Next Sustainability 6 (2025) 100201

Contents lists available at ScienceDirect

Next Sustainability

journal homepage: www.sciencedirect.com/journal/next-sustainability

Research article

Extracellular laccase production by bacterial isolates for sustainable treatment of palm oil mill effluents

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A R T I C L E I N F O

Keywords:
Laccase
Bacteria
Agrowastes
Corncob
Palm oil mill effluents
Pseudomonas aeruginosa

A B S T R A C T

Laccase is widely known to oxidize numerous substrates. The laccase-producing isolate used in this study was obtained from a snail gut. Agrowastes were processed using 1 % NaOH and hydrolysed with agrowastes produced by *Aspergillus niger*. The hydrolysate was used in place of fructose and optimized using Box-Behnken response surface methodology. Submerged fermentation in a Luria Bertani broth supplemented with 0.01 % guaiacol was used to produce laccase based on the optimal conditions. The laccase was partially purified using ammonium sulphate precipitation and used to treat palm oil mill effluents (POME). The laccase-producing isolate was identified as *Pseudomonas aeruginosa* (accession number OR687603). Corn cob had the highest capacity for laccase production (7.25 U/mL). The optimal production of laccase was achieved at 37°C, pH 9, 60 h and 3.3 % carbon source. Under these conditions, 60 mL laccase (10.76 U/mL) was produced from a 5 L medium. After treatment there were reductions in chemical oxygen demand (COD) (1875.10–1542.90 mg/L), biochemical oxygen demand (BOD) (23.67 mg/L to 7.33 mg/L) and pH increased from 3.57 to 3.73. The result showed that the laccase produced using corncob as a carbon source can be developed as an environmentally sustainable alternative in POME treatment.

1. Introduction

Laccase, an enzyme which is a blue copper oxidoreductase is responsible for converting molecular oxygen into water and oxidizing various substrates [38,66]. Laccase's capacity to oxidize lignin-related composite is of great significance. Unlike peroxidases, laccases do not require H_2O_2 for oxidation reaction as they oxidize wide range of chemical compounds such as aromatic amines, diamines diphenols, polyphenols, and benzenethiols [14]. Laccases were discovered in prokaryotes and some eukaryotes including fungi, insects and some plants signifying that laccase activity is prevalent. Majority of agricultural wastes and processing activities are currently being incinerated leading to air pollution and climate change [34]. The influence of agricultural wastes on human health, animal health, and the environment is significant, therefore necessitating a need to develop an environmentally friendly strategy in which this waste can be properly managed [4,55].

Palm oil processing discharges excessive quantity of oil-in-water viscous liquid as their main pollutant. The POME holds about 95–96 % H_2O, 0.6–0.7 % lubricant and oil, pH range of 4–5 and about 4–5 % of total solids consisting of great quantity of liquid and solid wastes and also consist of lubricant and oil of about 4000–6000 mg/L [13]. Palm oil mill effluent is the main by-product in the course of the palm oil extraction where about 1.5 tons of this POME generated from one tonne of fruit bunch processed. Palm oil mill effluent contains high COD, acidic pH, high BOD, oil, and grease and large amounts of total solids. Most times, the raw effluent is channeled to soil or water bodies in that vicinity owing to the cost of existing wastewater treatment (Fig. 1) [49]. Soil and water pollution caused by a non-selective release of untreated and partially treated POME is still the primary concern of the palm oil industry in terms of environmental issues.

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https://doi.org/10.1016/j.nxsust.2025.100201
Received 20 May 2025; Received in revised form 14 October 2025; Accepted 20 October 2025
Available online 25 October 2025

Aluminum sulphate and polyaluminium chloride are the most used coagulants in water treatment, especially in cases of overdose to improve coagulation efficacy. Although these coagulants generate activated sludge that carries poisonous levels of aluminum, which can cause adverse consequences if released into water marine environment. Not only that, there is also a theory that consuming excessive amounts of aluminum salt can result in Alzheimer's disease. It is therefore paramount to develop an effective environmentally safe method, hence the use of microorganisms and their products as seen in Table 1[13].

Laccases, which are flexible biochemical catalysts and are known for their environmentally friendly enzymatic breakdown, have shown several biotechnological applications, including the conversion of complex xenobiotics into innocuous compounds [21]. Innovative strategies in wastewater management, have been developed including the use of laccase, which is known to have a large specificity of substrate, great unchanging catalyst, and utilizes atmospheric O_2 as the next substrate, this is in a bid to curb the increasing trends of environmental pollution and emerging contaminants from anthropogenic activities [56]. Laccases have shown potentials in eliminating various pharmaceutical products from pharmaceutical effluents, including antibiotics, analgesics and hormones. The degradation competence is mostly dependent on the structure of the compounds. The formation of di-, oligo-, and polymers of the initial compounds, often enabled by laccase mediators, can help in their removal in wastewater treatment plants. Estrogen and tetracycline have shown significant breakdown or with laccase treatment [23]. Laccases have also been reported to be used in the textile industry for decolorization of dyes. Successfully breaking down dyes, which are often recalcitrant to orthodox treatment methods. Bacterial laccases, have been reported to be advantageous due to their stability at high temperatures and high pH levels which are common in textile wastewater [58]. Researches from the pulp and paper industry, have also documented laccase ability for detoxification, delignification and bio-bleaching of effluents [58]. Bacterial laccases are known to be inexpensive, as they can be cloned and expressed in an acceptable microbial host with little energy. In addition, laccase from bacteria provides a wide level of biological cleanup potentials based on bacterial capacity to produce optimum nutritional and environmental state *via* microbial enhancement [58].*Pseudomonas*, *Comamonas*, *Pandoraea*, and *Novosphingobium*, which are from the genera Proteobacteria, are known to exhibit significant laccase activity and are often isolated from various environments such as wastewater, compost, decaying biomass, soil and bamboo materials [39]. Species such as *Bacillus ligniniphilus*, *Bacillus amyloliquefaciens*, and *Brevibacillus thermoruber*, which are Firmicutes are usually isolated from environments such as marine sediments, soil, and sludge. These taxa are known for their production of laccase and other lignin-degrading oxidative enzymes, some of which show

Fig. 1. Production of palm oil and effluent discharge, A = Threshed palm fruits from bunches, B = Pressing process (extraction of palm oil from fruit), C = Crude palm oil after pressing, D = Effluent disposal site, E = Effluent disposal site.

Table 1
Molecular Identification of Bacterium Isolated from Snail Gut.

Sample identity	Scientific name	Maximum score	Total score	Query cover	E Value	Percentage identity	Accession
Select seq OM665413.1	*Pseudomonas aeruginosa*	2492	2492	0.99	0 %	0.9985	OR687603

thermostable features [32]. Some other laccase producing bacterial species include *Yersinia pestis, Pseudomonas aeruginosa Escherichia coli, Caulobacter crescentus, Bacillus subtilis, Bordetella campestris*, *Azospirillum lipoferum* and *Pseudomonas putida* [58].

Although, *Pseudomonas aeruginosa* is reported to be a laccase producer, its applications, particularly those involving large-scale, requires attention because of its antibiotic-resistant and pathogenic nature. The possibility for unintentional release of these organisms, or their genetic material, into the environment requires strict containment and safety protocols. Researchers using *Pseudomonas aeruginosa* for industrial purposes must device management strategies to lessen these environmental and public health risks [40]. *Pseudomonas aeruginosa* is known to cause a range of acute and chronic infections, especially in immune compromised individuals. These infections can affect various parts of the body, including the lungs and the urinary tracts [57]. The presence of this pathogen in nature including those producing beneficial enzymes like laccase, poses a risk. If these strains are released into the environment through effluent discharge, they could contaminate water sources, agricultural products, or even enter the food chain, which would ultimately lead to public health risks. The versatility of *Pseudomonas aeruginosa* allows it to adjust to various habitats, and these environmental strains can exhibit pathogenic properties similar to the clinical isolates [40].

Using non-pathogenic strains could be an alternative. Prioritizing the use of genetically modified non-pathogenic laccase-producing microorganisms. Also immobilizing the laccase enzyme itself, rather than using live cells, can decrease the risk of microbial spread [40].

The influence of agricultural wastes on soil health and the atmosphere is significant, therefore necessitating a need to develop an environmentally friendly strategy in which these wastes can be properly managed [4,48]. Poisonous mixes are freed directly into the atmosphere from the method of incineration employed in agro-waste management such mixes include volatile organic compounds (VOC), nitrous oxide, carbon monoxide (CO) and other matters which can cause harmful effects to the atmosphere. Also, this agro-wastes can further block canals, and drainage systems eventually giving rise to erosion and flood [27]. The large quantity of agro-wastes starts cluttering the environment until it gradually becomes irritating as they add significantly to environmental toxic waste including polluting numerous bodies since they are not effectively exploited. Oyewole et al., [55]. The fact that most agro-wastes are lignocellulosic makes them suitable for the production of laccase [47]. Vasudhevan et al. [74] in their study confirmed that the fact most agro-wastes are lignocellulosic makes them suitable for the production of laccase as they recorded an exceptionally high laccase activity (623 U/mL) produced from *Aspergillus oryzae* under optimized conditions in comparison to submerged fermentation yields much lower than $<$ 100 U/mL in standard synthetic media using corn cobs as substrate. Another study carried out by Abdelgalil et al. [1] also recorded *Lysinibacillus macroides* LSO on sugarcane bagasse yielding ~7653 U/L/min in a 10 L bioreactor after optimization—over 70 × higher than basal medium.

Both agro-wastes and industrial wastes such as from the paper and textile sectors offer important potential as cost-effective and sustainable substrates for laccase production. These wastes are abundant, often discarded, and therefore represent low-cost raw materials, considerably reducing the production cost of laccase likened to using synthetic media [65]. Using these wastes aligns with circular economy principles, converting environmental burdens into valuable bio-products. This decreases waste disposal problems and promotes sustainable industrial practices [61]. The lignin component found in both agro-wastes and industrial wastes can act as a natural inducer for laccase production, potentially leading to higher enzyme yields. Although agro-Wastes have been recorded to have consistent chemical compositions within a specific type such as corncob. However, the overall diversity of agro-wastes means that pretreatment approaches need to be design to each biomass [36,71]. Industrial effluents, may exhibit significant variability in composition depending on the industrial processes involved and also raw materials used. Laccase production can become challenging because of these and may require more adaptable microbial strains and sophisticated pretreatment step [3]. If properly pretreated, agro-wastes can provide an excellent balance of carbon sources and lignin inducers, which will therefore lead to robust microbial growth and high laccase yields. Since the agro-waste hydrolysates are relatively clean compared to most industrial effluents, can often support higher microbial viability and enzyme activity [70]. While Paper and textile industrial wastes can induce laccase production, the presence of diverse inhibitory compounds might require the use of highly tolerant or genetically modified strains. The laccase activity observed might be influenced by the pollutants present, since these laccases are also used for their degradation. The dual purpose of using these industrial wastes for laccase production and bioremediation can sometimes lead to trade-offs between maximizing enzyme yield and achieving effective pollutant removal [69].

Laccase produced from agro-wastes is usually used for biofuel production and bioremediation. The purity requirements for laccase will dictate the extent to which it can be used. Whereas laccase produced from these wastes often finds direct application within the industries for processes like pulp bleaching, dye decolorization, or delignification. This can simplify the overall process, as the enzyme is produced where it is needed, therefore reducing purification costs [69].

