falade-1 peroxidase activity was optimum at 30 °C and further declined at 35–40 °C incubation conditions [36]. *Bacillus* sp. WT isolated from Urmia Lake showed maximum laccase activity at 20–45 °C [37]. Temperature is an essential component in a fermentation process and potentially influences the expression of extracellular laccase extracellularly. The increase in temperature of the fermentation medium might have produced an unfavourable condition for *Bacillus* sp. NU2 to efficiently secrete quantifiable laccase. Unfavourable temperature hampers cell regulatory activities and alters microbial function.

Enzyme activity was detectable at the initial pH value of 3 (20.411 U mg^{-1}) followed by a remarkable increase in enzyme activity (35.82, 40.501 and 38.103 U mg^{-1}) at pH 4, 5 and 6, respectively. The difference in enzyme activity was not statistically significant ($\alpha = 0.05$), but pH value of 5 was optimal for laccase production. The laccase specific activity was >14.23 U mg^{-1} within the pH range of 7–9, while further incubation at pH 10 drastically reduced laccase activity (Figure 3(b)). The broad pH range supporting laccase production by the NU2 strain suggests its suitability for bioprocess application without stringent pH controls, which could be cost-effective on a commercial scale. *Trichoderma harzianum* similarly showed optimum laccase activity at pH 5 for laccase production [26], and *Bacillus* sp. CF96 showed a broad pH range of pH 3–8 for laccase production [38]. Unfavourable variations of the hydrogen ion concentration in a fermentation medium may adversely influence organismal growth and impair the conformational structure of the secreted enzyme, thereby impairing its activity. That may have accounted for the resultant reduction in the enzyme activity at pH 10.

The agitation speed during fermentation was optimal at 100 rpm and yielded laccase activity of 41.26 U mg^{-1} (Figure 3(c)). By implication, the oxygen quotient for laccase production by *Bacillus* sp. NU2 may be stringent as 100 rpm gave the best yield. Low and high agitation speeds resulted in lower laccase production. High agitation speed may decrease the nutrient uptake through undesired shear forces, diminishing the microorganism's ability to biosynthesize the enzyme of interest; as a result, leading to a reduced enzyme yield at a high agitation speed (150–200 rpm) [39]. Thus, an optimized process is crucial for enzyme production in an industrial system.

The time course assay showed a gradual increase in laccase production from day 5 up to day 14 of incubation. The optimum laccase yield was observed on day 12 with a specific activity of 36.52 U mg^{-1}; however, a considerable specific activity of >22–23 U mg^{-1} was also observed on day 13 and 14, respectively (Figure 3(d)). Nevertheless, further incubation up to day 15 drastically decreased the laccase activity to almost non-existent (Figure 3(d)). This might have occurred as a result of cell lysis liberating the protein

