Original research paper

Opuntia extractable and non-extractable (NEP) residues as co-carbon sources for improved extracellular laccase production: partial purification and characterization

Running title: Cladode residues and laccase production

Authors’ contributions: This work was carried out in collaboration with all authors.

Abstract

Aim: Laccases are copper-containing enzymes involved in the degradation of lignocellulosic materials, biocatalytic reactions, and in treatment of phenol-containing wastewater. In this study, the effect of submerged fermentation with copper and xylidine supplementation on laccase activity and production by *Trametes pubescens* grown on cladode extractive residues alone, and as co-carbon source with wheat was investigated.

Place and duration of study: Study was carried out at the Durban University of Technology Biotechnology laboratory from April to September 2017.

Methodology: Plant extraction, submerged fermentation, enzyme activity and characterization techniques were utilized.

Results: The highest laccase activity was observed at 60-80% wheat:residue ratio combinations under submerged conditions with copper and xylidine supplementation. Partially purified enzyme fractions showed similar characteristics at different temperatures and pHs. There was good retention of relative enzyme activity at temperatures near 60°C, and pH stability from pH 4.0-6.0. At optimal culture conditions laccase activity was highest at 49.01±0.21 U/ml (80% wheat:20% residue ratio), and less at 10.02±0.51 U/ml (80% wheat:20% NEPP residue). Optimum temperature for laccase fractions was 25°C, and pH optimum at 5.

Conclusion: These results show the potential of cladode residues as an essential and economic growth medium for laccase production, offering a new alternative use for this common agro-industrial by-product.

Keywords: Laccase, *Trametes pubescens*, Cladode residues, NEPP, Wheat bran
1. Introduction

Lignin, cellulose, and hemicellulose are the major compounds present in plant residues. Among them, cellulose and hemicellulose can be decomposed by a large number of aerobic and anaerobic microorganisms through the action of hydrolytic enzymes [1]. Conversely, lignin biodegradation occurs at a lower rate than plant cell wall polysaccharides [2]. Certain white- and brown-rot fungi, are known to be able to degrade lignin from lignocellulosic biomass [3]. The extracellular enzymatic system responsible for lignin degradation consists of lignin peroxidase (LiP, E.C. 1.11.1.14), manganese-dependent peroxidase (MnP, E.C. 1.11.1.13), and laccase (para-benzenediol: oxygen oxidoreductase, EC 1.10.3.2) [2,3,4].

In white-rot fungi, laccases are typically produced as multiple isoenzymes [5]. In several organisms, extracellular laccases are constitutively produced in small amounts [5]; however, their production can be considerably enhanced by a variety of substances such as aromatic/phenolic compounds (xylidine or p-anisidine) [6], aliphatic alcohols [7], aqueous plant extracts [8], and metal ions, especially of copper [9]. Copper is also able to regulate the synthesis of several different laccase isoforms at the level of gene transcription. It is an essential micronutrient for most living organisms [10], and copper requirements by microorganisms are usually satisfied by very low concentrations of the metal, in the order of 1–10 μM [11].

Laccases are copper-containing glycoproteins with molecular weight between 60 and 80 kDa and with carbohydrate content between 15% and 20% [12, 13]. Enzyme production is an expanding field of biotechnology and laccases can catalyse the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water [14]. The most important source of laccases are fungi (particularly basidiomycetes). They are however present also in other natural sources e.g. plants, insects and bacteria [14]. The white-rot fungi are the most efficient microorganisms capable of extensive aerobic lignin degradation. Compared to bacterial laccases, fungal laccases show higher redox potential, hence their use in a variety of biotechnological/industrial applications e.g. synthesis of organic chemicals [15], biosensors [16].

Laccase expression in fungi is influenced by different cultivation modes, culture conditions such as nature and concentration of carbon and nitrogen sources, media composition, pH, temperature etc [17]. The optimal temperature and pH are mainly dependent on the substrate
for high yield of laccase [18]. The pattern of laccase expression by the wood degrading fungal species is an inconsistent process and vary one from another [19]. Most fungal laccases have been expressed in low quantities in the culture fluid [5], hence the use of laccase inducers [9, 11]. The implementation of many applications attributed to laccase is possible only through production increase with low production cost. Laccase production from lignocellulosic agro-industrial wastes valorises these wastes and reduces the problems associated with their disposal [18]. Moreover, these substrates provide a favourable natural habitat for the secretion of lignocellulolytic enzymes in larger amounts [18].