The choice of substrate significantly impacts the bioprocess design, even though agro-wastes and industrial wastes present compelling opportunities for sustainable laccase production. Agro-wastes, such as corncob that are known to respond well to pretreatment, often provide an easy path to high laccase yields due to their relatively simpler composition and less inhibitory compounds. While offering the dual benefit of waste treatment and enzyme production, Industrial wastes, still demand sophisticated approaches to manage their characteristic variability and complex inhibitory profiles [69].

Thus, the replacement of complex nutrients using agro-wastes in laccase production will not only reduce environmental pollution but also provide an easily available and affordable laccase for industrial applications ([67]; El-Ramady *et al.*, 2020).This replacement will also bring about the achievement of goals 6, 8, 9 and 12 (SDG 2030) for sanitation and clean water, economic growth, industry, innovation and production and also goal 7 (Agenda 2063) for environmental sustainability. Laccases catalyse breakdown for broad diversity of inorganic and organic compounds, such as aromatic amines, phenolic substrates, phosphates, ketones, and ascorbates. They can convert phenolic compounds in POME into simpler ones by one-electron oxidation [66]. This investigation was aimed at producing laccase from bacterial isolates by utilizing agro-waste as carbon source for treating palm oil mill effluents.

2. Materials and methods

2.1. Samples collection

Exudates were collected from guts of snails (*Cornu aspersum*) sourced from a home-based garden in Bosso Estate, Bosso Local Government in

Minna, Niger State, Nigeria, while soil samples were gathered from dumpsites, automobile workshops, carpentry workshops, rhizosphere of plantain and rice within Minna, Niger State, Nigeria. The snails were first stored inside sterile plastic jars, identified by a zoologist from the Department of Animal Biology, Federal University of Technology, Minna, Niger State, Nigeria and further transferred to Microbiology laboratory, Federal University of Technology Minna, Niger State, Nigeria for microbial analysis. Corncob, sugarcane bagasse and plantain peduncle which were the agro-wastes used were obtained from Kaswua-Gwari market Minna, Niger State, Nigeria and POME was collected raw, directly discharged from a local palm oil milling site located in Anyigba Local Government of Kogi State, Nigeria (Fig. 1).

2.2. Evaluation of bacteria with potential for laccase production

Isolation of laccase-producing bacteria was done according to methods of Sheikhi et al. [67]. Each Ten grams (10 g) of each sample of the soil was poured into sterile distilled water of 100 mL. This created a 1:10 soil-to-water suspension. The 250-mL Erlenmeyer flasks was incubated for 60 min with a continuous agitation at 120 rpm and at 40 °C. A 1 mL aliquot was aseptically transferred from each of the soil suspension into a 9 mL each of sterile distilled water. This served as the initial 10^{-1} dilution. Successive serial dilutions were carried out by transferring 1 mL from the previous dilution tube into 9 mL of sterile distilled water, continuing up to a 10^{-5} dilution. From the 10^{-3} and 10^{-5} dilutions of each soil sample, 0.1 mL aliquots were aseptically spread onto the surface of fresh nutrient agar plates. The plates were then incubated at 37°C for a period of 24 h.

Snails obtained were cut open using sterile forceps, and exudates from the snail gut was streaked on the surface of fresh nutrient agar plates using a sterile swap stick. These plates were subsequently incubated under 37°C for 24 h.

2.3. Primary screening for laccase production (quantitative/plate analysis)

Laccase production was identified primarily from the isolated samples through quantitative assay using Luria-Bertani agar augmented with (0. 01 %) guaiacol. Isolates from the nutrient agar plates were subcultured onto the prepared LB agar plates. These plates were then incubated under 37°C, for 48 h. Isolates that showed the presence of brown colour growth were taken as laccase producers [9].

2.4. Secondary screening for laccase production (qualitative/laccase activity assay)

Qualitative assay was done by inoculating a loopful bacterial suspension prepared from a 24 h old culture grown on nutrient agar. The cells were harvested and re suspended in sterile saline to a standardized optical density OD600 of 0.6 using Shimadzu-UV-1800 series spectrophotometer (Japan). This was carried out for each bacterial isolate positive to the primary screening. A 1 mL of each bacterial suspension was inoculated into a 50 mL sterile Luria Bertani broth that contained 0.01 % (w/v) $CuSO_4$ and 1 % (w/v) fructose. The broth was incubated for 96 h (4 days) at 125 rpm and at 30°C. Laccase activity was examined every 24 h according to the methods of Alkesh and Jaydip [9]. To determine the laccase activity a 5 mL of each sample was carefully removed and then centrifuged for a period of 10 min at 10,000 rpm. Laccase activity was measured using a substrate called guaiacol. A reactive mixture consisting of 2.0 mL guaiacol (0.5 mM), 1.5 mL NaOAc buffer (pH 5.0, 1 mM), and also 1.5 mL cell-free supernatant was prepared. A blank sample was also prepared alongside to subtract background absorbance. The blank contained all components of the reaction mixture except the cell-free supernatant and was treated identically to the experimental samples. This reactive mixture stood for a period of 30 min at 28°C and the enzyme activity was halted using 0.1 mL of 10 % trichloroacetic acid. A UV/Visible Shimadzu-UV-1800 series spectrophotometer (Japan) was used to measure the absorbance at 420 nm. The equation: E. A. = A x Vt x e x v was applied for calculating the activity of laccase in U/mL. E. A: Enzyme activity A: Absorbance at 420 nm; t: time of incubation; v: volume of enzyme (mL); V: total mixture volume (mL); e: extinction coefficient for Guaiacol (substrate) (26.6 mM^{-1} cm^{-1}).

2.5. Analysis of bacterial isolates producing laccase

Isolated bacteria with laccase activity were subcultured on a nutrient agar plate and recognized based on their Gram stain, biochemical test including catalase test, citrate utilization test,oxidase test, indole test, methyl red-Voges Proskauer test, urease test and various carbohydrate fermentation tests,cultural characteristics such as colony morphology pigmentation, and growth patterns., [22], and also their molecular features including DNA Extraction, 16S rRNA gene amplification, agarose gel electrophoresis, DNA sequencing, sequence analysis and phylogenetic analysis [51]. The isolate which exhibited the highest laccase production capacity was submitted to the GeneBank at NCBI and was provided with an accession number.

2.6. Pre-treatment, enzymatic production of cellulase, and agro-wastes hydrolysis

The agro-wastes were pretreated and hydrolysed as follows:

2.6.1. Agro-wastes pre-treatment

The procedure was carried out as described by Makut and Ekeleme [42], which involved washing the agro-wastes with uncontaminated running H_20, then leaving to dry for a period of 5 days and then carefully milling each of them into powder using a blender. The milled agro-wastes were soaked in 200 mL of prepared NaOH solution (1:10) (w/v) for 10 min. Microwave treatment was carried out for about 5 min at 2450 Mhz to delignify the agro-wastes. A neutral pH was reached when the agro-wastes were washed severally after microwaving with clean H_2O.

2.6.2. Cellulase production from Aspergillus niger

The cellulase enzyme was locally produced in the laboratory of the Microbiology Department Federal University of Technology Minna. Cellulase was produced following the method of Omonije et al. [52]. A loopful of the *A. niger* was placed in a basal medium used for cellulase production containing, yeast extract, 2.0; $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; and $FeCl_3$, 0.001 all in mg/L. Carboxymethyl cellulose (CMC) was added at 0.5 mL of 1 % concentration and the culture was grown for 7 days at 25ºC. The culture medium was filtered to obtain the filtrate using Whatman No. 1 filter paper and the culture filtrate was centrifuged using a speed cold centrifuge at a speed of 8000 ×g, 5000 RPM, 4ºC for 10 min, and the supernatant served as the crude enzyme solution [54]. Cellulase assay was done according to the method of Omonije et al. [52]. One millilitre (1 mL) of 1 % CMC in 0.1 M citrate buffer (pH 5.5) was placed in a test tube and 1 mL of crude enzyme was added. The reaction mixture was incubated at 50°C for 30 min and the reaction terminated by adding 1.5 mL of 3,5-dinitro salicylic acid (DNSA) reagent and placing in a boiling water bath at 100ºC for 5 mins, The absorbance was obtained at 540 nm. Enzyme activity expressed as U/mL.

2.6.3. Agro-waste hydrolysis using cellulase

Hydrolysis of agro-waste was conducted by dispensing 20 g of each pre-treated agro-waste into an Erlenmeyer flask. Subsequently, a 20 mL of the cellulase was added to each agro-waste, which was then securely corked prior to utilisation. The Erlenmeyer flasks were immersed into water bath for a period of 48 h at 60 °C, with continuous agitation for even enzyme circulation. To prevent further hydrolysis, the temperature was raised to about 100°C for 48 h, which completely suppressed cellulase activity. Hydrolysed agro-wastes were sieved with the aid of a muslin cloth and further clarified using Whatman filter paper after which hydrolysates were obtained [52].

2.6.4. Evaluation of hydrolysate for laccase production

A Luria-Bertani medium (LB) was made by measuring the following components: yeast extract (5 g), copper sulphate (0.001 g), sodium chloride (10 g), and tryptone (10 g) for screening of the hydrolysate. A 10 mL of each agro-waste hydrolysate was used as a replacement for fructose (the fermentable carbohydrate) and the control contained fructose. After this, 100 mL of each medium (LB and hydrolysates) was poured into 250 mL Erlenmeyer flasks, corked firmly, and allowed to autoclaved at a 121°C for a period of 30 min. After the medium was autoclaved it was then allowed to cool, then 1 mL of bacterial inoculum was poured into each medium then incubated for a period of 48 h at 30°C and 150 rpm. Control sample was prepared without isolate which served as a blank for spectrophotometric reading [64].

2.6.5. Evaluation of agro-waste for laccase production

The agro-waste (corn cob) was analysed using Fourier-transformed infrared spectroscopy, scanning electron microscopy, and x-ray diffraction before and after pre-treatment [12].

2.7. Optimization of conditions for laccase production

The Box Behnken design was used to generate a statistical model for optimization of fermentation conditions using a one-level four-variable full factorial design with 27 fermentation experimental runs where different input values were produced and executed. Several parameters were optimized for laccase production using bacterial isolate with agro-waste as substrate (Supplementary Data (SD) 1).

Andler et al. [10] demonstrated that carbon source, incubation time, pH and temperature are important factors in determining the optimal state for producing and obtaining a high yield of laccase. Every parameter was modified to ensure needed variants. Erlenmeyer flasks holding 100 mL each of the medium were utilized to establish experimental batch cultures. The parameters were optimized by adding a 100 mL of fermentation medium to the required number of flasks, 1 mL of bacterial inoculum and incubating them at 200 rpm. After each run, samples were collected from the flasks, measurements were taken to determine enzyme activity.The volume and method of collection were consistent with the secondary screening for laccase production (qualitative/laccase activity assay) using a spectrophotometer Modelled UV-1800, Shimadzu UV, Japan at a 420 nm.