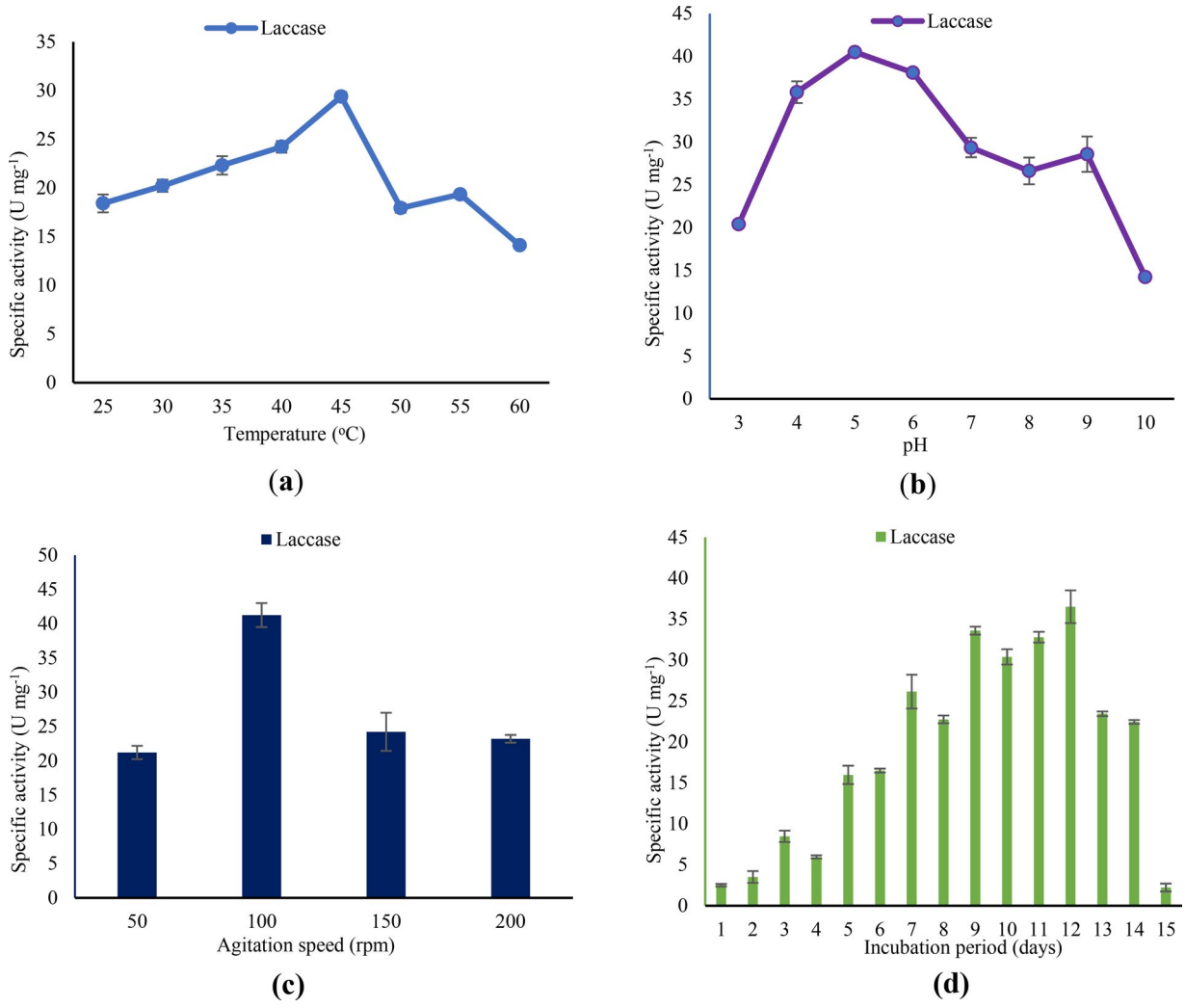

Figure 3. Influence of temperature (a), pH value (b), agitation speed (c) and incubation period (d) on laccase production by *Bacillus* sp. NU2.

contents into the medium. *Bacillus subtilis* MTCC 2414 and *Bacillus* sp. B16 maximally produced laccase at an incubation period of four and five days, respectively [30, 40].

Enzyme purification

The crude supernatant from the growth medium of *Bacillus* sp. NU2 was purified to homogeneity using ammonium sulphate, which resulted in a 1.28-fold purification factor and a total protein recovery of 28.69% from the 200 mL culture filtrate harvested. This process decreases the unwanted protein and allows for smoothness of further purification steps (Table 1) by increasing the specific activity of the enzyme and the magnitude of purification [41]. The DEAE-Sepharose column resulted in a specific laccase activity of 108.98 U mL^{-1}, 3.35% laccase recovery and 2.35 purity fold. Finally, the pooled fractions loaded into the Sephacryl S-200 column were purified to 2.38% laccase yield with a purification fold of 7.55 and specific laccase activity of 349.94 U mg^{-1}. In a similar manner, Olajuyigbe et al. [42] reported a 2.92% purification yield of *Cyberlindnera fabiani* laccase from an initial crude enzyme of 1920 mL. However, the initial crude enzyme (200 mL) used in this study could have resulted in the low laccase yield obtained after a series of purification steps involved. On the contrary, Zhang et al. [43] reported an extracellular laccase from *Bacillus vallismortis* fmb-103, giving a purification fold of 15.2 and a specific laccase activity of 389.9 U mg^{-1}. Pawlik et al. [44] purified *Sinorhizobium meliloti* laccase to an 81-fold purification factor and had a laccase recovery of 19.5%. Another reason for the low laccase yield (2.38%) observed could be due to the loss of enzyme activity at every purification step. The enzyme activity could decrease as a result of the manipulations at each purification step, leading to denatured proteins. Another factor is the

