



Production of polyextremotolerant laccase by *Achromobacter xylooxidans* HWN16 and *Citrobacter freundii* LLJ16

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ABSTRACT

Given the upwelling of a variety of potential applications laccases could participate in, it would be fitting to equally make available laccases that are well suited for the aforementioned. Therefore historical understanding of the catalytic and physicochemical properties is desirable. Owing to this, the biochemical properties of the crude laccases from *Achromobacter xylooxidans* HWN16 (Hb9c) and *Citrobacter freundii* LLJ 16 (Ie1c) were assessed. Furthermore, a hint of the molecular basis for their production from respective organisms was presented. Results showed that both laccases were tolerant, and sometimes had their activities improved by the set of parameters tested. They were active at broad range of temperature (0–90 °C), pH (3–11), and were equally thermo- and pH-stable. Their activities were either improved, or left unabated by cations, detergents, and chloride (5–40%), however, the highlight of the study was their augmented activity, when they were incubated with certain concentrations of fluoride (2–20%), a potent inhibitor. They were depicted to have multiple homologous laccase encoding genes, on molecular evaluation, which may be responsible the conferral of these remarkable qualities they possess. Therefore, the laccases might be beneficial, if employed in formulations for a wide range of environmental and biotechnological applications. Moreover, the molecular machinery of their production be exploited for economical benefits in the immediate future.

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1. Introduction

Laccases are dimeric or tetrameric glycoproteins that contain at least two copper atoms distributed in redox sites generally having fewer saccharide compounds of about 10–25% in fungi and bacteria than in the plant enzymes [1,2]. It has been observed that hexoamines, glucose, mannose, galactose, fucose and arabinose are constituents of their carbohydrate compound [1]. Most laccases exhibit mobilities equivalent to molecular weight of 60–100 kDa on SDS-PAGE, 10–50% may be attributed to glycosylation, which is responsible for secretion, proteolytic susceptibility, activity, copper retention and thermal stability [3].

Laccases do not have crucial requirement of H₂O₂ or Mn²⁺ for substrates oxidation; a property that gives them an edge over other ligninolytic enzymes, and have broader substrate spectrum than peroxidases [2,4]. However, this feat has been made possible by

redox mediators, which serve as electron shuttles to further oxidize high molecular weight phenols, aromatics, even inorganic compounds with a concomitant four-electron reduction of atmospheric molecular oxygen to water. Consequently, laccases have been used to oxidize mediator compounds such as phenol, aniline, 4-hydroxybenzoic acid, and 2,2' - azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), to the end, chemical pollutants, which were once passively disintegrated and degraded, were interestingly modified on treatment [5,6]. Since laccases are constitutively consistent on molecular oxygen as an electron acceptor and do not require any other co-substrate, they are the most promising enzymes of oxidoreductases group for industrial applications [7,8].

These properties so far mentioned have made laccases become industrially relevant due to a number of diverse applications they can be designed to achieve. Typical examples are biocatalytic purposes such as delignification of lignocellulose and cross-linking of polysaccharides, bioremediation applications, such as; waste detoxification and textile dye transformation, food technological uses, personal and medical care applications, and biosensor and analytical applications [9–12]. Most of the environmental

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conditions, which laccases may be exposed to during their applications could be harsh e.g. high pH, high salt concentration, intense temperature, presence of heavy metals *inter alia*, therefore, different biotechnological applications will require different laccases with properties more suited to those applications.

A recent compilation of studies has highlighted the thermostability and pH stability of bacterial laccases, with a few mentions on halotolerant bacterial catalytic proteins. Correspondingly, laccases from bacteria have been proposed to be highly active and much more stable at high temperatures and high pH value [13,14]. In the light of this, our current study served to add to the growing chain of information regarding halotolerant laccases. A memorable statistic was recorded for pH- and thermo-stability in *Achromobacter xylosoxidans* HWN16 and *Citrobacter freundii* LJ16, and their corresponding data on halotolerance might be the most significant ever reported on bacterial laccase. Moreover, antithetical to the general opinion on the effect of fluoride on laccase activity, the positive to seemingly benign influence the aforementioned halide has on the bacterial extracellular secretions was reported in our study. Ratiocinations on possible reasons for this outcome led to the brief examination of its molecular basis. The rationale behind this trend was the possible secretion of constitutive and inductive laccase at different stages during fermentation, which could be regulated by different laccase gene loci on the respective bacterial genomes. It has been confirmed that multiple laccase genes could be found in many organisms, albeit most data presented have addressed their presence in fungi [15–19]. Conversely, heuristic studies depicting the corresponding discovery of multiple laccase genes in bacteria taxa, which have only been reported so far by Kellner et al. [20], Ausec et al. [21] and Wang et al. [22], to our knowledge, bestows a prospect worth exploiting in the near future. In the light of this, an investigative approach was designed to provide a molecular snapshot behind the interesting properties of extracellular laccase of a woodland isolate, as well as a semi arid soil denizen.