2.8. Laccase production using the submerged fermentation method

Laccase was synthesized from the best value obtained from the optimization process. Five litres (5 L) of LB broth was used with the following compositions (g/L): yeast extract (5 g), copper sulphate (0.001 g) tryptone (10 g),and sodium chloride (10 g) for laccase production, this represents a scale-up production and a 1 mL of bacterial isolate was inoculated to every 100 mL of the production medium, a 50 mL of bacterial inoculum was prepared and used from a 24 h old culture at 200 rpm, temperature 37℃, pH 9 and carbon concentration of 3.3 % [64].

2.9. Partial purification of laccase

The culture medium now containing laccase was centrifuged at 4°C for a period of 10 min at a 10,000 rpm. Supernatant was further obtained through fractionation using 80 % ammonium sulphate saturation to concentrate by slowly adding ammonium sulfate to the supernatant while stirring continuously on ice, to ensure complete dissolution and to prevent localized high concentrations that could denature the enzyme. Phosphate buffer of 100 mL was poured into the Erlenmeyer flask after centrifugation, this samples were then further subjected to high dialyzing in phosphate buffer (50 mM, pH 8.0).This was done by placing the protein solution inside a semi-permeable dialysis membrane and immersing the membrane into a 100 mL of dialysis buffer for 12 h. Protein concentration was determined at the stages of purification using the Bradford assay with bovine serum albumin (BSA) as a standard. Laccase activity was checked when the crude enzyme was dissolved in phosphate buffer as described in secondary screening of laccase production [9].

2.10. Determination of physico-chemical parameters of palm oil mill effluent

POME samples were collected in clean sampling bottles and transported to the laboratory at 4°C. Effluent samples were analysed for this physicochemical parameter (chemical oxygen demand, pH total dissolved solids total suspended solids, biochemical oxygen demand, and turbidity) using American Public Health Association (APHA) Standard Methods for the Examination of Water and Wastewaters [11].

2.11. POME treatment

The POME was treated with partially purified laccase, according to the procedure of Mehandia et al. [43] with slight modifications, to assess its potential for industrial uses. A constant volume of enzyme at an activity of 10.76 U/mL was optimized with variable measurements of POME in the following ratio, 1:1, 1:10, 1:100, 1:1000, 1:10000 and 1:100000. This preliminary study was followed by measuring absorbance at 272 nm at 0 h and after 24 h at 28°C. A ratio of 1:100 was chosen as the most effective as regards to this setup based on its highest absorbance difference after 24 h. A large-scale treatment was performed in 1000 mL conical flasks containing 505 mL final reaction volume. Five milliliters (5 mL) of partially purified laccase and 500 mL POME were contained in the reaction blend, these were incubated for 24 h at temperature 28°C.Treatment of POME was followed by measuring absorbance at 272 nm before and after 24 h. This POME was further analysed for its physicochemical parameters after 24 h using APHA Standard Methods for the Examination of Water and Wastewaters and matched with the physicochemical parameters of the untreated POME.

Calculations for Pollutant Reduction and Enzyme Efficiency:

Total Laccase Units Applied: volume of laccase used(mL) × enzyme activity U/mL

Total Parameter Reduced: initial value(untreated) − final value (treated) = Total reduction

Laccase Efficiency: Total Laccase Units Applied/Total reduction

2.12. Data analysis

Data acquired from these studies were assessed using linear model analysis of variance to find the mean differences at 5 % (0.05) significant level [50].

3. Results

3.1. Laccase production performance of bacteria after secondary screening

The bacterium colonies visualized by appearance of brownish coloured growth indicating substrate oxidation when cultured on Luria-Bertani medium in the presence of 0.01 % guaiacol were selected as laccase producing isolates. Out of the 35 bacteria isolated, five appeared positive to this quantitative screening with isolates Sn2 and D3 showing a deeper colour intensity and C3 showing the faintest brownish growth amongst the five positive isolates

These five isolates demonstrating brownish growth were further subjected to secondary screening. Among them, the bacterial isolate obtained from the snail gut, designated as Sn2 displayed the maximum activity of 6.4 U/mL. This isolate was taken as the best competent laccase producer. Fig. 2 shows the secondary screening result of the isolates.

3.2. Identity of laccase-producing bacteria

Isolates identified were species from genus *Bacillus* (2) and *Pseudomonas* (3). The molecular character of the isolate with the best laccase production ability showed that it had a linear DNA fragment of size 1500 bp (Fig. 3). The sequence revealed the isolate to be 99 % similar to *Pseudomonas aeruginosa* with an accession number of OR687603 (Table 1). The phylogenetic tree (Fig. 4) showed that it is related to other Gram negative bacteria including *Proteus vulgaris*, *Escherichia coli, Citrobacter freundii*, *Klebsiella pneumoniae* and *Alcaligenes faecalis*.

3.3. Hydrolysate and Laccase production

Hydrolysates screening for the production laccase revealed that corn cob showed the maximum laccase activity, by a recorded value of 7.25 U/mL. In contrast, sugarcane bagasse displayed the lowest activity, with a measurement of 6.65 U/mL, while plantain peduncle recorded 6.83 U/mL activity.

3.4. Optimized laccase production conditions

Optimized laccase production conditions results are presented for pH, incubation time, temperature and carbon source. Details are shown in **SD 2.** An increase in the carbon concentration showed a decrease in laccase activity. When carbon concentration was 3 % a laccase activity of about 4.7 U/mL was obtained but at a higher concentration of carbon at 7 % a lower laccase activity of 4.5 U/mL was obtained. A negative interaction was obtained between carbon concentration and laccase activity (Fig. 5).

The results in Fig. 6 showed a positive interaction between pH and laccase activity. Less laccase activity (3 U/mL) was obtained when the pH was low (pH 5), while the highest laccase activity (6.5 U/mL) for this interaction was obtained when the pH was 9. This shows a change in pH notably influences laccase activity. Incubation time has an insignificant effect on the activity of laccase, as incubation time increased from 60 h to 96 h the value for laccase activity remained constant. The outcomes showed that there was no form of interaction between temperature and laccase activity. An increase in temperature neither increased nor decreased laccase activity.

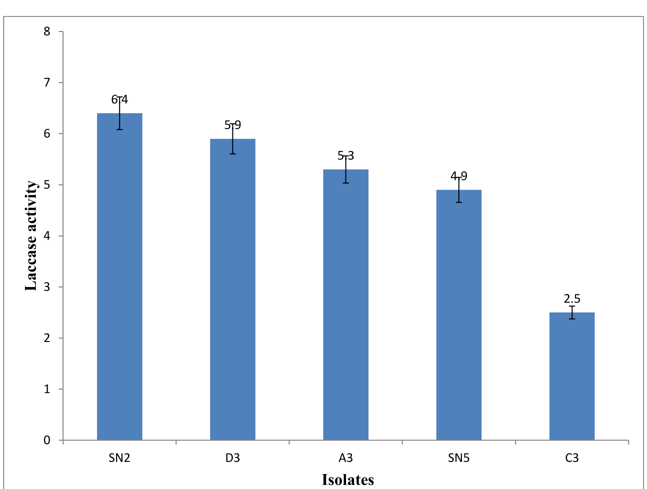

Fig. 2. Laccase production performance of bacteria after secondary screening, SN2 = Snail 2, D3 = Dumpsite soil 3, A3 = Automobile workshop soil 3, SN5 = Snail 5, C3 = carpentry workshop soil 3.

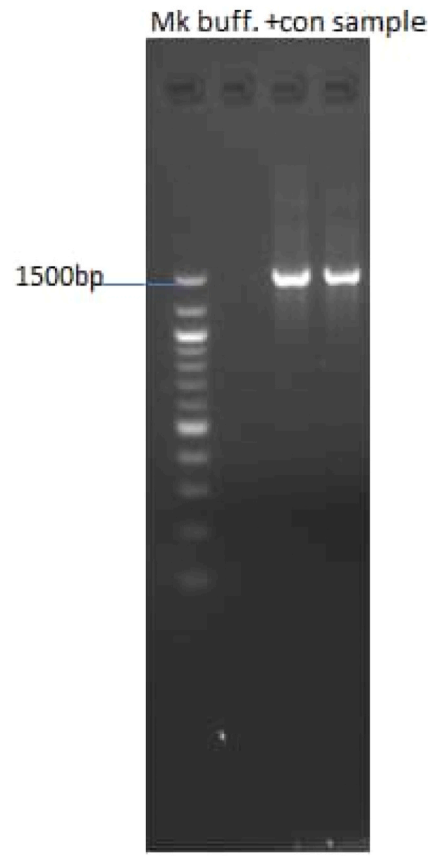

Fig. 3. Agarose gel electrograph of the positive amplification of the 16S rRNA partial gene of selected bacterial isolate.

3.5. The effect of temperature and pH on laccase activity using using *Pseudomonas aeruginosa* and corncob

In the experimental setup Fig. 6 revealed that, an activity of 4 U/mL was obtained when temperature was between 25 °C to 37 °C and pH of 5.7. Temperature remained between 25 °C to 37 °C as pH of the medium increased to a 7.4. In contrast, activity increases from 4 to 7 U/mL. This therefore indicates that pH is the factor of influence in this interaction, and although pH had a notable influence, the interaction between temperature and pH on activity was not significant ($p > 0.05$).

3.6. The effect of incubation time and pH on laccase activity using *Pseudomonas aeruginosa* and corncob

The result presented in Fig. 7 shows that at an incubation time of 75 h and a pH of 5.7, an activity of about 3 U/mL was obtained. As the time increased to 87 h and pH increased to 6.5 activity also increased to a 4 U/mL. Although at a pH of about 8.5 and reduction in incubation time to 75 h, the optimum activity of the enzyme was 7 U/mL. Therefore this result shows that the relationship concerning incubation period and pH shows a significant influence on activity of laccase ($p < 0.05$).