Table 1. Purification steps of laccase obtained from *Bacillus* sp. NU2.

Purification steps	Total volume (mL)	Total activity (U)	Total protein activity (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification fold
Crude enzyme	200	2500.86	53.991	46.32	100	1
Precipitation	19	717.495	12.059	59.49	28.69	1.28
DEAE-Sepharose	2.9	83.818	0.77	108.98	3.35	2.35
Sephacryl S-200	11.1	59.49	0.17	349.94	2.38	7.55

type of buffer used, as some buffers contain detergent that could denature proteins [45]. Moreover, potassium phosphate buffer entails the ability to inhibit enzymatic activity. Therefore, further studies need to focus on an appropriate buffer solution with the desired ionic strength and employing a large amount of crude supernatant.

Enzyme characterization

The molecular weight of the purified laccase in this study was ~55 kDa (Figure 4). Similarly, Muthukumarasamy et al. [30] reported molecular weight of 52 and 55 kDa of laccase extracted from *Bacillus subtilis* MTCC 2414. Other studies [46, 47] reported laccase with molecular weight within the range of 24–80 kDa. This might be an enzyme with the same catalytic sites but different structural conformation. It is also possible for the laccases produced to exhibit the same catalytic action but to be structurally different. Therefore, several enzymes might have evolved, bearing the same activity as laccase.

Effects of temperature and pH profile on laccase activity

The optimum temperature for the activity of the purified laccase was 60 °C, while further incubation above 60 °C showed a reduction in laccase activity (Figure 5(a)). The decrease in enzyme activity may be attributed to the changes in the conformational structure of the protein, which altered the catalytic sites because of the increased heating process. In addition, the effect of pH on the purified laccase showed two optimal pH values for laccase activity: pH 6 and 8 (Figure 5(b)), respectively. The two pH (6, 8) optima observed might have resulted from the measurement of laccase activity using the same potassium phosphate buffer at different pH values. The laccase characteristics suggest a potential for application in mildly acidic conditions and influence enzyme activity in alkaline environments. The optimum pH and temperature conditions for *Sporothrix carnis* CPF-05 laccase were pH 7.0 and 50 °C [48].

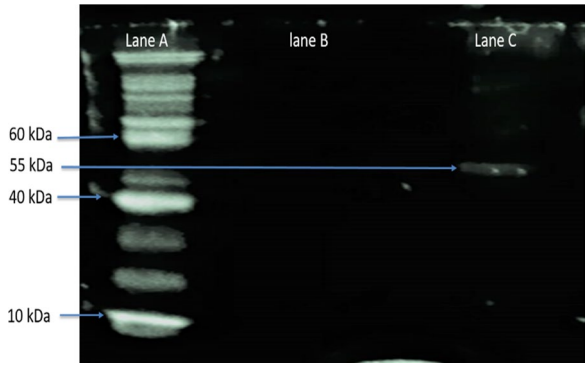

Figure 4. SDS-PAGE gel electrophoresis analysis of purified protein (laccase) extracted from *Bacillus* sp. NU2. Lane A: protein marker (BioLabs – P7717S), lane B: no band, distilled water only, and lane C: purified laccase with the band at ~55 kDa.