2. Materials and methods

2.1. Strains and culture conditions

Laccase-producing bacteria, coded Hb9c and Ie1c, employed in this study, were isolated from wood marsh in Hogsback Forest and semi arid soils in Amathole District Eastern Cape. They were recovered from the Biocatalysis chest in AEMREG culture collection. They have been identified molecularly using 16S rRNA sequence analysis as *Achromobacter xylosoxidans* HWN16 and *Citrobacter freundii* LJ16 with accession numbers MF073257 and MF073260 respectively from GenBank, National Center for Biotechnology Information (NCBI). Appropriately standardized cultures of the aforementioned isolates were inoculated in a basal medium with individual preferences for carbon and nitrogen sources, as well as inducers (Hb9c: mandarin peelings; 0.25%, NaNO₃; 1.25 g, 0.05% acetaminophen and Ie1c: maize stover; 1.0%, KNO₃; 0.25 g, 0.05% 4-nitrophenol), according to Unuofin et al. [23, 24], and the following composition (g/L): KH₂PO₄; 0.514, K₂HPO₄; 0.32, KNaC₄H₄O₆.4H₂O; 0.32, NaCl; 0.08, MnSO₄.H₂O; 0.032, MgSO₄.7H₂O; 0.192, CaCl₂.2H₂O; .008, CuSO₄.5H₂O; 0.0008, FeCl₃.7H₂O; .0008, ZnSO₄; 0.0008. (Sigma-Aldrich, South Africa) in pH 5, Citrate buffer, and the shake flasks were incubated in an orbital shaker at 30 °C, 100 rpm.

2.2. Extraction, protein analysis and activity assay

Aliquots of crude extracts were harvested post-incubation and centrifuged at 15,000 rpm for 12 min at 4 °C using a benchtop centrifuge (SIGMA-1-14k). The resultant supernatant was used to assay for protein concentration and laccase activity, respectively.

The concentration of protein was determined using the Folin-Phenol reagent, and our method was consonant with Lowry et al. [25], using bovine serum albumin as standard. Laccase activity was interpreted to be the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), on monitoring, according to the method of Unuofin et al. [26]. Briefly, a 50 µL aliquot of appropriately diluted crude laccase was reacted with 2 mM ABTS in potassium phosphate buffer (pH 6) at 30 °C, and the reaction was terminated after 10 min with 40 µL 20% TCA. The changes in absorbance due to oxidation of ABTS were monitored spectrophotometrically at 420 nm ($\epsilon = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$) using a SynergyMX 96-well microtitre plate reader (BioTeK Instruments). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of ABTS per minute under the aforementioned conditions.

2.3. Biochemical characterization of the laccases

The optima for laccase activity was evaluated at 30 °C for 30 min. in buffers with pH values ranging from 3 to 11. Conversely, the temperature optima was assessed at temperature ranging from 0 to 90 °C in phosphate buffer (pH 6) for 30 min. However, for stability studies, crude laccase were incubated in different buffers over a period of 455 min., while temperature stabilities for 40–90 °C were measured over 1440 min. Furthermore, the effect of selected metal ions (Mn²⁺, Cu²⁺, Fe²⁺, Fe³⁺, Ba²⁺, Zn²⁺, Mg²⁺, Co²⁺.) and surfactants (EDTA, benzoic acid, SDS, urea) were assayed after 30 min. of preincubation with crude laccases at the concentrations 1 mM, 2.5 mM, 5 mM and 7 mM, respectively. The effect of varying concentrations of tungstate was also evaluated. Other surfactants (DMSO and Tween 20) and NaCl was assayed for at concentrations of 5%, 10%, 20%, and 40%, respectively. However, NaF, which has been purported to have strong inhibitory activity on laccases was evaluated at 2%, 5%, 10%, and 20% concentrations, respectively. The aforementioned studies were conducted with 2 mM ABTS as assay substrate. Furthermore substrate specificity studies were conducted using the following substrates; ABTS, guaiacol, 1-naphthol, 2,6 - Dimethoxyphenol (DMP), potassium ferrocyanoferate (PFC), pyrogallol, and syringaldazine, while the kinetic values were determined for the best substrate. Assays were carried out in triplicates.

2.4. Detection of laccase-encoding genes

2.4.1. Extraction of DNA by lysis of axenic cultures

The genomic DNA of the bacterial cultures were extracted according to the method of Queipo-Ortuno et al. [27], with modifications. Actively growing axenic cultures were harvested from nutrient broth and centrifuged at 15,000 × g for 10 min. (HERMLE Z 233 M-2, Lasec, SA). The cellular pellets were vortex-washed in microbiological saline, twice. The washed cells were then resuspended in molecular grade nuclease free water, twice after spinning at 15,000 × g for 10 min., and were boiled in an AccuBlock Digital dry bath (TECHNE, Lasec, SA) at 100 °C for 10 min. Thereafter, the lysates were quickly but briefly cooled on ice, and then spun at 13,500 × g for 5 min. to separate the cellular debris from the clear supernatant containing the genetic material. The clear supernatant was aseptically decanted and stored at –20 °C. Aliquots of 5 µL of template DNA were thereafter used for PCR.

2.4.2. PCR and gel electrophoresis

The primers used (Table 1) were specifically designed for this study using the NCBI primer-BLAST tool, and they were further synthesized by Integrated DNA Technologies® Whitehead Scientific (Pty) Ltd. The PCR mixture contained 5 µL template DNA, 12.5 µL 2× OneTaq PCR MasterMix (Biolabs, South Africa), 1 µL (10 µM) of each forward and reverse primer, and was adjusted to a total volume of 25 µL with nuclease-free sterile water (Life

Table 1
set of primers designed for the study.

Primer	Sequences	Cycling conditions
CueOP	F: 5'- CGA GAC CTA CGA CGT GAT CG -3' R: 5'- GTG GGT CAT CAT GGT GTC GT -3'	5 min at 94 °C 35 cycles (40 s at 93 °C, 1 min at 52.4 °C, 1:50 min at 72 °C) and a final extension of 7 min at 72 °C.
MCOS _{stm}	F: 5'- AAG GCG TGG TAC ACC TCT TC -3' R: 5'- CCA GTT CAA CCT GGG CAA CC -3'	5 min at 94 °C 35 cycles (40 s at 93 °C, 1 min at 54 °C, 1:50 min at 72 °C) and a final extension of 7 min at 72 °C.
CueOC _{it}	F: 5'- TAT AAC GGC AAC CTG CTG GG -3' R: 5'- GCT GAT CCG GGG TAA AGG TC -3'	5 min at 94 °C 35 cycles (40 s at 93 °C, 1 min at 64.6 °C, 1:50 min at 72 °C) and a final extension of 7 min at 72 °C.