3.7. The effect of pH and carbon concentration on laccase activity using *Pseudomonas aeruginosa* and corncob

As shown in Fig. 8 at a carbon concentration of 5.9 and pH of 6.2, an activity of 4 U/mL was obtained as carbon concentration decreased to 5.3 and pH increased to 7.3 an activity of 6 U/mL was obtained. The result shows that the relation between carbon concentration and pH has

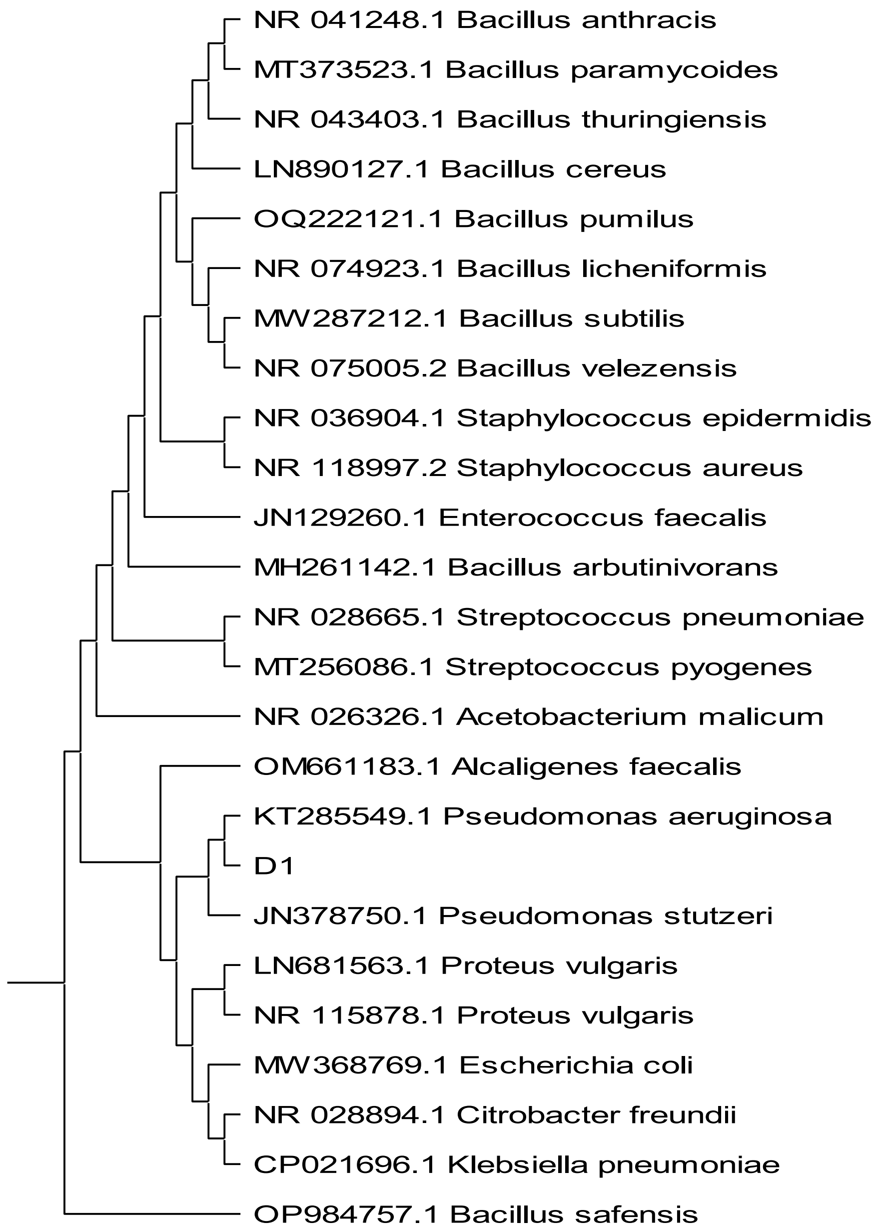

Fig. 4. Phylogenetic tree of the relationship among the bacterial isolates.

a significant effect on laccase activity as regards to this experimental setup ($p < 0.05$).

3.8. The effect of temperature and incubation time on laccase activity using Pseudomonas aeruginosa and corncob

Fig. 9 shows a rise in activity (4.8–5 U/mL) from an incubation time of 85–96 h and a temperature of 29–35 °C, the increase of laccase activity is minute and hence can be said that the interaction concerning temperature and incubation time does not have a notable influence ($p > 0.05$) on the activity.

3.9. Effect of carbon concentration and temperature on laccase activity using Pseudomonas aeruginosa and corncob

The result in Fig. 10 shows an increased laccase activity with a decrease in carbon concentration at a decreasing temperature. The relationship between temperature and carbon concentration as regards this set-up is not significant as the activity is minute (4.5–5.0 U/mL) ($p > 0.05$).

3.10. Effect of carbon concentration and incubation time and on laccase activity using Pseudomonas aeruginosa and corncob

Carbon concentration of 7 and an incubation time of 96 h resulted in an activity of 4 U/mL was obtained (Fig. 11). At a steady decrease in the carbon concentration and change in incubation time there is an increase in activity. The result therefore shows that the interaction between carbon concentration and incubation time has a significant effect on laccase concentration ($p < 0.05$).

3.11. Desirability responses generated from evaluated data for validation of laccase production

One hundred (100) desirability solutions were generated (**The details are presented in SD 2)** for the effects of pH, incubation time, carbon source and temperature on laccase activity from the 27 runs carried out (**The details are presented in SD 3).** The best desirable

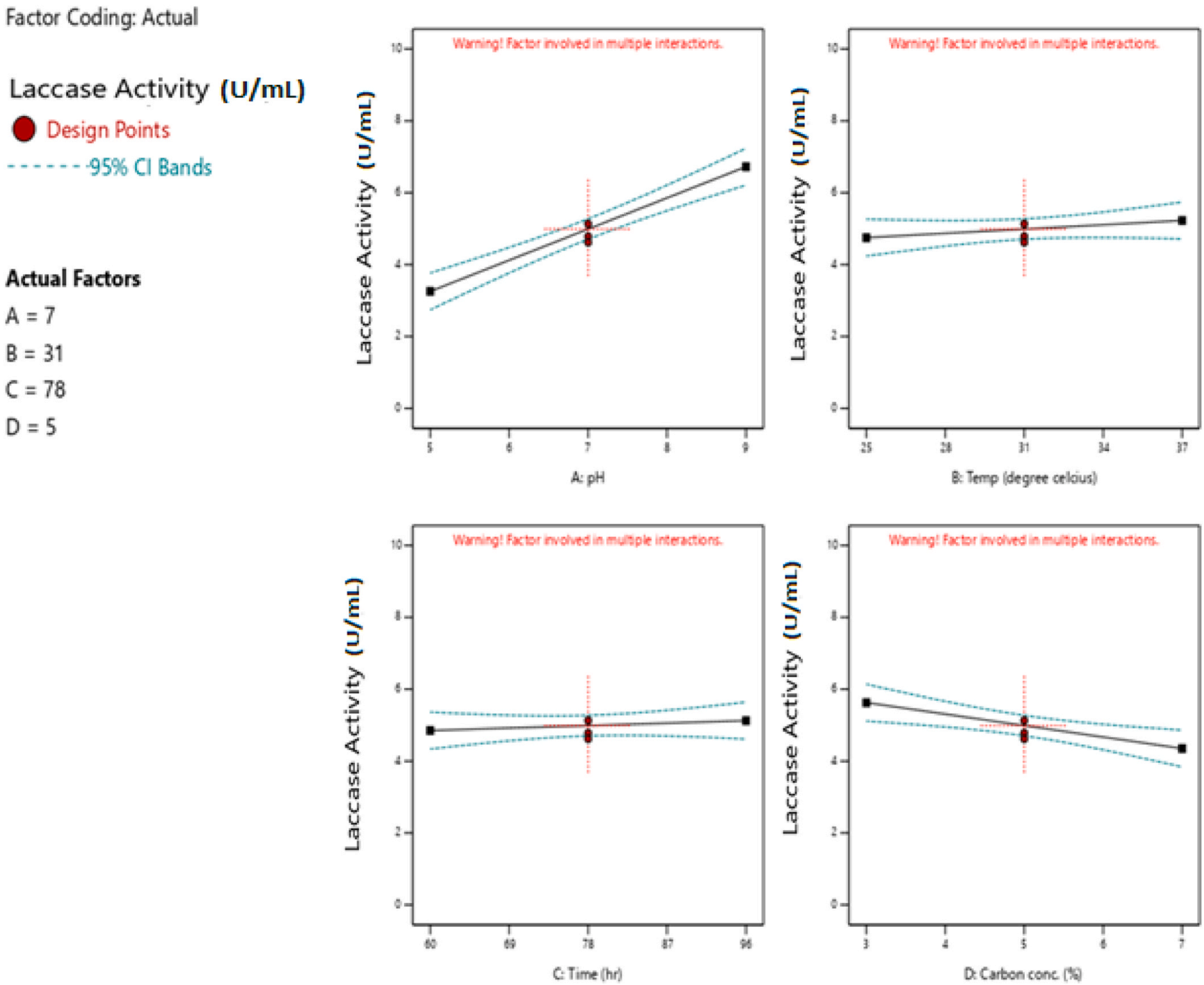

Fig. 5. Response surface for the effect of temperature, pH, incubation time and carbon concentration on laccase activity using *Pseudomonas aeruginosa* and Corncob.

optimum condition was chosen from the 100 solutions generated to validate the optimization process. For this experiment, the following conditions were selected: pH 9, incubation time of 60 h, a carbon concentration of 3.3 %, and temperature of 37 °C. The desired objective was 0.916, and the predicted activity was 8.96 U/mL.

3.12. Validated optimized process

Validation of the optimised process (Table 2) indicated a predicted activity of 8.96 U/mL under the conditions of 60 h incubation time, a 3.3 % carbon concentration, temperature of 37°C and a pH of 9, while the experimented value was observed to be 8.49 U/mL within exact conditions.The predicted values are seen in **SD 2**.The variance between the predicted and experimented laccase activity was a 0.47 which represented 5.2 % deviation, an acceptable deviation range as it is within the limit of ±8.4 %.

3.13. Laccase activity after partial purification

Table 3 shows the special effects of the ammonium sulphate precipitation and subsequent dialysis on laccase activity and also the protein concentration. Following the step of precipitation, the activity of laccase was measured at 10.50 ± 0.04 U/mL. The concentration of the protein at this stage was 1.65 ± 0.13 mg/mL. Following dialysis, laccase activity exhibited a slight increase to 10.76 ± 0.11 U/mL, while concentration of the protein also rose a little to 1.86 ± 0.12 mg/mL ($p < 0.05$).

3.14. Physicochemical parameters of POME after treatment

After optimizing the laccase-to-effluent ratio needed for POME treatment **(SD 9),** a substantial difference was observed between the control group and treatment group ($p < 0.05$) using a *t*-test. The most effective ratio for effluent treatment, with a % difference of 108.51 ± 0.50, was observed at 1:100 while 1:100000 recorded the lowest (36.33 ± 0.40).

Before treatment, the physicochemical properties of the raw effluent were as follows: pH (3.57 ± 1.33), BOD (23.67 ± 0.55 mg/L), COD (1875.10 ± 0.07 mg/L), TDS (25.94 ± 0.78 mg/L), TSS (1.41 ± 0.09 mg/L), and turbidity (204.27 ± 1.49 NTU). Following laccase treatment, these parameters were re-analysed, yielding the following results: pH (3.73 ± 0. 75), BOD (7.33 ± 1.33 mg/L), COD (1542.90 ± 0.05 mg/L), TDS (19.62 ± 0.54 mg/L), TSS (0.72 ± 0.05 mg/L), and turbidity (195.11 ± 1.76NTU). The assessment of physicochemical analysis between untreated POME and laccase-treated is presented in Table 4 while Table 5 presents the laccase treatment efficiency of palm