Specificity and kinetics parameters of bacillus sp. NU2 laccase

The activity of the purified laccase on various phenolic and non-phenolic substrates was 31.21, 28.1, 17.13and 14.416 U mL^{-1} for ABTS, PFC, guaiacol and 2,6 DMP, respectively. The highest enzyme activity was observed on ABTS and PFC substrate (Figure 6). Similarly, *Sporothrix carnis* CPF-05 showed a broad substrate spectrum laccase activity on different phenolic and non-phenolic compounds [48]. Furthermore, the Lineweaver–Burk plot of the *Bacillus* sp. NU2 laccase activity kinetics on various ABTS concentrations showed the goodness of fit at R^2 = 0.958, while the K_m and V_{max} values were 0.19 mmol L^{-1} and 50 µM min^{-1} mL^{-1}, respectively (Table 2). However, the purified laccase catalytic efficiency (K_{cat}/K_m) was 23.92 mM^{-1} min^{-1}, which best describes the conversion rate to a product. Likewise, it proves a quicker product formation when it is involved in a large catalytic reaction cycle. Jaiswal et al. [49] revealed that the K_m and V_{max} values of the purified laccase from *Leucaena leucocephala* were 1.92 mmol L^{-1} and 0.088 µM min^{-1} mL^{-1} with ABTS catalytic efficiencies (K_{cat}/K_m) value of 1.83 mM^{-1} min^{-1}. Neelkant et al. [19] showed that *Sphingobacterium* sp. ksn-11 displayed high catalytic strength on the ABTS compound. On a similar note, *S. carnis* CPF-05 laccase kinetics showed high affinity on ABTS parameters [48]. The result from the kinetic analysis shows that *Bacillus* sp. NU2 had a strong affinity for ABTS with high catalytic activity.

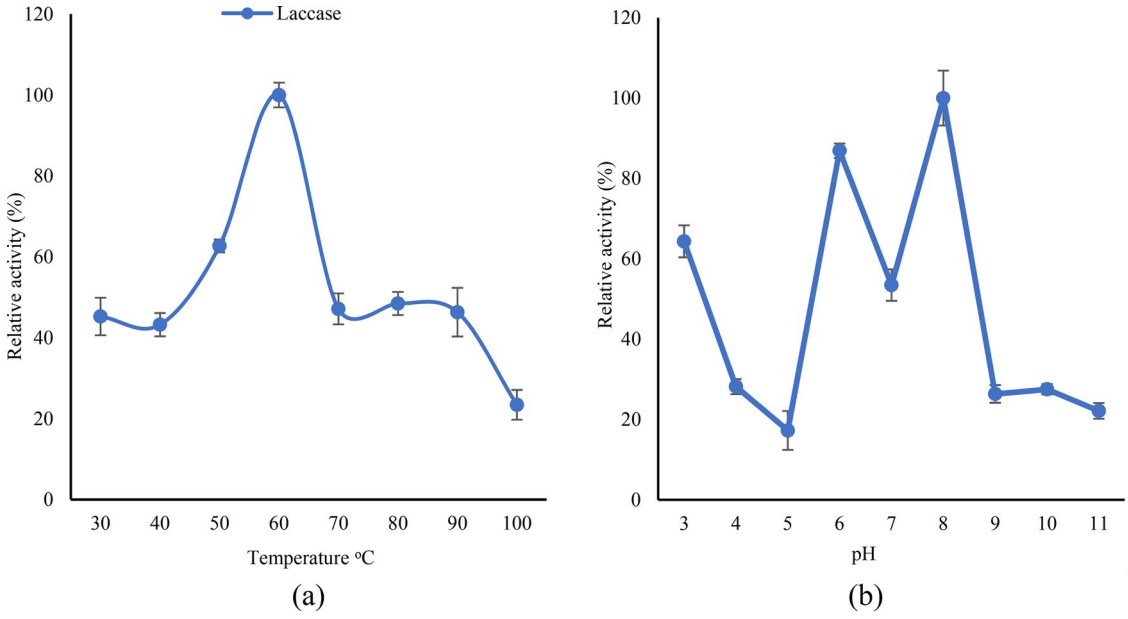

Figure 5. Temperature (a) and pH (b) profile of the purified *Bacillus* sp. NU2 laccase.