Science). PCR amplification was run on a thermal cycler (G-STORM, UK) using the respective conditions mentioned in Table 1 (*supra*). The amplicons were run on 1.7% agarose gel (Merck, SA) in a Submarine Electrophoresis System (Mupid-One, Takara, ADVANCE Co., Ltd. Japan) for 45 min. at 100 V, and thereafter had their bands visualized with the aid of ethidium bromide stains (Sigma-Aldrich, South Africa) in a transilluminator (ALLIANCE 4.7, France) with the UVITEC Cambridge software.

3. Results and discussion

In a bid to answer the critical demand for precise and efficient catalytic activity of laccases in industrial and biotechnological applications, an idea of the range of suitable environmental conditions for respective laccase biotechnological applications must be explored and exploited. This could be achieved by characterizing the proteinaceous exudates, in order to identify their properties and specifications, so that they can be bespoke for the aforementioned applications. The characterization of laccases have been reported by several authors, where necessary features highlighted range from thermotolerance to halotolerance and molecular features [28–31] *inter alia*.

In this investigation, laccases to be characterized were produced from the bacterial strains, Hb9c and Ie1c grown on lignocellulosic agroindustrial residues, which had been highlighted *supra* (see methods). The annals of laccase production by the two isolates have been portrayed by Fig. 1. The result of increased laccase production at longer incubation periods may be due to the unleashing of simpler intermediates from the biomaterials after continuous consistent attack by the bacteria, which could have triggered the secretion of greater volumes at the late idiophase. This may appear ambiguous, however, the basis for our ratiocinations are corroborated by the review of Li et al. [32], and references therein.

3.1. Effect of pH on laccase activity and stability

It was observed that both enzymes were remarkably active over a broad range of pH, displaying over 90% relative activities in buffers spanning pH 6–10 in Hb9c, and pH 4–8 in Ie1c, respectively (Fig. 2). Both isolates however displayed different pH optima (Hb9c; pH 8, Ie1c; pH 6). Although the range outside the optima were regarded as relatively low in activity, they still afforded a considerable measurement of relative activity, with Ie1c presenting a minimum of 84.27% at pH 11. The broad pH range displayed by the examined isolates is similar in pattern to the one reported by Si et al. [33], where their purified laccase from *Trametes pubescens* displayed tremendous activity over a range of 4.5–11.0, however their optimum was recorded at pH 5.0. As opposed to fungal laccases, bacterial laccases usually have an optima toward the neutral to alkaline range, and this is consistent with our study, just as with the work of Niladevi et al. [34], where they observed an optima at pH 8.5 and a relative activity of 97% at pH 9 in their study on *Streptomyces psammoticus*. Conversely, acidic optima have recently been observed by bacterial secretions. This is evident in

the study conducted by Ausec and co-investigators [35], where they recorded an acidic optima (pH4) on all substrates evaluated.

Stability studies (Fig. 2) showed that both enzymes were stable for over 455 min., with Hb9c showing a residual activity of over 100% for all the pH ranges assessed (pH 3–11). Although residual activities of Ie1c laccase dipped towards terminal incubation (455 min.), lowest residual activity recorded was approximately 100% (pH 10). Individual studies have shown bacterial laccase stability at acidic pH regimes [35,36], and alkaline pH regimes [34]; however, the unique broad pH stability observed in our study coincides with the work of Zhang et al. [37]; also, Das et al. [38] observed successive increases in laccase residual activity over 5 days in pH 3.0 and 6.8 buffered solutions respectively, albeit these were achieved by different substrates as opposed to the single substrate (ABTS) employed in this study. Hence, this report might be a pioneer on such capability of bacterial laccases. There are different factors responsible for this phenomenon, some could be; available oxygen, source of enzymes, spectral and biomolecular characteristics of the active sites, and the differences between redox potentials of substrates and the enzymes. However, the exact logic for this phenomenon might be undecipherable, at the moment; therefore, further studies using state-of-the-art analytical techniques are anticipated in the future to elucidate this claim.

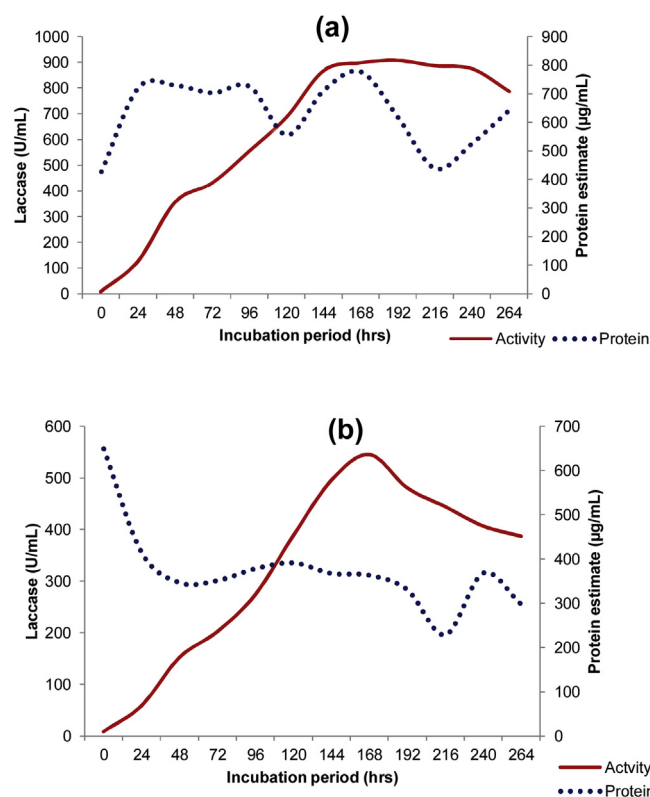


Fig. 1. Time course for extracellular laccase secretion from; (a) *Achromobacter xylosoxidans* HWN16 (Hb9c), (b) *Citrobacter freundii* LJ16 (Ie1c).