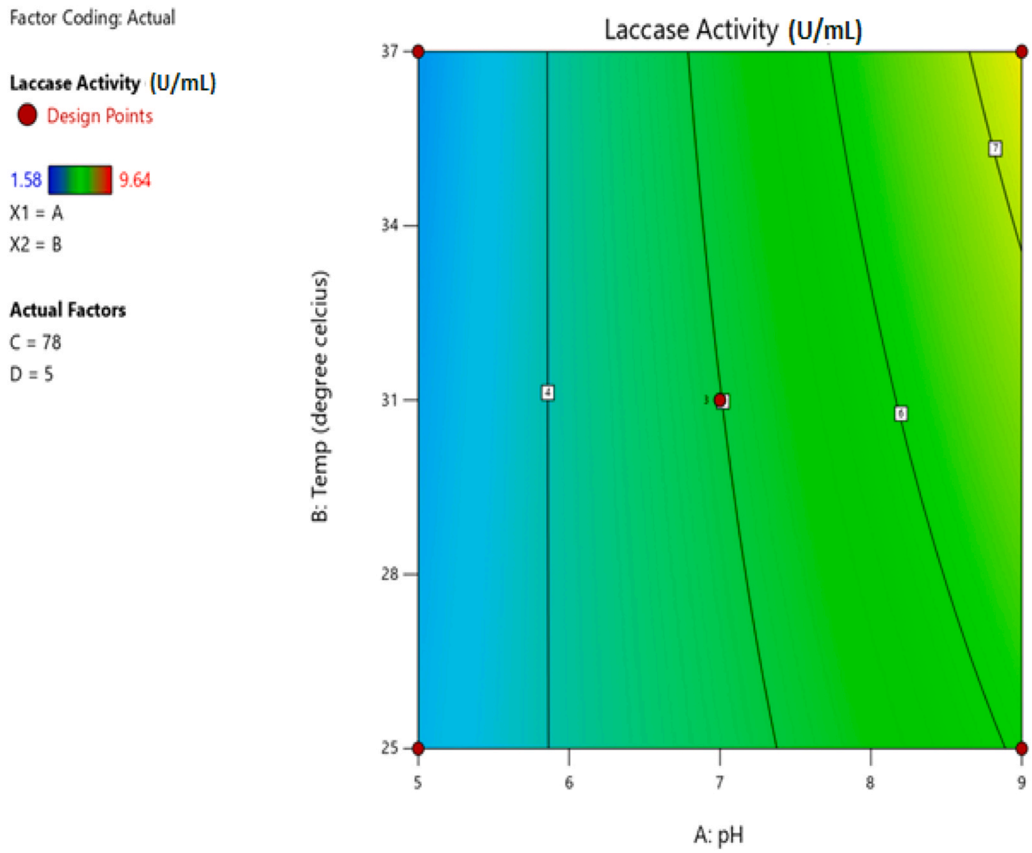

Fig. 6. The effect of temperature and pH on laccase activity using *Pseudomonas aeruginosa* and corncob.

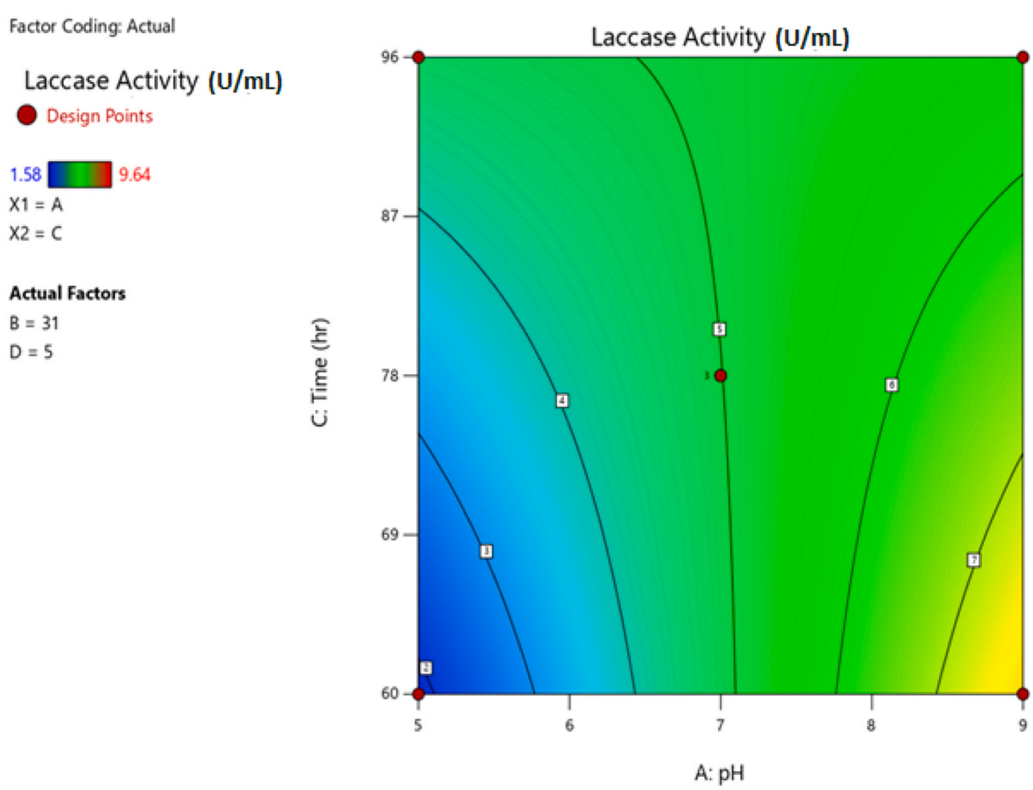

Fig. 7. The effect of incubation time and ph on laccase activity using *pseudomonas aeruginosa* and corncob.

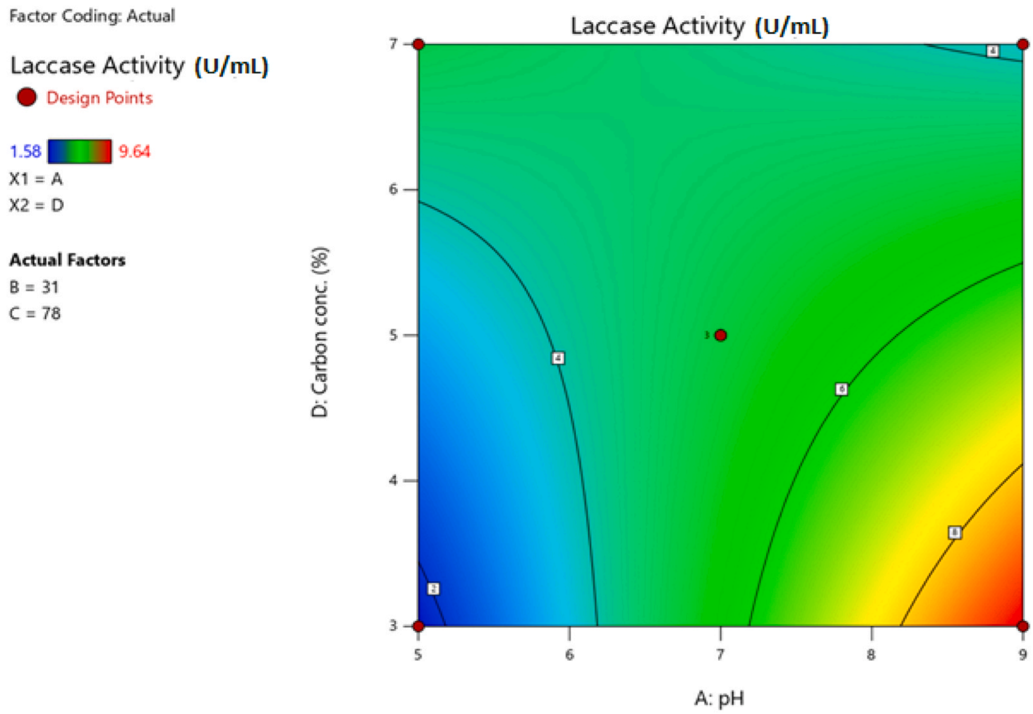

Fig. 8. The effect of carbon concentration and ph on laccase activity using *pseudomonas aeruginosa* and corncob.

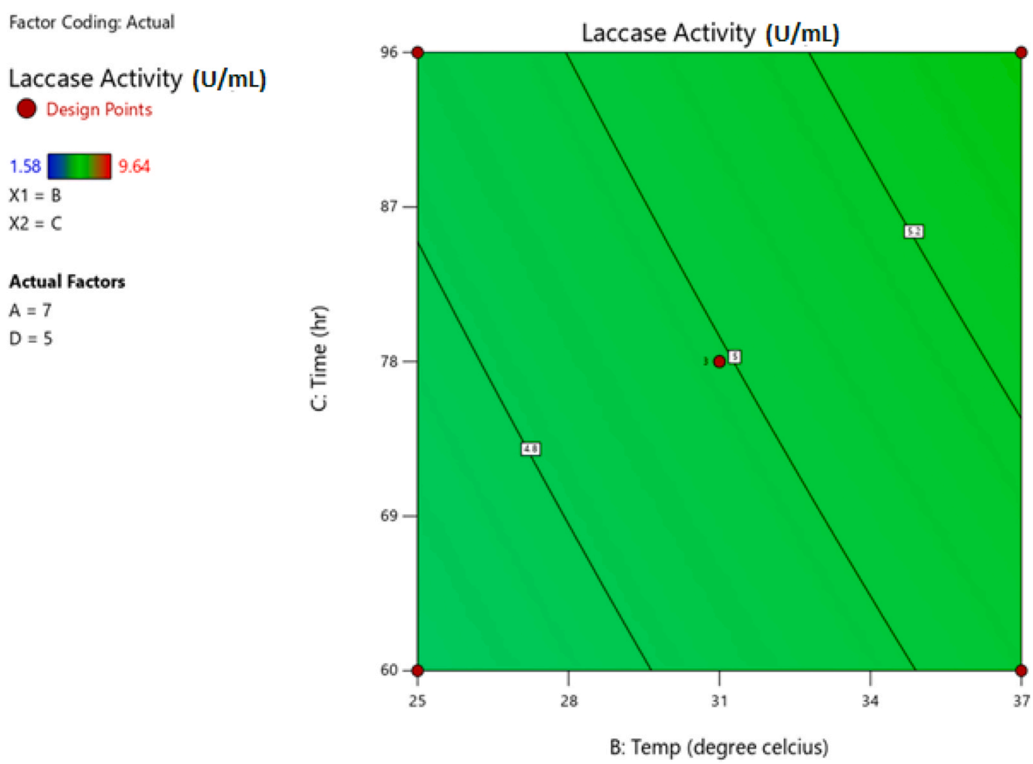

Fig. 9. The effect of temperature and time on laccase activity using *pseudomonas aeruginosa* and corncob.

oil mill effluent.