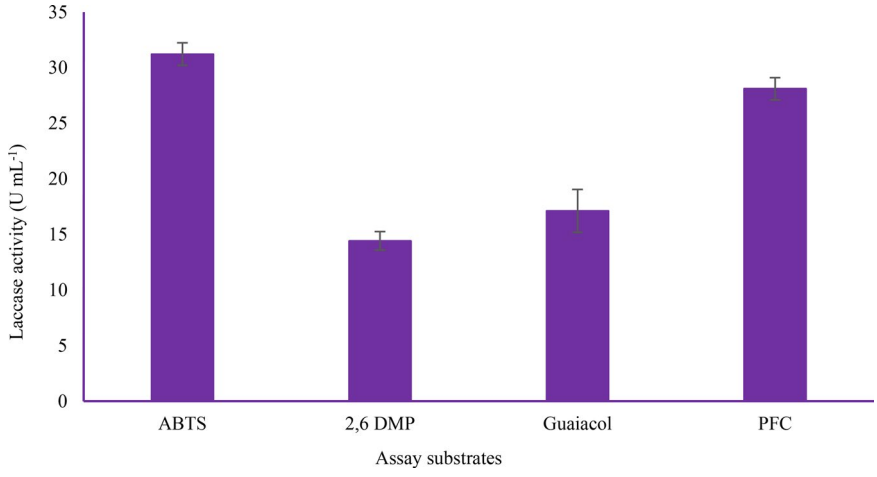

Figure 6. Substrate specificity of the purified laccase on various phenolic and non-phenolic substrates: 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,6-dimethoxyphenol (2,6-DMP) and potassium ferrocyanoferrate (PFC) in pH 6 phosphate buffer solution at 30 °C.

Table 2. Laccase kinetic activity on ABTS substrate.

Laccase producing strain	K_m (mmol L^{-1})	V_{max} (μM min^{-1} mL^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
Bacillus sp. NU2	0.19	50	4.53	23.92

Dye decolourization assay

The purified laccase showed a decolourization potential of >50% on all applied dyes (Figure 7). However, optimum decolourization efficiency was observed on CR (87.63 ± 2.33%) followed by MO (70.63 ± 0.88%). The decolourization efficiency on RBBR, RB4 and MG was 65 ± 0.47%, 63.33 ± 0.94%, 51.15 ± 0.54%, respectively. The maximal dye decolourization potential of *Bacillus* sp. NU2 laccase is an indication for its prospects for industrial applications. *Bacillus amyloliquefaciens* can produce laccase with efficient dye decolourization prospect [50]. Similarly, *Oudemansiella canarii* produced laccase with about 80% decolourization efficiency on Congo Red [4]. The application of laccase as an azo-reductase is topical [9, 51]. Consequently, enzyme application for the decolourization of industrial dyes portrays an efficient treatment and management system on diverse dye effluents in industrial