Lignocellulosic agro-wastes, extraction residues and/or plant by-products such as Opuntia cladodes (easily available raw materials) could facilitate such economical and high yield. The Opuntia plant is an interesting plant which has come under the radar of scientist for its far reaching and ever evolving applications in many industries. Cactus (Opuntia) cladodes have useful content of total soluble solids [20], pectic polysaccharides [21], and wide array of phenols [22]. Just like in the wide variety of agro-industrial waste/substrates which have been extensively studied, these naturally available cladode phenol constituents may serve as inducers, along with soluble carbohydrates which may serve as good carbon sources [23] (Table 1). While they also contain inorganic constituents (Ca, Mg, P), they possess little or no copper [24].

Table 1. Proximate composition of cladodes (Opuntia ficus indica) (spiny and spineless cladodes).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wheat bran</th>
<th>Spiny cladodes</th>
<th>Spineless cladodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>8.1–12.7</td>
<td>90.67 ± 0.75</td>
<td>91.04 ± 0.53</td>
</tr>
<tr>
<td>Total protein b</td>
<td>9.60–18.6</td>
<td>8.74 ± 0.51</td>
<td>8.88 ± 0.74</td>
</tr>
<tr>
<td>Total ash b</td>
<td>3.9–8.10</td>
<td>25.65 ± 0.94</td>
<td>23.3 ± 0.78</td>
</tr>
<tr>
<td>Total carbohydrates b</td>
<td>60.0–75.0</td>
<td>60.36 ± 1.13</td>
<td>60.93 ± 0.99</td>
</tr>
<tr>
<td>Total dietary fiber (TDF) a</td>
<td>33.4–63.0</td>
<td>51.24 ± 2.12</td>
<td>41.83 ± 2.98</td>
</tr>
<tr>
<td>Phenols and flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vitamins and minerals</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Significant difference between samples (P < 0.05), b No significant difference between samples (P < 0.05). (g/100 g of dry matter); ** % of Total carbohydrates, + = present

Based on the striking biotechnological applications of laccase, extraordinary efforts have been devoted towards the optimization of process for improving the laccase production using various microorganisms and agro-wastes as substrates. However, insoluble dietary fibres
(IDF) and/or extraction residues have not been utilized in production of laccase green enzyme. Dietary fibre (DF) is considered as an important element in nutrition and health. By this reason, there is need to identify new sources of DF that contributes to improving human and animal health and nutrition; and with potential industrial and economic applications. The present work aimed to ascertain the reliability of natural, insoluble dietary fibres/residues from conventional cladode extractions as substrates for enhanced laccase enzyme production using *Trametes pubscens* under submerged fermentation condition. The stability of laccase enzymes at different pH, temperature was also demonstrated for better industrial application. Moreover, partial purification, and characterization of laccase were also carried out to study their potential for wider biotechnology applications. The present work therefore was focused on the first time use of cladode extraction residues for laccase production. Wheat bran was also used as carbon source for purpose of comparison to achieve economical and improved laccase activity and production.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this experimentation were purchased from Hi-Media Limited, Mumbai, and were of the highest purity available. 2, 2’-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was obtained from Sigma.

