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

Abstract
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. The highest specific laccase activity was achieved when the culture was conducted under 60-80% wheat:residue ratio combinations under submerged conditions supplemented with copper (1 M, to final concentration of 2 mM) and xylidine (1 mM, to final concentration of 10 µM). Regardless of the residue and wheat:residue co-substrate ratios, enzymatic partially purified fractions behaved similarly at different temperatures and pHs, most of them presented the maximum activity at 25 - 50 °C, with good retention of enzyme activity at temperatures near 60°C; and a pH range between 3 and 6. Fractions also showed similar stability profiles. At optimal culture conditions laccase activity was highest at 49.01±0.21 U/ml (80% wheat:residue ratio), and lowest at 10.02±0.51 U/ml (80% wheat:NEPP) of protein for partially purified/crude extract. In summary, 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

1.0 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 (Vandamme, 2009). Conversely, lignin biodegradation occurs at a lower rate than plant cell wall polysaccharides (Guillén et al., 2005). Certain white- and brown-rot fungi, are known to be able to degrade lignin from lignocellulosic biomass (Sánchez et al., 2011). 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 1.10.3.21) (Guillén et al., 2005; Sánchez et al., 2011; Gonzales et al., 2013).

In white-rot fungi, laccases are typically produced as multiple isoenzymes (Bollag and Leonowicz 1984). In several organisms, extracellular laccases are constitutively produced in small amounts (Bollag and Leonowicz 1984); however, their production can be considerably enhanced by a variety of substances such as aromatic/phenolic compounds (xylidine or p-anisidine) (Mansur et al. 1998), aliphatic alcohols (Lee et al., 1999), aqueous plant extracts (Ardon et al., 1996), and metal ions, especially of copper (Palmieri et al. 2000). 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 (Cervantes and Gutierrez-Corona 1994), and copper requirements by microorganisms are usually satisfied by very low concentrations of the metal, in the order of 1–10 μM (Galhaup and Haltrich, 2001).

Laccases (E.C. 1.10.3.2, p-benzenedial: oxygen oxidoreductase) are copper-containing glycoproteins with molecular weight between 60 and 80 kDa and with carbohydrate content between 15% and 20% (Arora et al., 2010; Mathur et al., 2013). Enzyme production is an expanding field of biotechnology and laccases can catalyze the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water (Abd el-Raheem and Shearer, 2002). 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 (Abd el-Raheem and Shearer, 2002; Urairuj et al., 2003). 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. biopulping and bleaching (Thurston, 1994), textile industry (Setti et al., 1999; Harazono et al., 2005), food industry (Selinheimo et al., 2006), synthesis of organic chemicals (Karamyshev et al., 2003), biosensors (Jarosz-Wilkolazka et al., 2005; Chawachart et al., 2004).

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 (Bakkiyaraj et al., 2013). The optimal temperature and pH are mainly dependent on the substrate for high yield of laccase (Muthukumarasamy et al., 2015;
Nyanhongo et al., 2002). The pattern of laccase expression by the wood degrading fungal species is an inconsistent process and vary one from another (Jang et al., 2002; Jiang-Ping et al., 2001). Most fungal laccases have been expressed in low quantities in the culture fluid (Bollag and Leonowicz, 1984), hence the use of laccase inducers (Hess et al., 2002). 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 (Muthukumarasamy et al., 2015; Poonam and Pandey, 2009). Moreover, these substrates provide a favourable natural habitat for the secretion of lignocellulolytic enzymes in larger amounts (Muthukumarasamy et al., 2015; Revankar and Lele, 2006; Chawachart et al., 2004).

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 (Jun et al., 2013), pectic polysaccharides (Bayar et al., 2016), and wide array of phenols (Avila-Nava et al., 2014). Just like in the wide variety of agro-indusrial 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 (Songulashvili et al., 2007) (Table 1). While they also contain inorganic constituents (Ca, Mg, P), they possess little or no copper (Contreras-Padilla et al., 2015).

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 (%)</td>
<td>9.60–18.6</td>
<td>8.74 ± 0.51</td>
<td>8.88 ± 0.74</td>
</tr>
<tr>
<td>Total ash (%)</td>
<td>3.9–8.10</td>
<td>25.65 ± 0.94</td>
<td>23.3 ± 0.78</td>
</tr>
<tr>
<td>Total carbohydrates (%)</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) (%)</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>
<tr>
<td>(vitamins B and E; P, Mg, Fe, Zn, Cu etc.)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Source: Adapted from Ayadi et al. (2009) and Onipe et al. (2015)

* Significant difference between samples (P < 0.05), ** 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.