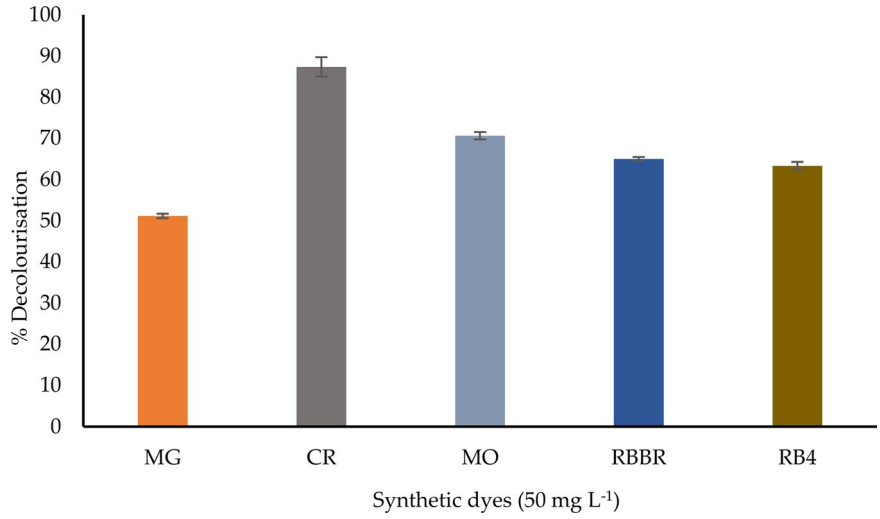

Figure 7. Potential of the purified laccase from *Bacillus* sp. NU2 to decolourize Malachite Green (MG), Congo Red (CR), Methyl Orange (MO), Remazol Brilliant Blue R (RBBR) and Reactive Blue 4 (RB4) at pH 6 incubated at 30 °C for 1 h treatment time.

discharge that impairs the quality of the environment [12].

Residual toxicity of detoxified dye on bacterial growth

As a next step, we assayed the toxic potential of untreated dyes (MG, RB4, RBBR, CR and MO) on *Bacillus* sp. NU2 growth. There was a high per cent growth inhibition (%GI) of 70.07, 56.59, 48.79, 41.91 and 31.45, respectively. However, there was an increase in the number of viable bacterial cells upon incubation with the laccase treated dye solution (Table 3). A decrease in the toxicity of all the treated dyes was observed, showing a %GI of 38.41, 36.75, 28.14, 17.19 and 12.57 scored against RBBR, MG, RB4, MO and CR, respectively. Younes et al. [52] reported a 99% increase in *Bacillus megaterium* growth after incubation in the purified laccase treated MG solution. Forootanfar et al. [17] reported a decreased toxicity of laccase treated azo dyes solution, with an increased viable cell counts observed after incubation. Overall, the laccase produced by *Bacillus* sp. NU2 effectively degraded the azo dyes and holds good potential for application in the industrial sector.

Table 3. *Bacillus* sp. NU2% growth inhibition on exposure to laccase-treated and untreated MG, CR, MO, RBBR and RB4 solution.

Dyes	Untreated dye	Treated dye
Malachite Green	70.07 ± 0.70	36.75 ± 1.41
Congo Red	41.91 ± 2.12	12.57 ± 1.41
Methyl Orange	31.45 ± 2.12	17.19 ± 0.71
Remazol Brilliant Blue R	48.79 ± 0.71	38.41 ± 0.70
Reactive Blue 4	56.59 ± 0.71	28.14 ± 2.12

Conclusions

The agro-waste biomass serves as an effective organic substrate for the growth of *Bacillus* sp. NU2 and can also be useful in the commercial production of laccase on an industrial scale for low-cost enzyme production. The process conditions for the laccase production and the characteristics of the purified laccase indicate that the suitable conditions for the effective activity of the enzyme would be mesophilic but some tolerance to mild thermal conditions is possible. The purified laccase showed remarkable laccase activity both at acidic and alkaline conditions, signifying its potential in effluent treatment disposal. The catalytic strength of dephenolizing the five synthetic dyes indicates its potential for environmental remediation and industrial application. The detoxification effects as revealed by exposure of *Bacillus* sp. NU2 strain to the laccase-treated dye indicate that NU2 laccase has high catalytic efficiency and bioprospects application. In addition, this serves as the first report on laccase-producing *Bacillus* sp. NU2 isolated from hospital wastewater from Eastern Cape Province, South Africa, and applied for dye decolourization. The properties exhibited by the *Bacillus* sp. NU2 laccase motivate a further study on the immobilization of NU2 laccase and potential application for degradation of other synthetic dyes.

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Author's contribution

CEE executed the research and prepared the first manuscript draft. UUN conceived and planned the research, funded and supervised the research. CEE prepared the final draft of the manuscript.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Disclosure statement

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