2.2. Extraction of total polyphenols of nopal/cladodes

Cladode *(from a garden in Durban, Kwazulu-Natal, South Africa)* polyphenols extraction was done according to a modification of the method described by Waterman and Mole (1994). Briefly, 10 g of dehydrated nopal was added to 50 ml of a methanol/water solution (50/50, v/v). This mixture was stirred for 1 h and filtered using a Whatman No. 1 filter paper. The resulting filtrate stored at -20°C. The residue was subjected to a second extraction with 50 mL of acetone/water (70/30, v/v) solution and agitation for 60 min. The mixture was filtered and stored as indicated earlier. A third extraction was performed stirring the residue in 50 mL of distilled water for 15 min. The resulting filtrates were pooled, and extraction solvents removed by rotary evaporation for use in another experiment. Residue from this extractive process is regarded as the first cladode residue (R) used in this study for laccase production. A part of this residue was further subjected to acid hydrolysis according to Durazzo *et al.* [27]. Residue from this hydrolytic reaction were dried and termed cladode non-extractable
polyphenol (NEPP) residue or residue of residue (RR) in this study. Both R and RR were thereafter applied in laccase production through submerged fermentation.

2.3. Support substrate

Cladode (*Opuntia ficus-indica*) extraction residue and residue of residue where dried into fibre/flake form at 25°C. Wheat (*Triticum aestivum* L.) bran flakes (purchased from Alnatura GmbH (Bickenbach, Germany), were also used as a support substrate for laccase production under submerged conditions. Chemical composition of wheat bran, as indicated on the label of the product, was 14.9% protein, 20.5% carbohydrates and 4.7% fat. Before use, the flakes and residues were autoclaved at 121°C for 20 min.

2.4. Microorganism

The white rot fungus *T. pubescens* was procured from microbial type culture collection (MTCC) of the Cape Peninsula University of Technology (CPUT), South Africa, and maintained in potato dextrose agar (PDA) and sub cultured every 30 days and maintained at 4°C.

2.5. Inoculum preparation

The five-day old culture of *T. pubescens* grown on potato dextrose agar (PDA) at 25°C was used to inoculate the laccase fermentation media.

2.6. Fermentation for laccase production

Fungal disks (two 1.5 cm disks) taken from the active borders of PDA cultures of *Trametes pubescens* were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of sterilized (at 121°C for 15 min) production medium having the following composition (in g/ml): potato dextrose broth (20% potato extract and 2% dextrose); wheat bran flakes rationed with cladode residue as well as residue of residue as follows: 20%, 40%, 60% and 80% wheat to residue ratios), and separate flasks containing 100% wheat bran and 100% residues only, at 1 g/100 ml. 1 M CuSO4 and 1 mM Xylidine were used as laccase synthesis inducers. Enzyme activity in the broths was monitored daily for fourteen days. Flasks were incubated at 28°C with shaking at 160 rpm [28]. Experiments were carried out in three replicates.
2.7. Analytical methods

2.7.1. Extracellular laccase assay
Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2-
Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS $\varepsilon_{420} = 36,000$ M$^{-1}$cm$^{-1}$) as the
substrate. Assay mixture contained 0.330 ml ABTS (5 mM), 2.5 ml 0.1 M sodium acetate
buffer (pH 5.0), and 0.17 ml laccase extract. ABTS oxidation was monitored by measuring
the increase in absorbance at 420 nm. One unit of laccase activity was defined as the amount
of enzyme required to oxidise 1µmol of ABTS per minute at 25°C [29]. Experiments were
carried out in three replicates.

2.7.2. Protein concentration estimation
The concentration of protein was estimated with the Lowry assay, and using bovine serum
albumin BSA) as a standard [29].

2.7.3. Partial purification of enzyme
Ammonium sulphate was added to the cell free filtrate obtained from fermentation broth. The
filtered broth obtained from the final optimized production was subjected to ammonium
sulphate precipitation (50% and 80% saturation) at 4°C. The precipitate was collected by
centrifugation at 10000 rpm for 15 min under refrigerated condition. The production fraction
precipitates were dissolved in 0.1 M sodium acetate buffer (pH 5.0). Enzyme activity and the
total protein content for the solutions obtained were measured. Enzyme activity and total
protein content were determined for the ammonium sulphate fractions [30].

2.8. Enzyme characterization

2.8.1. Effect of temperature on laccase activity and stability
The effect of temperature (temperature profile) on activity of laccase fractions/extracts were
studied according to the method of Kumar et al. [29] by measuring the activity in a range of
30 - 55°C. The partially purified enzyme solutions were incubated in 0.1 M sodium acetate
buffer (pH 5.0) at different temperatures (25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C) and the
activity was determined with ABTS as substrate.