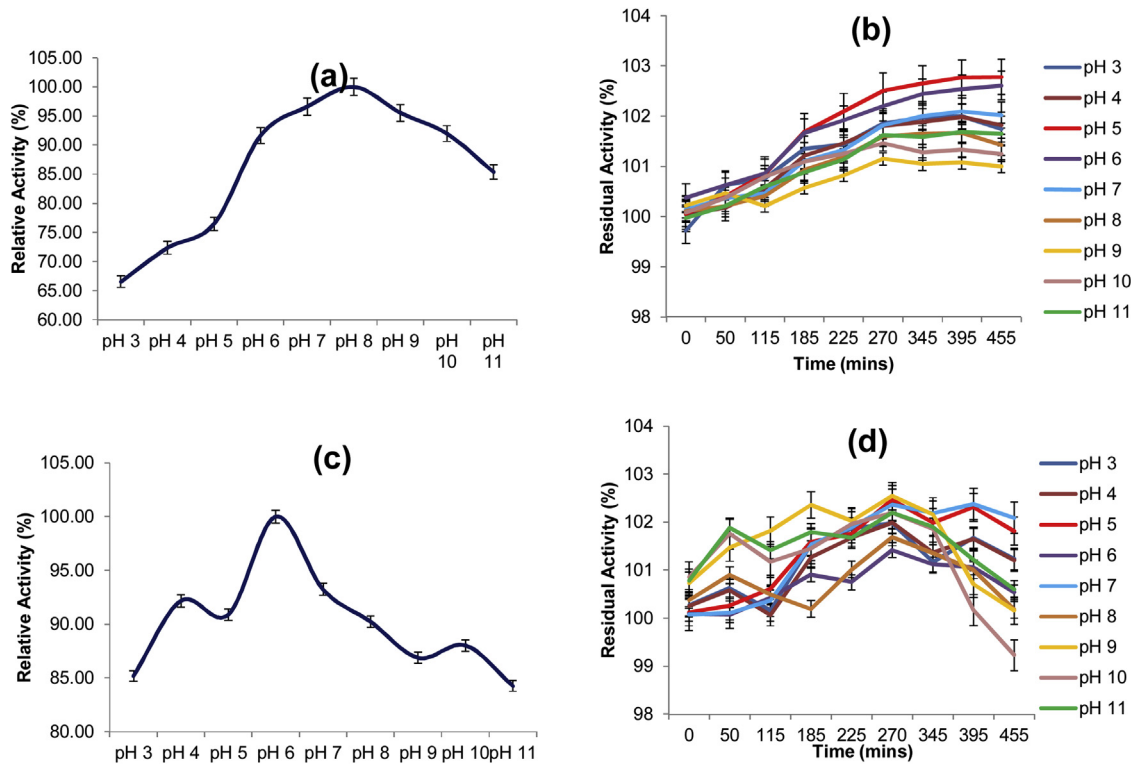


Fig. 2. Effect of pH on laccase activity and stability; a&b (Hb9c: activity & stability), c&d (Ie1c: activity & stability).

Ultimately, the most interesting highlight of our stability study was the discovery of at least 88% residual activity from the laccases after they had been incubated at refrigeration (4 °C) for 23 weeks in the respective pH buffers (3–11) (data not shown), suggesting they could be stored for longer periods without appreciable loss in activity.

3.2. Temperature optima and thermostability

Fig. 3 reflects the effects of temperature on laccase activity, which was determined using 2 mM ABTS as substrate. It was observed that both laccases were active at all the ranges of temperature assessed (0–90), thereby demonstrating over 90%