2.0 MATERIALS AND METHODS

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.

Extraction of total polyphenols of nopal/cladodes
Cladode 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 was saved and 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. (2016). 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.

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), was 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.

Microorganism
The white rot fungus T. pubescens was gotten 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.

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.

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 Xyldine 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 (Khan et al., 2016).

Analytical methods
Extracellular laccase assay
Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS $\varepsilon_{420} = 36,000 \text{ M}^{-1}\text{cm}^{-1}$) 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 1 µmol of ABTS per minute at 25°C (Kumar et al., 2016; Sivakumar et al., 2010).

**Protein concentration estimation**

The concentration of protein was estimated with the Lowry assay, and using bovine serum albumin BSA) as a standard.

**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 (El-Batal et al., 2015).

**Enzyme characterization**

**Effect of temperature on laccase activity and stability**

Environmental temperature is a factor to which the biomass is an inescapable subject, since cell temperature must become equal to the temperature of culture medium. Temperature affects the rate of cell reaction, nature of metabolism, nutritional requirement, and the biomass concentration. The effect of temperature (temperature profile) on activity of laccase fractions/extracts were studied according to the method of Kumar et al. (2016) 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 at optimum pH and temperature after pre-incubation of laccase at 25 - 55°C. Enzyme
solutions were incubated in water bath in the range from 30°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.

**Effect of pH on laccase activity and stability**

The influence of hydrogen ions on biological activities is related to their hydrogen ion concentration on enzyme activity. 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 (Kumar et al., 2016).

**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.

**Results and Discussion**

![Laccase enzyme activity (U/ml) vs Fermentation (day)](chart.png)

**Fig. 1:** Lacsse filtrate activity of wheat:cladode residue during fermentation
Fig. 2: Lacase filtrate activity of wheat:cladode residue-residue during fermentation

Fig. 3: Effect of pH on laccase fractions activity at optimum assay conditions (25°C; 0.1M sodium-acetate buffer)
Fig. 4: pH dependence of laccase fractions in buffers (pH 3-8) using different buffers in laccase activity assay

Fig. 5: Temperature profile of laccase fractions at optimum assay conditions (25°C; 0.1M sodium-acetate buffer)

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
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/prod
broth with high wheat ratio and less of residues as substrate demonstrated highest laccase activity. Production broths with wheat alone as substrate had the lowest enzyme activity; while the 100 % cladode residue (R) broth showed higher activity compared to the 20% wheat to residue ratio, but lower than activities recorded for 40-80% wheat to residue ratios. 80% wheat to residue ratio had the highest activity of substrate to co-substrate mixture (Fig. 1). More wheat and less residue as co-substrate 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. The high laccase activity observed for substrate and co-substrate combinations could be associated with their content of useful carbohydrates for sustenance of fungal growth through the production period (Gonzales et al., 2013), presence of some amount of copper (He et al., 2014; Strong, 2011), as well as phenolic and non-phenolic compounds that may act as innate inducers for laccase production (Stevenson et al., 2012). Phenolics and other inherent substances may act as potent inducers of laccase gene expression (Xiao et al., 2006; Galhaup et al., 2002). 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 (Galhaup and Haltrich, 2001; Crowe and Olsson, 2001). Similar results were also reported for the production of Coloriopsis gallica laccase (Yagüe et al., 2000). Laccase gene expression is mainly up regulated by Cu^{2+}. Nevertheless, Mn^{2+}, Fe^{3+}, heavy metals, 2,6-

**Fig. 6e:** Thermostability profile of laccase produced from 80% wheat:20% cladode NEPP residue
dimethoxy-1,4-benzoquinone, amphotericin B, syringic acid, tannic acid, aromatic compounds, and microclimatic changes (i.e. lower temperature and osmotic pressure) are also recognized as potent inducers (Dekker et al., 2007; Xiao et al., 2006; Galhaup et al., 2002).

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 (Strong, 2011). 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 presented in the agro-industrial waste and the copper availability in the medium (Gonzales et al., 2013).