The thermal stability of the enzyme was determined by following the oxidation of 5 mM
ABTS after pre-incubation of laccase at 25-55°C. Enzyme solutions were incubated in water
bath in the range from 25°C to 55°C with 5°C increments for (15 min). Aliquots were removed after an hour interval of incubation and assayed at optimum pH and temperature. **Experiments were carried out in three replicates.**

### 2.8.2. Effect of pH on laccase activity and stability

The pH dependence on the activity of partially purified enzymes was carried out at 25°C using 5 mM ABTS in different buffer solutions by incubating it with the following buffers: citrate phosphate buffer for pH (3–5) and sodium phosphate for pH (6–8). For pH stability, enzyme extracts were pre-incubated at room temperature in different buffers at pH 3–8. Aliquots were removed after incubation and assayed at optimum temperature [29]. **Experiments were carried out in three replicates.**

### 2.9. Statistical Analysis

The experimental results were expressed as mean±standard deviation (SD). Mean separation was done using Duncan’s Multiple Range test in Statistical Package for Social Sciences (SPSS) version 16 programme. *P* values < 0.05 were regarded as significant.

### 3. Results and discussion

![Graph](image.png)

**Fig. 1:** Laccase filtrate activity of wheat:cladode residue during fermentation

*W = wheat, R = residue; Value represents a mean ± SD (n=3) at *p* < 0.05*
Fig. 2: Lacasse filtrate activity of wheat:cladode residue-residue during fermentation

W = wheat, RR = NEPP residue; Value represents a mean ± SD (n=3) at p < 0.05

Fig. 3: Effect of pH on laccase fractions activity at optimum assay conditions (25°C; 0.1M sodium-acetate buffer)

W = wheat, R = residue, RR = NEPP residue; Value represents a mean ± SD (n=3) at p < 0.05

Fig. 4: pH dependence of lacasse fractions in buffers (pH 3-8) using different buffers in laccase activity assay

W = wheat, R = residue, RR = NEPP residue; Value represents a mean ± SD (n=3) at p < 0.05
Fig. 5: Temperature profile of laccase fractions at optimum assay conditions (25°C; 0.1M sodium-acetate buffer)

W = wheat, R = residue, RR = NEPP residue; Value represents a mean ± SD (n=3) at p < 0.05

Fig. 6a: Thermostability profile of laccase from 100% wheat

Fig. 6b: Thermostability profile of laccase from 80% wheat:20% cladode residue
**Fig. 6c:** Thermostability profile of laccase produced from 100% cladode residue

**Fig. 6d:** Thermostability profile of laccase produced from 60% wheat:40% cladode NEPP residue

**Fig. 6e:** Thermostability profile of laccase produced from 80% wheat:20% cladode NEPP residue
Substrate source (and amount of each residue co-substrate used) for laccase production showed to be an important factor for laccase production (Figs. 1 and 2). Cultures/production broth with high wheat ratio and less of residues as substrate demonstrated higher laccase activity. Production broths with 100% wheat alone as substrate had lower enzyme activity, but increased in 100% cladode residue (R), 60W:40R, 80W:20R production broths while 20W:80R and 40W:60R showed the lowest enzyme activity (Fig. 1). 80% wheat to residue (80W:20R) ratio had the highest activity of the wheat to residue (W:R) ratio combinations (Fig. 1). More wheat (60%-80% W:R) combined with low residue ratio resulted in higher laccase enzyme activity.