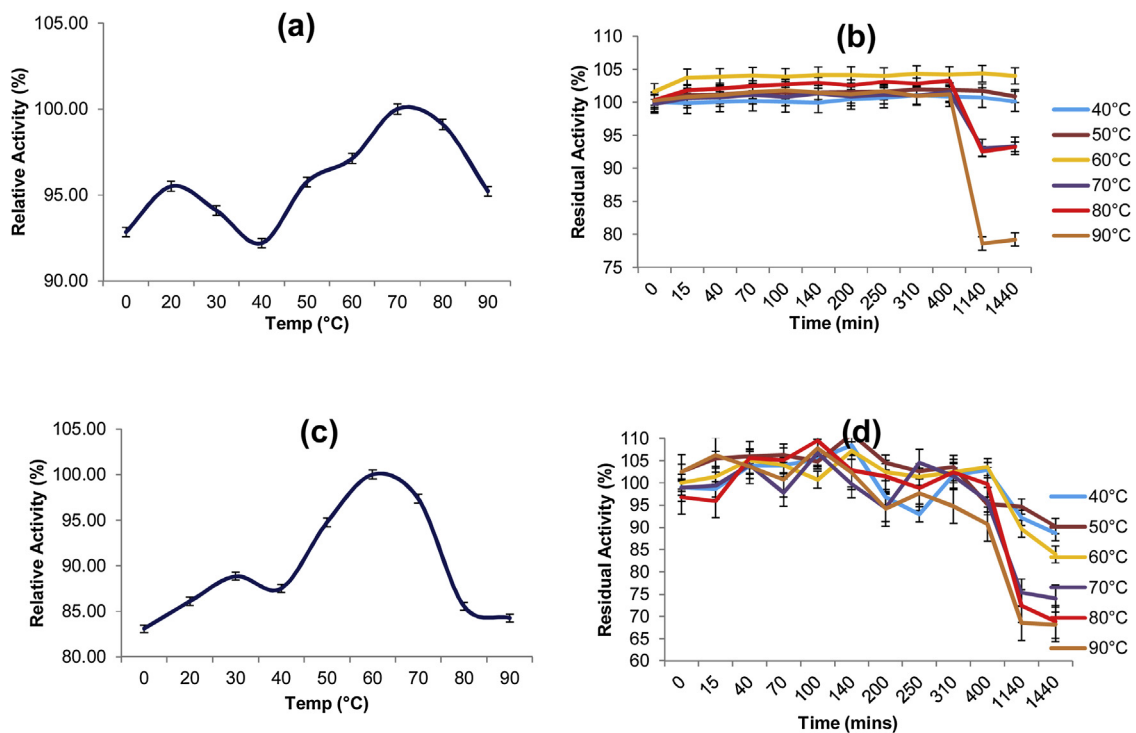


Fig. 3. Effect of temperature on laccase activity and stability; a&b (Hb9c: activity & stability), c&d (Ie1c: activity & stability).

relative activity in Hb9C and 80% in Ie1c, respectively. The activities of both laccases plummeted at 40 °C, but had individual temperature optima (Hb9c; 70 °C, Ie1c; 60 °C); albeit they were both regarded as thermotolerant. Bacterial laccases have been observed to have a high temperature optima, Zhang et al. [37] had an optimum reaction temperature at 85 °C, as did Sun et al. [39], while Sondhi et al. [40] reported 90 °C. Consistent with our study, Singh et al. [36] recorded an optima of 70 °C from a recombinant laccase of *Yersinia enterocolitica*, the same was reported by Odeniyi et al. [31] for *Enterobacter ludwigii*. From the representation (Fig. 3), both bacterial laccases were recorded as thermostable; however, Hb9c was more resilient than Ie1c in this case. Although our experiment was initially planned for 400 min., a fortuitous inspection after 1140 and 1440 min., respectively, showed they could still elicit a bare minimum residual activity of cca. 78% (Hb9c; 90 °C) and cca. 68% (Ie1c; 90 °C), thereby suggesting they could partake in high-temperature-long-time catalytic reactions in the industry. Further corroborations and juxtapositions of our finds have been identified [28,29,33,35,37,40–42].

3.3. Effect of potential inhibitors and halides

Result portrayed in Fig. 4 showed that, irrespective of the extracellular secretion, or the concentration of the salts, solvents or surfactants tested, at least 95% residual activity was recorded, sequel to incubation of reactants. Although both laccases performed comparably well, favourable responses were mostly recorded in Ie1c laccase, and with surfactants (urea: cca. 105–113%; SDS: cca. 107–114%), generally. This remarkable feat posits them as favourable candidates for real wastewater treatment, among other environmental applications. The possible effects of some of these compounds have been highlighted by Ausec et al. [35], which is seemingly antithetical to our finds, whereas Saito et al. [43] surmised the inconsequentiality of EDTA at high concentrations (25 mM); however, Niladevi et al. [34] did not fail to expose the inhibitory effect of Co^{2+} and EDTA, among other notable inhibitors mentioned, while a recent report highlights the improvement of

laccase activity, when incubated with Mn^{2+} , Cu^{2+} and Mg^{2+} , respectively [39]. After perusal and deliberations on reports of high tolerance of bacterial laccase secretions towards chloride [28,41,44], an investigation of the effects of increasing concentrations (5%, 10%, 20% and 40%) of chloride and other detergents was carried out. It observed that, at all levels tested, residual laccase activity either remained unaffected (100%) or was further improved. A similar outcome was observed by Brander et al. [28] and Li et al. [45], where bacterial laccase were either tolerant to increasing levels of chloride, or were induced, thereby suggesting them as salt-activated proteins. A possible reason for this phenomenon might be due to transformation of the proteins from monomeric (unfolded) suppressed forms to oligomeric (folded) catalytically active forms, on interaction increasing salt concentrations. This is characteristic of proteins that contain large amounts of negatively charged amino acid residues (Aspartic acid), and a few positively charged ones (Lysine), where proper folding is promoted by interaction of acidic residues of proteins with hydrated ions to stabilize a folded orientation [46]. This implies that both enzymes could thrive well as part of active ingredients for commercial formulations for detergents.

A typical example of such is DeniLite®, a product of Novozymes, has been used in several environmental applications, ranging from textile bleaching to the modification of pharmaceuticals. However, an upgrade of their laccase to a Denilite Cold, which is active at colder temperatures is congruent with our discovery of laccase stability over prolonged period of refrigeration, earlier mentioned in our study. Quite a number of studies have discussed the inhibitory effects of detergents and solvents [34,35]. Correspondingly, a recent study reported an increase in relative activity elicited by increasing concentration of DMSO (10%, 20%, 30%), however, at 40%, only affordable units of activity could be detected [47]. Conversely, matching reports on the positive effects of Tween 80 have been identified [48], albeit higher concentrations of Tween 20 were evaluated in this study. Finally, with consideration of the famously reported inhibitory activity of NaF [3,49], we pensively evaluated the effects of its increasing