4. Discussion

The recorded laccase activity ranged from 2.5 to 6.38 U/mL. Previous research has highlighted laccase synthesis by bacteria. Rajeswari and Bhuvaneswari [59] screened about twenty-two bacterial isolates for laccase synthesis ability after using a fresh nutrient agar media with a 0.1 % guaiacol for screening, they noted the production of reddish-brown colouration as positive for laccase production. Similarly, Shukur. [68] employed syringaldazine as an indicator and substrate for the determination of laccase activity to screen over 50 *Bacillus* isolates, among which only three were positive. The isolates were also examined for the production of laccase quantitatively by measuring their laccase

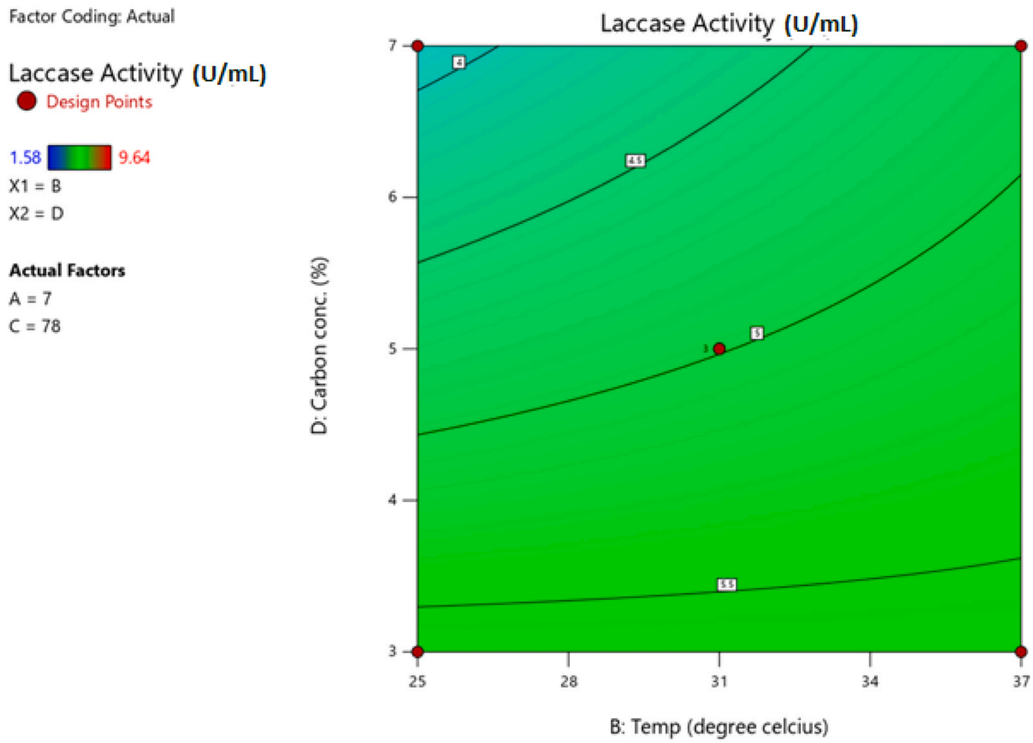

Fig. 10. The effect of carbon concentration and temperature on laccase activity using *pseudomonas aeruginosa* and corncob.

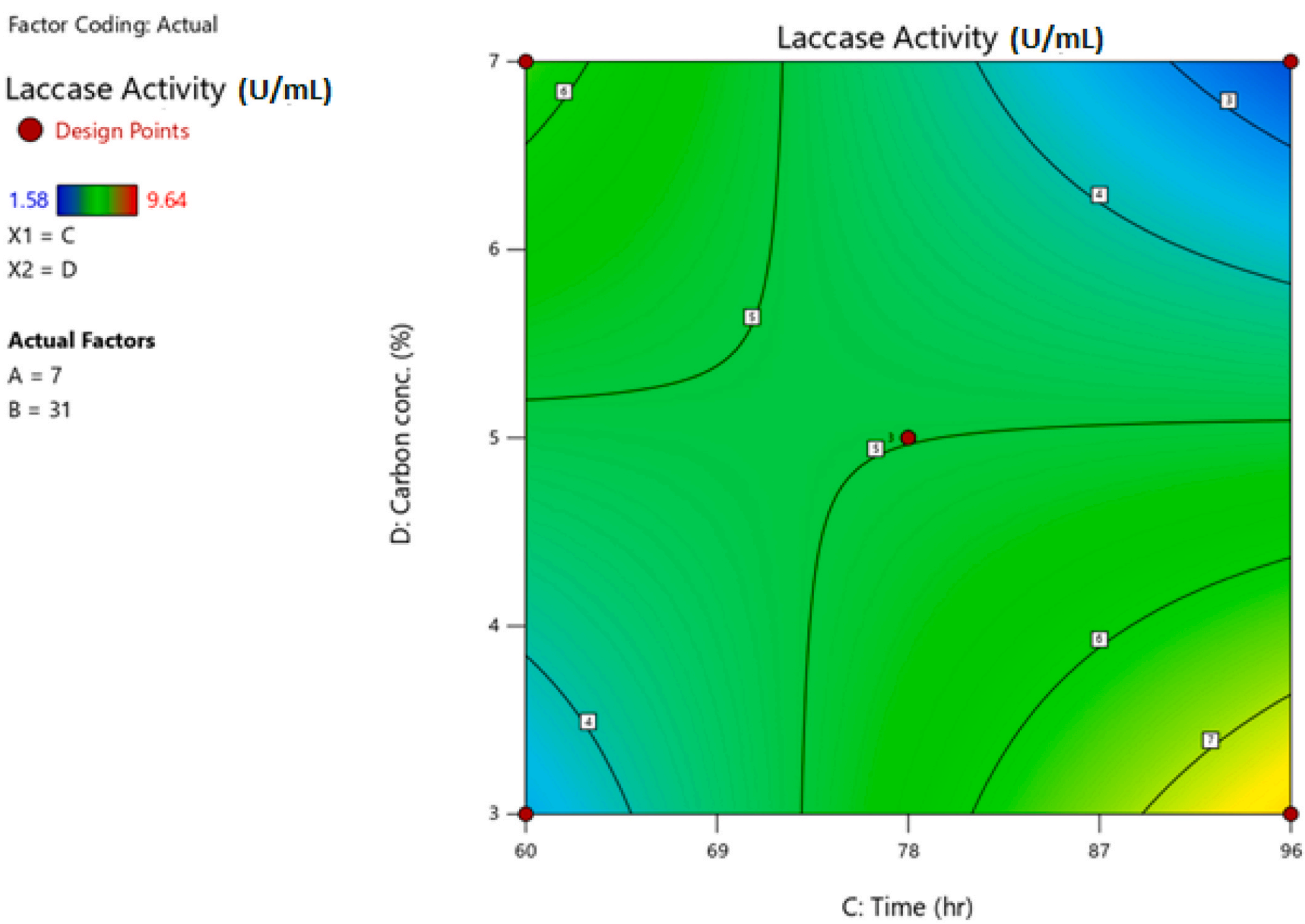

Fig. 11. The effect of carbon concentration and incubation time on laccase activity using *pseudomonas aeruginosa* and corncob.

Table 2
Validation of the optimization process.

Factors	Values	Predicted	Experimented	Difference	% change
Carbon conc (%)	3.6				
Incubation Time (h)	64	8.96	8.49	0.47	5.2
Temperature (° C)	37				
pH	9				

Table 3
Laccase activity and protein concentration after partial purification (T-test).

	After ammonium sulphate precipitation	After dialysis
Laccase activity (U/mL)	10.50 ± 0.04^a	10.76 ± 0.11^b
Protein concentration (mg/mL)	1.65 ± 0.13^a	1.86 ± 0.12^a

KEY: Value Mean ± Standard Deviation of mean (N = 3). ($p < 0.05$)

Table 4
Physicochemical Parameters of treated and untreated Palm oil Mill Effluent (T-test).

	Treated	Untreated	Environmental protection agency (EPA) limit
pH	3.73 ± 0.75^b	3.57 ± 1.33^a	6–9
BOD (mg/L)	7.33 ± 1.33^a	23.67 ± 0.55^b	50.00
COD (mg/L)	1542.90 ± 0.05^a	1875.10 ± 0.07^b	250.00
TDS (mg/L)	19.62 ± 0.54^a	25.94 ± 0.78^b	2000.00
TSS (mg/L)	0.72 ± 0.05^b	1.41 ± 0.09^a	30.00
Turbidity (NTU)	195.11 ± 1.76^b	204.27 ± 1.49^a	75.00

KEY: Value Mean ± Standard Deviation of mean (N = 3). Mean within rows with the same superscripts are not significantly different ($P > 0.05$).

Table 5
Laccase Treatment Efficiency of Palm Oil Mill Effluent (T-test).

Parameter	Initial Value (500 mL POME)	Final Value (500 mL POME)	Total Reduction	Laccase Efficiency (U/unit reduced)
COD (mg)	937.55 ± 0.95^b	771.45 ± 1.20^a	166.10	0.324 U/mg COD
BOD (mg)	11.84 ± 0.21^b	3.67 ± 0.05^a	8.17	6.585 U/mg BOD
Turbidity (NTU)	204.27 ± 1.50^b	195.11 ± 1.45^a	9.16	5.873 U/NTU

Note: Total Laccase Units Applied = 53.8 U (5 mL of 10.76 U/mL laccase). ($P > 0.05$).

activity, with the highest levels of laccase activity recorded being 1.84 U/mL.

The screening method employed, which involved a plate assay containing LB medium supplemented with 0.01 % guaiacol, provided a conducive environment for identifying laccase-synthesing strains. The guaiacol used serves as a substrate for laccase activity, undergoing oxidation in the presence of the enzyme to produce a characteristic colour change (brownish growth) which serves as a visual indicator of laccase production potential among the microbial isolates [73]. The detected laccase synthesis might be affected by the growth conditions and metabolic capabilities of the microbial isolates [35].Certain microbial strains may possess competent metabolic pathways for laccase biosynthesis or may flourish under precise conditions such as temperature, oxygen levels, pH and nutrient availability used in the screening process [16].

A total of 35 bacterial isolates were obtained from the bacteriological analysis of soil and snail gut exudate, among which 5 showed capability for laccase synthesis. The isolates were acknowledged to belong to the genera *Pseudomonas* (3 isolates) and *Bacillus* (2 isolates) with one *Pseudomonas* specie exhibiting the maximum laccase synthesis capability and was designated for molecular analysis. From the NCBI blast data, the isolate was identified as *Pseudomonas aeruginosa* (OR687603). Our finding aligns with Verma et al. [75] who in his study isolated *Pseudomonas putida* LUA15.1 from soil samples, which demonstrated the capability of producing extracellular laccase enzyme. Also, Verma et al. [75] isolated *Pseudomonas putida* LUA15.1 from soil samples, which demonstrated the capability of producing extracellular laccase enzyme. The occurrence of the observed bacteria species in soil as stated in the various studies could mainly be ascribed to the increased level of organic matter, moisture and other favourable conditions that support microbial growth that are present in such environment [76].In addition, the quantity and kind of microorganisms present in a specific soil would be significantly influenced by geographical setting, organic matter contents, soil temperature, soil pH, soil type, cultivation, aeration and moisture content [45].

In the present study, hydrolysates obtained from the cellulase hydrolysis of different agricultural residues (plantain peduncle, sugar cane bagasse and corn cob) were screened for hydrolysates for laccase production.

The result showed significant variations in activity levels among the different agricultural residues. Specifically, corn cob had the highest laccase activity at 7.25 U/mL, followed by plantain peduncle with a recorded activity of 6.83 U/mL and sugarcane bagasse demonstrated the lowest activity, measuring 6.65 U/mL. The production of laccase enzyme by these organisms can be associated to the fairly high ash content, crude protein content and carbohydrate content of the agro-wastes. The nutritional contents of the agro-wastes serve as source of minerals, nitrogen and fermentable sugar carbon source respectively for the development of the bacteria and subsequent synthesis of laccase [15]. Agro-wastes has a compact structure that restricts the access of microorganisms to the cellulose components. NaOH pretreatment effectively disrupts the lignin-hemicellulose matrix, which leads to the creation of new pores and an expansion of the overall surface area. A large surface area can provide more attachment sites for microbial cells, easing the breakdown of cellulose into fermentable sugars. These sugars then serve as carbon sources for microbial growth and also inducers for laccase production. Higher porosity also aids in efficient nutrient uptake by microbes and higher product release into the medium [20,46,7]. These agro-wastes also contains crystalline and amorphous regions of cellulose. Crystalline cellulose is highly ordered and resistant to enzymatic degradation, while amorphous cellulose is more readily hydrolyzed. NaOH pretreatment can reduce the crystallinity of cellulose by disrupting hydrogen bonds. A decrease in crystallinity therefore makes the cellulose more susceptible to enzymatic attack by cellulases and hemicellulases. This enhanced digestibility provides more readily available sugars for microbial metabolism and enzyme synthesis([60]). Particle size reduction such as through milling, as used in this study also significantly impacts microbial access. Smaller particle sizes increase the overall surface area and reduce the diffusion limitations for enzymes and microorganisms [6].