The observed differences in laccase activity for each wheat and residue ratio combinations could be attributed to the chemical composition of each substrate, as well as the influence of laccase inducers utilized in the fermentation process [4]. The high laccase activity observed for the substrate and co-substrate combinations could be associated with their content of useful carbohydrates for sustenance of fungal growth through the production period [4], presence of some amount of copper [31, 32], as well as phenolic and non-phenolic compounds that may act as innate inducers for laccase production [33]. Phenolics and other inherent substances may act as potent inducers of laccase gene expression [34]. Copper sulphate and xylidine are also widely used laccase inducers for laccase production by not only by T. pubescens, but also by Rhizoctonia solani, obtaining an increase of 4.1-fold in laccase activity [11, 35]. Similar results were also reported for the production of Coloriopsis gallica laccase [36]. Laccase gene expression is mainly up regulated by Cu^{2+}. Nevertheless, Mn^{2+}, Fe^{3+}, heavy metals, 2,6-dimethoxy-1,4-benzoquinone, syringic acid, tannic acid, aromatic compounds, and microclimatic changes (i.e. lower temperature and osmotic pressure) are also recognized as potent inducers [34, 37]. In general, Cu^{2+} supplementation has been reported to increase the laccase activity in both submerged and solid-state fermentation processes. Addition of inducers counter growth repression of laccase synthesis and improve laccase production substantially [32]. Also, a combination of two or more inducers work synergistically in increasing laccase synthesis as is the case in this study. However, the copper ion concentration required to obtain the highest laccase activity may vary depending on agro-industrial waste, and which may be attributed to the copper present in the agro-industrial waste and its availability in the medium [4].
On the other hand, only 60W:40RR and 80W:20RR substrate ratio showed no significant difference in laccase activity during fermentation. 100% NEPP residue (100RR), 20W:80RR and 40W:60RR wheat to NEPP residue substrate ratios showed little or no enzyme activity (Fig. 2). Generally, in this study as wheat fraction increased (majorly 60-80%) in relation to the cladode residue co-substrates, enzyme activity also increased. Wheat chemical composition may play a key role in the observed enzyme activity during growth when used with NEPP residues. Wheat bran has been reported to contain minerals, vitamins, polyphenols, phenolic acids [33]. Also, laccase activity during growth in wheat:NEPP residue (W:RR) substrate combination was lower (7.21 U/ml) (Fig. 2) compared to those recorded for wheat:residue (W:R) substrate combinations (11.8 U/ml) (Fig. 1). This may be due to the possible depletion or significantly reduced amounts of phenols, carbohydrates and other fungal growth inducers/nutrients in NEPP residues (RR) compared to the unhydrolyzed cladode residue (R) [38]. This may also explain the attainment of low enzyme activity within a shorter fermentation period (10-12 days) before a fall in enzyme activity was observed (Fig. 2), compared to higher activity for unhydrolyzed cladode residue over a longer fermentation period (14 days) which may be linked to availability of more nutrients for fungal growth sustenance (Fig. 1). Although simple phenols and other inorganic components have been reported to be associated with cladode residues (R) and NEPP residues (RR) [4, 39], other factors like inoculum size, availability of more nutrients, some batch variance and other intrinsic factors may play a role in determining recorded laccase enzyme activity during fermentation/production [40, 41].

Best combination of wheat and cladode residues (60-80% wheat:40-20% R), or the use of cladode residues (R) alone as shown in this study lead to higher laccase activities. Extraction residues are obtained in very small quantities after plant extractive processes. Since only small amount are required for increased laccase activity, these residues may yet find use in small or medium scale production processes for production and laccase enzyme activity optimization. In addition, since these residues are spent remains from extractive processes, use of enzyme inducers may be required to increase enzyme activity for optimal use of both cladode residues in ratios with wheat.

3.2. Effect of pH on laccase fractions activity

Although pH optima may also depend on the substrate used [29], most laccases in other fungi also have an optimum pH of around 3 with ABTS as a substrate [42, 43]. The maximum
relative activity for the enzyme fractions with highest activity after precipitation from production broth was observed at a pH from 4.0 to 6.0 (Fig. 3), with a monotonical decrease at pHs above this range. Enzyme fraction had pH optimum at 5. The relation between pH and type of electron donor molecule present in production substrates/batch may be utilized in explaining this performance on laccase activity. It has been reported that when the substrate is an organic hydrogen donor, i.e. phenols, the optimal pH ranges from 3.5 to 6.0, while the laccase activity monotonically decreases from pH 2.5 to 7.0, if the substrate is an electron donor like ABTS [44]. Despite that at different pHs, most of the purified laccase fractions from the rationed substrates presented a similar performance.