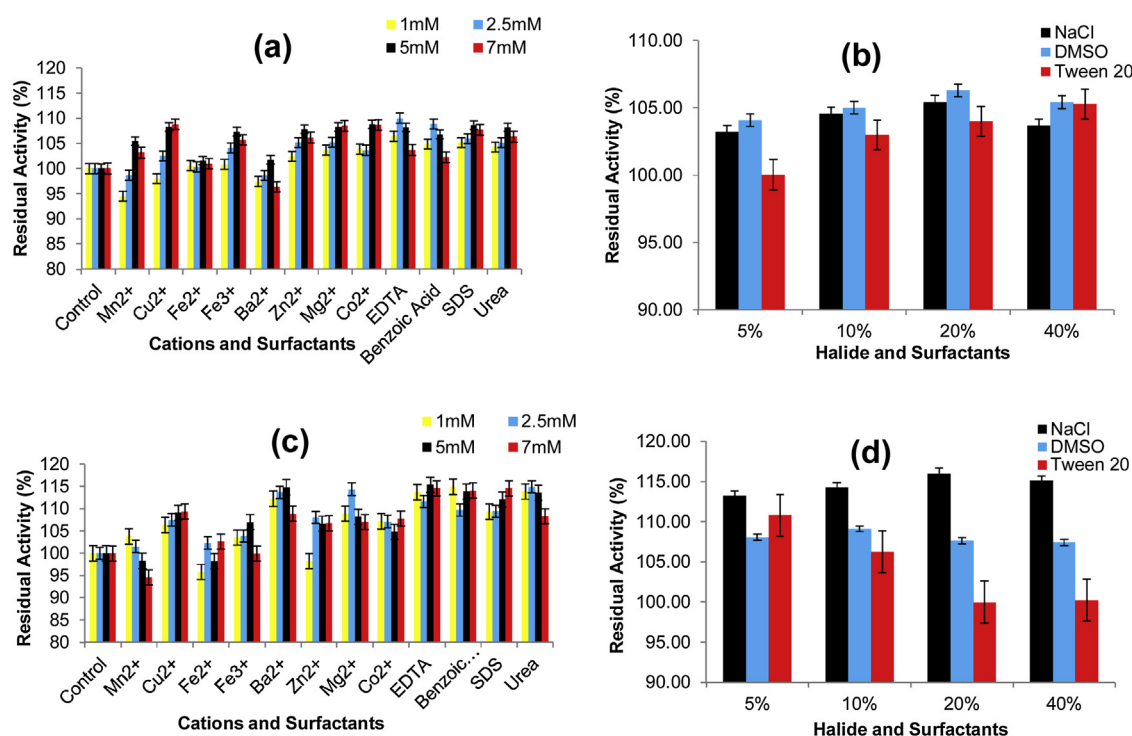


Fig. 4. Effect of potentially inhibitory compounds and halide on laccase activity in; a&b (Hb9c), c&d (Ie1c).

concentration (2%, 5%, 10%, 20%) on laccase activity. However, appreciable units of activity were measured across all concentrations assayed (Fig. 5), with the bare minimum, overall, observed in 20% (Ie1c; cca. 98%). A reason for this might not be exactly decipherable, but it would be an area worth exploring in no distant future.

3.4. Substrate specificity and laccase energetics

ABTS was the most suitable substrate for the laccase from the strains studied (Hb9c and Ie1c) (Fig. 6). In addition, PFC, an inorganic substrate, was second most suitable substrate, which depicts the laccases have a predilection for non-phenolic compounds. A possible reason for this could be: the laccases prefer the electron transfer mechanism, due to high redox potentials of the substrates that aid in the efficient reduction of oxygen, hence their rapid oxidation and generation of radicals, which afford these compounds the status of excellent mediators for laccase catalyzed reactions. Another reason could be the type of buffer used and the assay pH, among other assay conditions; however, an adept explanation for this scenario has been provided Brander et al. [28].

Interestingly, this does not imply the other substrates are not fitting for laccase detection, as Ihssen et al. [50] acquaints us with the vast amount of substrates available for laccase catalysis. Conversely, pyrogallol and 2,6-DMP were equally good phenolic substrates, yielding moderately appreciable amounts of activity; all other substrates could afford only detectable amounts of activity. Niladevi et al. [34] reported pyrogallol as the most suitable substrate for a laccase secreted by *Streptomyces psammoticus*, while

in another study, 2,6-DMP was regarded the most suitable, over ABTS [51]. Seeing the most suitable substrate was ABTS, we proceeded to estimate the energetics of laccases from both strains thereupon, the results are communicated in Table 2.

The statistic displayed in Table 2 were obtained from a Lineweaver-Burk plot ($R^2=0.9181$; Hb9c and $R^2=0.934$), which was plotted using varying reactions of varying concentrations of ABTS. Both isolates were portrayed to have the same estimate of specificity constant (K_{cat}/K_m), which is a measure of their catalytic efficiency, and would show its relative affinity to the substrate in comparison with other substrates or enzymes assessed. Relatively low K_m value for both laccases (Hb9c; $0.625 \mu\text{M}$, Ie1c; $0.484 \mu\text{M}$) show they have a strong interaction with their substrate to form an enzyme-substrate complex [ES]. Since a lower K_m would imply a stronger bond, Ie1c laccase interacted better with ABTS. The conversion of the substrates in the [ES] to product is best described by their K_{cat} values, albeit the rate of conversion was faster for Hb9c laccase since it was able to partake in a larger number of catalytic cycles, this outcome could be ascribed to the higher velocity (V_{max}) of the laccase, coupled with the absence of potential uncompetitive inhibition. In other studies conducted with ABTS, Rezaei et al. [42] reported a K_m value of $39.2 \mu\text{M}$ and a K_{cat} of 2150.0 s^{-1} from a laccase secretion of *Aquisalibacillus elongatus*. A secretion from *Bacillus* sp. had a K_m of $132.7 \mu\text{M}$ and a K_{cat} of 309 s^{-1} [52], while a fungal laccase had a K_m of $105.0 \mu\text{M}$, and a K_{cat} of 876 s^{-1} [33], which was further described as being better than values of fungal laccases reported by Guo et al. [53] and Zhuo et al. [54], respectively. However, the values observed by the aforementioned investigators has been eclipsed by results reported in this study, which imply their high catalytic rate