Corncob which is observed to yield higher laccase activity in this study, is likely to be due to a more favorable combination of these physical characteristics after NaOH pretreatment compared to sugarcane baggase and plaintain penducle.

Kumar et al. [37] evaluated numerous varieties of agro-wastes for laccase production from *Bacillus* sp. AKRC01. Their results noted highest production of laccase (1.954 U/ mL) in rice bran then by wheat bran (1.625 U/mL), followed by sawdust (0.954 U/mL), and potato peel (1.326 U/mL), orange peel (0.864 U/mL), pea peel (1.448 U/mL), banana peel (1.322 U/mL) and lastly sugarcane bagasse (1.365 U/mL). Corn cob, having the highest laccase activity, is likely due to its high cellulose content and porous structure which facilitates microbial activity and enzyme production. In contrast, sugarcane bagasse, although rich in lignocellulosic material, displayed comparatively lower laccase activity, which could stem from factors such as its lignin content or structural intricacy [24].Wang et al. [76] noted that, beyond the conventional cellulose, hemicellulose, and lignin components, additional factors like sugar, crude protein, and metal ions present in the substrate may influence microbial growth and enzyme production, potentially contributing to the observed variations in laccase activity. Additionally, variations in nutrient availability, pH, and other environmental factors in the hydrolysates may also contribute to differences in laccase activity levels.

Microbial growth and metabolism are dependent on carbon availability [63]. Afreen et al. [5] reported that the concentration and the nature of carbon source used in the medium have a remarkable effect on enzyme synthesis. In this present study, an increase in the carbon concentration results in a decrease in activity. At a concentration of 3 % a laccase activity of 4.7 U/mL was recorded but at a higher concentration of carbon at 7 % a lower laccase activity of 4.5 U/mL was obtained. A comparable finding was stated by Elshafei et al. [28] in their work on laccase optimization from *Penicillium martensii* NRC 345. The study noted that the activity increased with increasing galactose up to 5 g/L, which was the ideal concentration for laccase formation. However, increasing galactose concentration above this value resulted in a steady decline in activity. The observed decline in activity with rising carbon concentration suggests a potential inhibitory influence of higher carbon levels on laccase synthesis. This finding may be attributed to factors related to the metabolic processes and environmental conditions within the microbial culture. High carbon concentrations, can inhibit laccase production by triggering carbon catabolite repression. This alters microbial metabolism toward growth, suppressing enzyme synthesis. Hence, laccase gene expression is repressed, reducing enzyme yields. Optimal laccase production has been recorded in previous studies to occur under low-carbon or stress conditions that favor secondary metabolism [80]. You et al. [79] in their study also stated that during rapid growth, bacterial cells prioritize primary metabolism by allocating proteomic resources to vital functions like replication and energy production. This therefore limits the production of secondary metabolites, such as enzymes,resulting in reduced enzyme production under nutrient-rich conditions favoring growth.

Results from this current study indicate a notable influence of pH on activity. The highest activity of 6.5 U/mL was recorded at an elevated pH of 9. This finding shows the impact of pH variations on laccase activity, suggesting that optimal pH conditions are crucial for maximizing laccase enzyme efficiency. This result was similar with the findings of Narayanan et al. [47] who also recorded that the optimal pH for production of laccase from *B. subtilis* MTCC 2414 using agro residues was 9.0 which yielded an enzyme activity of 266 ± 4.02 U/mL. However contrary findings were reported in various other studies. Albulaihed et al. [8] reported pH value of 6.0 as the best for laccase production from *Stenotrophomonas maltophilia* E1 by submerged fermentation. The observed variations in the findings of these studies could be attributed to dissimilarities in the microbial strains used, also variations in the component of the fermentation medium and cultivation conditions. Cultural and methodological variances among studies, such as inoculum size, also fermentation duration, and agitation rate, can also contribute to discrepancies in optimal pH values for laccase production.

The present study showed that incubation time has no substantial effect on activity, as laccase activity remained constant with increasing incubation time from 60 h to 96 h. The result noted that despite the prolonged duration, there was no notable increase in laccase activity. This plateau suggests the beginning of diminishing activity with extended incubation. This has been reported in other studies including Narayanan et al. [47] who reported optimal laccase production by *Bacillus subtilis* MTCC 2414 at 72 h, and stated that further increase in time did not increase laccase activity, implying a threshold period after which enzyme production stabilizes or drops.

Mohsen et al. [44] also in their report highlighted that extended incubation beyond optimal incubation time frames can lead to enzyme degradation, feedback inhibition, or accumulation of toxic metabolites. In their study they also reported optimal incubation time frames to be 72–96 h yielding a maximum laccase activity of 0.922–1.040 U/mL. The record for this inhibitory effects could be as a result of nutrient depletion. As the culture ages, nutrients essential for microbial growth and enzyme production are exhausted, reducing metabolic activity. This can also be attributed to proteolytic degradation that may occur during fermentation. As accumulated proteases may degrade laccase. In longer incubations, oxygen demand may be more than oxygen supply in submerged cultures, ultimately limiting oxidative enzyme activity for laccase [10,16].

The result from the current study indicated that within this specific temperature range, there was no notable influence of temperature on laccase activity. An increase in temperature from 25 °C to 37 °C neither increased nor decreased laccase activity. However, Kumar et al. [37] in their study noted that temperature is a crucial influence affecting enzyme production. They reported that increasing the temperature up to 35°C increased laccase production. However, beyond 35°C, laccase production decreased, which contrasts with our findings of no notable influence. Albulaihed et al. [8] reported 35°C as the optimal incubation temperature for laccase production in *Stenotrophomonas maltophilia* E1. Their report aligns with the upper end of our studied range and suggests that 35°C is a regularly observed optimal temperature for bacterial laccases. Also, Narayanan et al. [47] in their study achieved maximum laccase production at a temperature of 30°C.Which falls within our range and further supports the idea that the laccase in your study is active and stable within this temperature window. Enzyme activity is highly dependent on temperature, generally increasing with temperature up to an optimum, after which denaturation occurs. The lack of a significant effect on this temperature range (25–37°C) in our study may suggest that this range falls within the optimal or near optimal temperature for the *Pseudomonas aeruginosa* laccase used in this study. Many bacterial laccases have been reported to have a robust activity across a moderate temperature range [33]. This can be an advantage for industrial applications, since it implies less stringent temperature control, potentially reducing operational costs.

The present result indicated that pH significantly affected the laccase activity obtained, having the highest activity recorded at a pH 7.4 compared to a pH 5.7. Specifically, activity rose from 4 U/mL at a pH 5.7 to an activity of 7 U/mL at a pH 7.4 within the temperature of 25°C to 37°C. However, the temperature discrepancies in this range did not exert a significant effect on the activity. The observed increase in activity with an increase in pH is consistent with Narayanan et al. [47] who reported that a rise in pH resulted in an increase in laccase production from *B. subtilis* MTCC 2414. This shows the crucial role of pH in enzyme activity regulation. pH influences enzyme structure and function, affecting substrate binding and catalytic efficiency [33]. However, the lack of noteworthy interaction between temperature and pH on laccase activity suggests that temperature variations within this studied range did not exert a substantial influence on enzyme activity when pH was adjusted.

Results from the effect of pH and incubation time show discernible trends where alterations in these factors lead to corresponding changes in laccase activity levels. It was observed that at an incubation time of 75 h and pH of 5.7, an activity of 3 U/mL was recorded, indicating a baseline performance under these conditions. Conversely, at pH 8.5, coupled with a reduced incubation time of 75 h, the maximum laccase activity surged to 7 U/mL, showing the critical role of pH and incubation time in modulating enzyme activity, with certain combinations favouring enhanced activity. Furthermore, the results also show the dynamic interaction between incubation time and pH on laccase activity. The observed result showed how a change in one variable can influence the outcome when combined with changes in the other. Results showed a rise in laccase activity from about 3 U/mL to a 4 U/mL as pH transitions from a 5.7–6.5, accompanied by an extension in incubation time from 75 h to 87 h. These results demonstrated the synergistic impact of incubation time and pH on laccase enzyme activity. Kumar et al. [37] and Albulaihed et al. [8] recorded similar findings to those of the present study where both incubation time and pH impacted laccase activity. The observed increase in laccase activity with varying pH levels and incubation times suggests an interaction between these factors.

In this present study, carbon concentration and pH had a substantial impact on activity. At a carbon concentration of 5.9 and pH of 6.2 the activity was measured at 4 U/mL. This initial observation suggests a baseline level of enzyme activity under these specific conditions. As the carbon concentration decreased to 5.3 and pH increased to 7.3, the activity notably increased to 6 U/mL. This increase in enzyme activity with higher pH and lower carbon concentration indicates a significant effect of the interaction between pH and carbon concentration on laccase activity within the experimental setup.

The observed increase in laccase activity as pH rises and carbon concentration decreases aligns with known principles of enzyme kinetics and substrate availability [29]. Higher pH levels might enhance the stability and activity of the enzyme, while lower carbon concentrations could potentially alleviate substrate inhibition or competition, leading to increased enzyme-substrate interactions and higher catalytic rates [78]. Studies by Bi et al. [17], Umar and Ahmed [72] also observed that pH and carbon concentration had significant effect on laccase activity.

Results from this study revealed a slight increase in activity from about 4.8 to a 5 U/mL as the temperature increased from 29°C to 35°C, coupled with an extension in the incubation time from 85 h to 96 h. However, regardless of this rise, the amount of the change in laccase activity is minimal. This observation suggests that the effect of the interaction between temperature and incubation time on laccase activity is not substantial within the experimental parameters tested.

Result from effect of carbon concentration and temperature on laccase activity showed that as the carbon concentration decreases, activity tends to increase, mainly at lesser temperatures. The observed activity ranged from about 4.5 U/mL to 5.0 U/mL through varying temperatures and carbon concentrations. However, observed deviations in laccase activity are relatively minor, suggesting that the relationship between temperature and carbon concentration within this particular experimental setup is not significant. The slight increase in laccase activity with decreasing carbon concentration and at lower temperatures indicates a potential interaction between these factors as lower carbon concentrations may alleviate substrate inhibition or competition, allowing for more efficient enzyme-substrate interactions and higher catalytic rates [78]. Similarly, lower temperatures might enhance enzyme stability or reduce non-specific reactions, further contributing to the observed increase in laccase activity. The result suggests that while temperature and carbon concentration may influence laccase activity to some extent, their combined effect within the tested parameters is not substantial.