On the other hand, only 60 and 80% wheat to NEPP residue substrate ratio showed not so significant difference in laccase activity during fermentation. 100% NEPP residue, and 20% to 40% wheat to NEPP residue substrate ratios showed little or no enzyme activity (Fig. 2). Generally, in this study as wheat fraction increased in relation to the cladode residue co-substrates, enzyme activity also increased. This showed that wheat chemical composition played a key role in the recorded enzyme activities during growth, but not more than when 100% residue was used as substrate. Wheat bran has been reported to contain minerals, vitamins, polyphenols, phenolic acids (Stevenson et al., 2012). Also, laccase activity during growth in wheat:NEPP residue substrate combination was lower compared to those recorded for wheat:residue substrate combinations. This may be due to the possible depletion or significantly reduced amounts of phenols and other fungal growth inducers/nutrients in NEPP residues compared to the cladode extracted unhydrolyzed residue (Benayad et al., 2014). This may also explain the attainment of highest activity within a shorter fermentation period (10-12 days) before a fall in enzyme activity was observed (Fig. 2) compared to unhydrolyzed cladode residue (14 days) (Fig. 1). However simple phenols and other inorganic components have been reported to be associated with cladode NEPP residues (Gonzales et al., 2015; Mora et al., 2013). 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 (Ergun and Urek, 2017; Gonzales et al., 2013; Patel et al., 2009).

Best combination of wheat and cladode residues, or the use of cladode residues alone as shown in this study lead to higher laccase activities. This is an interesting find as extraction
residues are obtained in very low quantities after plant extractive processes. Hence, since only a little mount is required for increased laccase activity, these residues may yet find use in small, medium and large-scale production processes for production and laccase enzyme activity optimization. In addition, since these residues are spent remains from extractive processes, use of higher levels/concentrations of induction may be required to increase enzyme activity for optimal use of both cladode residues in ratios with wheat.

Although pH optima may also depend on the substrate used (Kumar et al., 2016), most laccases in other fungi also have an optimum pH of around 3 with ABTS as a substrate (Jung et al., 2002; Shin and Lee, 2000). The maximum relative activity for the enzyme fractions with highest activity was presented at a pH from 3.0 to 6.0 (Fig. 3), regardless of the wheat to residues ratio, 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 (Morozova et al., 2007). Despite that at different pHs, most of the purified laccase fractions from the rationed substrates presented a similar performance.

As regard pH stability in different buffers, 80% wheat:R, and 100% R laccase fractions retained 54.2% and 48.3% of their initial activity at pH 8, compared to 33% retention for 100% wheat laccase fraction. Also, 60 and 80% wheat: RR enzyme fractions retained 60.3% and 55.9% of their initial activity, respectively at pH 8 (Fig. 4). Rationed substrate fractions proved to be more pH stable and less pH dependent compared to fraction from 100% wheat and 100% residue 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 presented a sharp decreased 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. (2009) and Patel and Gupte (2016) 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 (Halaburgi *et al.*, 2011).

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 (Kumar *et al.*, 2016; Patel and Gupte, 2016); with exception of thermotolerant laccase produced from *A. tabescens* (optimum at 45°C) (He *et al.*, 2014), and *Trametes trogii* LK13 (retained 50% of its initial activity at 80°C after 5 min) (Yan *et al.*, 2015). 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). 60 and 80% wheat:RR laccase fractions retained 18.6 and 11.9% of initial activity after 4 h of heating, respectively; while 80% wheat:R and 100% R laccase fractions retained 13.4 and 12% of their activity respectively, with 100% whet 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. Although there are no reports of laccase stability under the selected conditions, 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 the growth of the source organism (Kumar *et al.*, 2016; Hildén *et al.*, 2009). In contrast, Kumar *et al.* (2016) 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 and 50 °C, respectively, but after 8 h at pH 6.0 (Gaitan *et al.*, 2011). Thereby, 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 and pH.

**Conclusion**

This study has shown the potential of cladode residues (including NEPP residues) as co-substrates with wheat for the optimization laccase activity and production. The generated volume of residues is high and generally disregarded for added functions on any scale of
production after usual/conventional extractive processes. The results showed that submerged culture of *T. pubescens* using cladode residues as substrates or co-substrates may serve to obtain essential amounts of laccase, which could be favoured by the phenolic/aromatic components, minerals and vitamins which may be present in different quantities, but working synergistically to boost enzyme activity. Further supplementation of the laccase production broth with copper and xyldine also favoured the enzyme activity and production. Cladode residues could be useful for laccase production on a small or pilot scale side by side with wheat bran, and other agro-industrial wastes.

Conflict of interest
Authors declare no conflict of interest.

References