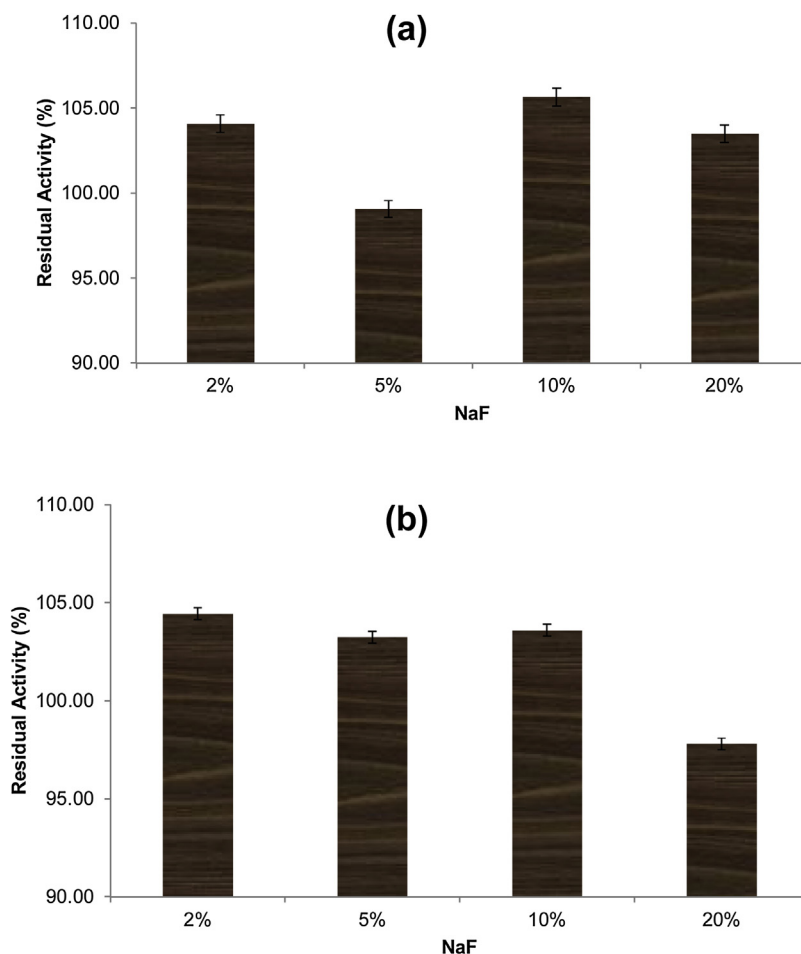


Fig. 5. Effect of different concentrations of a potent halide, NaF on laccase activity in (a) Hb9c, (b) Ie1c.

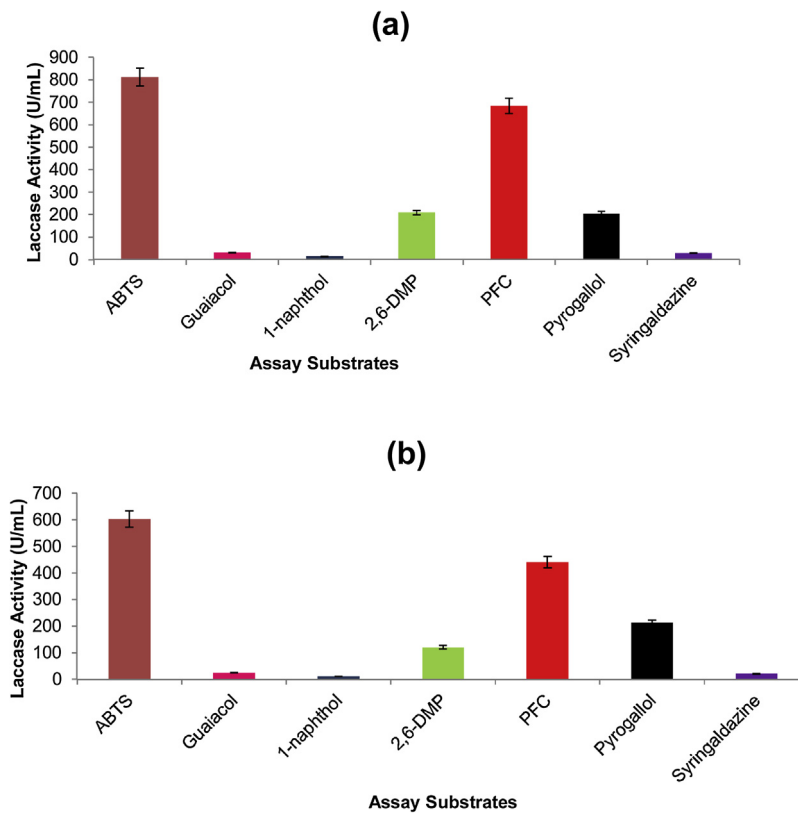


Fig. 6. Laccase substrate specificity studies of; (a) Hb9c, and (b) Ie1c.

Table 2
Kinetic properties of bacterial laccases.