Verification experiments were conducted to confirm the appropriateness of maximum response generation. The result shows the accuracy and consistency of the predicted activity within specific experimental conditions. The predicted laccase activity was 8.96 U/mL in the conditions of 3.3 % carbon concentration, 60 h incubation time, temperature of 37°C and pH of 9, while the experimented value was 8.49 U/mL under similar condition. The dissimilarity between the predicted and experimented laccase activity was a 0.47, which denoted 5.2 % deviancy, a satisfactory deviation range as it is within the limit of the ±8.4 %. This suggests that the experimented results are in unison with the predicted values and that the optimized process conditions are reliable and reproducible.

The results from the purification step showed the effectiveness of the purification process for separating laccase from the raw supernatant gotten after the large-scale production. At first, the raw supernatant, obtained after 5 L production had an activity of 8.54 U/mL. After its purification using 80 % saturated ammonium sulphate for precipitation, the activity increased to a 10.50 ± 0.04 U/mL, and signifying enhancement of the enzyme through precipitation. Additionally, the protein concentration was measured at 1.65 ± 0.13 mg/mL, suggesting that the purification process increased the concentration of the target enzyme relative to other proteins present in the crude supernatant. Subsequently, the laccase activity after dialysis was determined to be 10.76 ± 0.11 U/mL, indicating a slight increase compared to the post-precipitation activity. This increase in laccase activity after dialysis suggests the removal of contaminants or inhibitory factors that may have been present in the crude supernatant or precipitated fraction [25, 43]. Protein concentration after dialysis was found to be 1.86 ± 0.12 mg/mL, indicating a slight increase in protein concentration compared to the post-precipitation stage which suggests that while the dialysis process resulted in a further purification of the laccase enzyme, some non-specific proteins or contaminants may still be present in the purified sample.

A similar finding was reported by Umar and Ahmed [72] on the characterization, optimization, and purification of laccase from *Ganoderma leucocontextum.* The study noted that the top appropriate concentration for laccase purification was a volume of 80 % ammonium sulphate and this yielded 65 % laccase. Their study purified laccase by 4.5-folds from its first culture broth with a concluding yield of 82 %. The entire activity of the purified enzyme was 15,228.5 U/L. The observed increase in laccase activity reported in these studies could be attributed to the effectiveness of the ammonium sulphate precipitation method. The 80 % ammonium sulphate precipitation has been reported by various studies as a valuable yet non-specific technique for protein purification [26,77]. It is commonly utilized due to its simplicity, affordability, and ability to precipitate a significant number of proteins. These findings suggest that dialysis may have removed inhibitory molecules or impurities, leading to a small boost in laccase activity. The increase in protein concentration after dialysis is likely due to the removal of smaller molecules by the dialysis membrane, concentrating the protein solution overall.

According to Rodríguez-Couto [62] laccases are efficient biocatalysts for the removal of recalcitrant pollutants from wastewater. This study recorded physicochemical properties of the raw effluent pH (3.57 ± 1.33), BOD (23.67 ± 0.55 mg/L), COD (1875.10 ± 0.07 mg/L), TDS (25.94 ± 0.78 mg/L), TSS (1.41 ± 0.09 mg/L), and turbidity (204.27 ± 1.49 NTU) suggested poor water quality. The recorded values indicated acidic conditions, elevated levels of organic and inorganic pollutants, suspended solids, and turbidity.

The pH (3.57 ± 1.33) of the raw effluent indicates acidic conditions, which may have a negative impact on aquatic life and ecosystem health. A slight increase in pH (3.73 ± 0. 75) was observed after laccase treatment. Laccase catalyzing the oxidation of phenolic and aromatic compounds in POME, may be the reason for the slight pH increase recorded, converting these compounds into quinones and other less acidic intermediates. During this process, protons (H^+) are either consumed or neutralized as part of the oxidative reaction. This then leads to a gradual decrease in free hydrogen ion concentration, resulting in the slight increase in pH observed. Moreover, the precipitation of acidic intermediates reduces their buffering capacity, allowing the solution to shift toward neutral pH [62].

However, the observed pH value after laccase treatment still falls short of the standard pH limit typically required for wastewater discharge, which is set at 9. This discrepancy between the observed and standard discharge pH values may be due to incomplete oxidation of organic compounds, the presence of buffering agents in the effluent or low efficiency of the laccase enzyme in neutralizing acidic components. The pH values obtained from the study of Abioye et al. [3] before and after treatment of dye effluent waste water using laccase however differs from this result, as their study recorded a reduction in pH from 13.1 to 3.14, becoming more acidic.

In this study, elevated biochemical oxygen demand (23.67 ± 0.55 mg/L) level was observed in the effluent before laccase treatment suggesting a high organic load in the raw effluent, indicating the presence of biodegradable organic matter that consumes oxygen during decomposition. High BOD affects the ecosystem. According to Bulbul-Ali and Mishra, [19] high BOD depletes dissolved oxygen levels in water bodies, leading to hypoxia and harming aquatic life. After treatment, the biochemical oxygen demand reduced to 7.33 ± 1.33 mg/L indicating a decrease in the concentration of biodegradable organic pollutants. This result is in agreement with Abioye et al. [3] who recorded a decrease in BOD after treatment with laccase. This suggests that laccase effectively catalyzed the degradation of organic contaminants, improving water quality and reducing the oxygen demand of the effluent. Both the treated and raw effluent were found to be below the standard discharge limit of 50 mg/L. Before laccase treatment, the elevated COD (1875.10 ± 0.07 mg/L) level recorded suggest the presence of both biodegradable and non-biodegradable organic pollutants, as well as inorganic compounds that consume oxygen during oxidation. After laccase treatment, a slight reduction in COD (1542.90 ± 0.05 mg/L) was observed suggesting a partial degradation of organic pollutants and chemical compounds. The calculated efficiencies of about 0.324 U/mg of laccase per mg of COD reduced, 6.585 U/mg BOD, and 5.873 U/NTU can be linked to laccase acting on both phenolic and non-phenolic aromatic compounds found in the POME by catalyzing single-electron oxidation reactions. These reactions convert phenols and other aromatic compounds into reactive radicals, which undergo non-enzymatic coupling to form larger, more stable, and less reactive polymers such as quinones. This change reduces the solubility and reactivity of organic compounds in the effluent, thereby lowering their biochemical availability for microbial respiration and their oxygen-consuming potential during chemical oxidation [62].

The reactive intermediates from laccase oxidation form cross-links with proteins, polysaccharides, and suspended particles through covalent or hydrogen bonding. This enzyme-induced cross-linking leads to the formation of larger flocs, which settle out of solution under gravity, directly reducing the TSS. Some of these reactions also convert dissolved organics into larger, hydrophobic aggregates that become insoluble, thereby reducing TDS of the effluent [2].

Regulatory bodies set maximum permissible limits for COD in industrial wastewater discharge to protect aquatic environments. Environmental protection agency (EPA) discharge standards limit for POME, is 250 mg/L for COD. Hence reducing the COD of POME to meet these regulatory standards has implications such as protection of aquatic ecosystems. Since a reduction in COD means less oxygen consuming material is released into water bodies. This further helps maintain healthy dissolved oxygen levels, supporting diverse aquatic organisms and preserving the ecological balance of water bodies that these effluents are usually discharged into [41].

The BOD is an important parameter regulated for industrial wastewater discharge. The EPA also has limits for POME, with current standards often requiring BOD to be not more than 50 mg/L [30].

Reducing the BOD of POME to comply with these standards has significant environmental benefits such as prevention of anaerobic conditions. By removing biodegradable organic matter, the risk of anoxic or anaerobic zones forming in water bodies is reduced, preventing the associated problems of foul odors, habitat destruction and also protection of public health. Clean water bodies, free from organic pollution and foul odors, enhance public health and provide opportunities for recreational activities. This contributes to the overall well-being of communities living near palm oil operations ([18]).

Regulatory bodies also include turbidity or total suspended solids as key parameters in wastewater discharge standards. While specific turbidity limits might vary, they are usually indirectly controlled by TSS limits, generally. EPA standards for POME discharge typically set TSS limits at 35 mg/l, which directly correlate with turbidity often in the range of 75 mg/L [53].

Lower turbidity allows for greater light penetration which promotes photosynthesis and maintains healthy dissolved oxygen levels. This reduces pollutant transport,by removing suspended particles, the potential for transport and release of adsorbed pollutants is minimized, contributing to a clean water and reducing environmental contamination ([31]).

5. Conclusions

An extracellular laccase producing bacterial strain was isolated and identified using the 16rDNA sequence analysis as *Pseudomonas aeruginosa* (accession number OR687603). Corncob had a maximum capacity of producing laccase as compared to sugarcane bagasse and plantain peduncle when screened for its potential to be used as a carbon source for laccase production. The investigation revealed notable insights into the potential of microbial communities thriving in snail gut, particularly their role in laccase production.

The optimum laccase production of *Pseudomonas aeruginosa* was an alkaline pH of 9, 3.3 % carbon source at 37 °C for 60 h. The final volume, 60 mL of laccase was recovered from 5 L of the medium. The optimization of fermentation conditions highlighted the sensitive nature of producing laccase to factors such as carbon concentration, temperature, pH, and also incubation period. The purified laccase had an activity of a 10.76 U/mL and was employed for the treatment of POME for 24 h. This resulted in a reduction in TSS, TDS, COD and BOD, and a slight increase in the pH of the palm oil mill effluents.

Generally, this study revealed the potential of agricultural wastes as a valuable resource for industrial applications, particularly in enzyme production like laccase. Through the exploration of bacterial fermentation processes and optimizing cultivation conditions as revealed in this study, a significant advancement can be made toward sustainable and cost-effective methods for laccase production.

Scaling up the results of laccase treatment for POME from laboratory to industrial applications can be achieved through various ways such as the use of bioreactors and immobilization techniques such as alginate beads, activated carbon, or nanomaterials. Genetic engineering and strain improvement techniques such as overexpression are also promising strategies for industrial-scale enzyme production. The key bottlenecks in industrial conversion experienced over time includes enzyme stability and environmental sensitivity, Laccase is reported to be sensitive to pH, temperature, and inhibitors such as heavy metals that may be present in raw POME. Although thermostable and pH-tolerant laccases have been reported, many of them still perform optimally within narrow ranges. Maintaining optimal conditions on a large scale therefore presents a practical challenge. Also, at industrial scale, the inability to recover and reuse free enzymes increases operational costs. However, immobilized enzymes provides a solution but may suffer from mechanical degradation during long-term use. Despite being environmentally friendlier, enzymatic treatments has to compete economically with conventional coagulants like aluminium sulfate or polyaluminium chloride, which are cheaper and already well-integrated into industry practices to be fully accepted.

CRediT authorship contribution statement

Oluwafemi Adebayo Oyewole: Conceptualization, Methodology **Priscilla Yetu Tsado** Data curation, Writing- Original draft preparation **Priscilla Yetu Tsado; Konjerimam Ishaku Chimbekujwo:** Investigation **Olabisi Peter Abioye, Evans Chidi Egwim, Solomon Bankole Oyeleke:** Supervision, **Ummulkhair Salamah Ilyasu, Samia S. Alkhalil**: Writing- Reviewing and Editing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to acknowledge funding received from TETFund's Institutional Based Research Intervention (IBRI) scheme -TETFUND/FUTMINNA/2024/020.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nxsust.2025.100201.

Data Availability

The nucleotide sequences obtained were deposited in the NCBI database under accession number OR687603, this is available at the following URL: https://www.ncbi.nlm.nih.gov/nuccore/OR687603.1/

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