3.3. pH stability of laccase fractions

As regarded pH stability in different buffers, 80W:20R, and 100R laccase fractions retained 54.2% and 48.3% of their initial activity at pH 8, compared to 33% retention for 100W laccase fraction. Also, 60W:40RR and 80W:40RR enzyme fractions retained 60.3% and 55.9% of their initial activity, respectively at pH 8 (Fig. 4). Optimal pH for enzyme stability was observed at pH 5.0. Rationed substrate fractions proved to be more pH stable and less pH dependent compared to fractions from 100% wheat and 100% residue (R) as substrate. In addition, the utilization of 100% residue as laccase production substrate also showed highest enzyme activity (28.6 U/ml) compared to 100% wheat (17.7 U/ml) as substrate at pH 5 (Fig. 4). The chemical composition of these residues may have impacted on pH stability properties of the laccase fractions produced in this study.

For all tested enzyme fractions, the maximum activity was observed at pH 5.0, and despite that at acidic pH (≤ 3.0) the relative activity was higher than 80%. Nevertheless, at basic pHs the relative laccase activity showed a sharp decrease that is well correlate with the other laccase fractions. We are not able to attribute this observed trend to the presence of multiple laccase isoenzymes in the fraction. Patel et al. [41] and Patel and Gupte [45] recorded similar pH optimum for laccase produced from Pleurotus ostreatus and Tricholoma giganteum, respectively, although under solid state fermentation conditions. In another study however, a thermostable laccase produced from Cladosporium cladosporioides mold showed pH optimum of 3.5 [46].
3.4. Temperature profile and thermostability of laccase fractions

Optimum temperature for all laccase fraction were recorded at 25°C in 0.1M sodium-acetate buffer under standard assay conditions (Fig. 5). This is similar to other study observations for laccase produced from *T. pubescens* and other fungi [29, 45]; with exception of thermotolerant laccase produced from *A. tabescens* (optimum at 45°C) [31], and *Trametes trogii* LK13 (retained 50% of its initial activity at 80°C after 5 min) [47]. Enzyme activity however decreased proportionally with respect to increase in temperature.

Enzyme fractions were stable in a temperature range of 25-50°C (Figs. 6a-6e). 60W:40RR and 80W:20RR laccase fractions retained 18.6 and 11.9% of initial activity after 4 h of heating, respectively; while 80W:20R and 100R laccase fractions retained 13.4 and 12 % of their activity respectively, with 100% wheat (100R) fraction being less stable and retaining only 1.2% of its initial activity after 4 h (Figs. 6a-6e). Laccase from rationed substrate were more thermostable compared to whole wheat or residue fractions. Laccase activity reduction in the crude extracts did not seem to be the result of an additive effect of the activity loss of the purified fractions. These results agree with previous reports that state that fungal laccases rapidly decrease their activity at temperatures near 60 °C, and thermal stability correlates with the temperature range of growth of the source organism [29, 48]. In contrast, Kumar et al. [29] reported total loss of activity at 55°C for laccase produced from *Aspergillus flavus*. In other studies, more thermostable laccase crude extract obtained from culturing *T. pubescens* growing on coffee husk recorded an activity loss of 26% and 10% at 60°C and 50°C, respectively, but after 8 h at pH 6.0 [49]. The laccase fractions herein reported on are mesophilic in nature, but with good retention of enzyme activity at temperatures near 60°C; and were stable at their optimum temperature (25°C) and pH (5.0).

4. Conclusion

This study has shown that cladode residues as co-substrates with wheat have been beneficial for improving laccase activity and production. Extraction residues are generally disregarded for added functions in most production scale. Cladode residues as substrates or co-substrates with wheat using *T. pubescens* may serve for laccase production, thus valorising extraction residues. Further supplementation of the laccase production broth with copper and xylidine may also induce for better enzyme activities during fermentation production. Cladode
residues alone could be useful for small or medium scale laccase production, or side by side with wheat bran.

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Conflict of interest
Authors declare no conflict of interest.

References