Laccase type	V_{max} ($\mu M \text{ min}^{-1} \text{ mg}^{-1}$)	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\mu M^{-1} s^{-1}$)
<i>Achromobacter</i> sp. HWN16 laccase	416.66	0.625	6.94×10^3	1.1×10^4
<i>Citrobacter</i> sp. LLJ16 laccase	322.58	0.484	5.37×10^3	1.1×10^4

would be advantageous in environmental applications where a seamless, time lag free reaction is desirable.

3.5. Molecular snapshot of laccase production

The suspected laccase gene sequences of our test isolates (Hb9c and Ie1c) were amplified with the primers designed with NCBI primer-BLAST tool. The primers encoded copper oxidases (CueOP & CueOCit) and multicopper oxidase (MCOS_{tm}), respectively. The gel representations (Figs. 7 & 8) showed that both isolates were able to exhibit at least five distinct homologous gene sequences. However, our assertions remain suppositious till the amplicons are further analyzed. Therefore, an in-depth analysis of the fragments would be undoubtedly beneficial.

Multiple laccase genes have already been detected, mostly among fungi, and some investigators could investigate their uniqueness from one another based on their respective appearances on gel representations [55,56]. Sakamoto et al. [57] found 13 distinct multicopper oxidase genes in *Lentinula edodes*, while Wong and co-investigators [58] had found 11 laccase encoding genes in the same basidiomycete, prior to their investigation. Bacteria have been observed to possess multiple laccase genes, as this has been confirmed by reports of Kellner et al. [20] and Ausec et al. [21]. However, in the most recent report, high expression of about 16 Laccase-like multicopper oxidase (LCMO) genes were observed in

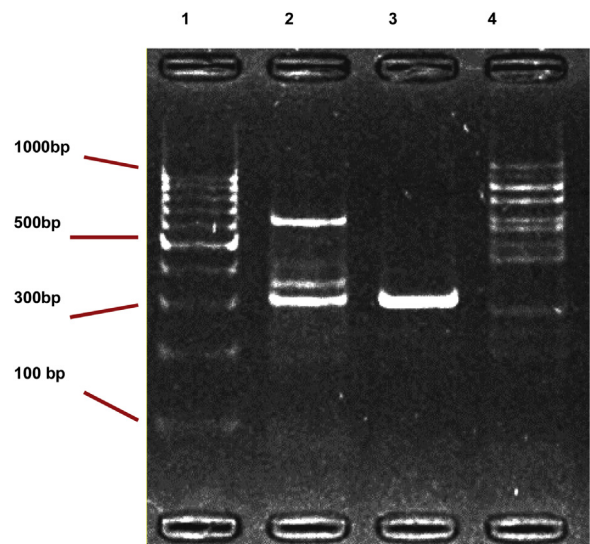


Fig. 7. Agarose gel electrophoresis (1.7% w/v) of fragments of laccase genes amplified by PCR on *Achromobacter xylosoxidans* HWN16 (Hb9c). Lane 1: ladder mix, lane 2: CueOP gene, lane 3: MCOS_{tm} gene, lane 4: CueOCit gene.

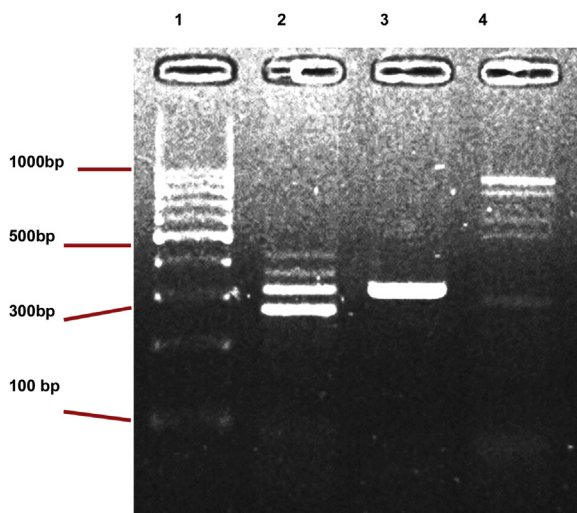


Fig. 8. Agarose gel electrophoresis (1.7% w/v) of fragments of laccase genes amplified by PCR on *Citrobacter freundii* LLJ16 (Ie1c). Lane 1: ladder mix, lane 2: CueOP gene, lane 3: MCOSm gene, lane 4: CueOCit gene.

13 bacteria taxa, which were grown in lignin supplemented basal medium [22]. Also, Feng et al [59] supposed bacterial laccase-like genes to be more involved in laccase activity than their fungal counterpart. The exact function of all the bacterial laccase genes discovered so far have not been ascertained; however, they have been linked with resistance to radiation and synthesis of pigments. Furthermore, the similar band positions of the respective genes may signify similar expression or role in the organism; notwithstanding, confirmatory experiments should be considered.

4. Conclusions

Extracellular laccases were produced by two bacterial strains, Hb9c and Ie1c from agroindustrial residues. They exhibited polyextremophilic tendencies that has eclipsed what has been reported so far, in literature, and this prompted us to decipher the basis for their hardiness. However, notable outcomes include their thermostability over 1440 min. of incubation (cca. 78%: Hb9c and cca. 68%: Ie1c) at 90 °C, and the unique halotolerance to 20% NaF (Hb9c: cca. 103%; Ie1c: cca. 98%) A molecular snapshot portrayed them to have more than one homologous laccase gene, which might either be constitutive or inductive, and whose expression might depend on the phase of growth and metabolism of the possessing strain. Therefore, the laccases evaluated in this study might be fitting for a wide range of environmental and biotechnological applications. Consequently, a deeper insight into the molecular machinery of their production should be conducted, so that they could be manipulated for economical benefits in the immediate future.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors

Conflict of interest

The author declare that they have no conflict of interest.

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