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Oxidation of 8-hydroxyquinoline catalyzed by laccase from *Trametes pubescens* yields an antioxidant aromatic polymer

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Abstract

We report for the first time the enzyme-catalyzed production of poly (8-hydroxyquinoline) using laccase from the white rot fungus *Trametes pubescens* (CBS 696.94). The oxidative polymerisation of 8-hydroxyquinoline was catalyzed by laccase in a reaction medium containing 8% acetone, 0.01–0.05 mg/ml 8-hydroxyquinoline and sodium acetate buffer (0.1 M, pH 5.0) at 30 °C. The average molecular weight of the polymeric product, as determined by matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF) analysis, was 789 *m*/*z*. Further structural characterisation of the product was achieved using Fourier transform infrared (FT-IR) spectroscopy. The potential for application of poly (8-hydroxyquinoline) as an antioxidant was investigated by evaluating its ability to quench the stable free radical 2,2′-diphenyl-1-picrylhydrazyl (DPPH). The antiradical efficiency (AE) was calculated to be 2×10^{-3}, a value which is comparable to the AE values for gallic acid (2.6×10^{-3}) and ascorbic acid (11.4×10^{-3}), and greater than that of polymeric lignin fractions of similar molecular weight (5×10^{-4}).
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Keywords: *Trametes pubescens* laccase; 8-Hydroxyquinoline; Organic medium; Antioxidants

1. Introduction

Biocatalysts are increasingly important in the chemical industry, for synthesis of products ranging from fine chemicals and pharmaceuticals to bulk chemicals including polymers. The potential benefits of utilizing enzymes and whole-cell biocatalysts in industrial processes arise from their activity under mild conditions of temperature, pH and pressure, compared to inorganic catalysts. In recent years there has been increasing interest in the production of polymers of economic importance using biocatalysts; the application of enzymes in polymerisation processes can lead to the synthesis of unique polymeric compounds with novel properties that could not be easily achieved by conventional methods [1,2]. Enzymes which have been used in polymerisation processes include, among others, laccase, tyrosinase and horseradish peroxidase [3]. Horseradish peroxidase, in particular, was used to synthesize poly (8-hydroxyquinoline-hydroquinone), a conjugated polymer that has been shown to have photovoltaic properties [4]. However, the disadvantage of using horseradish peroxidase is that the reaction requires hydrogen peroxide which can inactivate the biocatalyst [5] and also adds cost constraints in the application of the process. Laccases catalyze the one-electron oxidation of a wide variety of organic compounds, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate, with the concomitant four-electron reduction of oxygen to water [6]. They has also been used in biocatalytic oxidations of dyes, polymerisation of lignin and lignosulphonates, bioremediation and bleaching [7,8]. The laccases produced by *Trametes* strains are generally stable and some are readily commercially available, but these enzymes can also be produced simply by fermentation on a laboratory scale [6].

This study reports for the first time the enzymatic synthesis of poly (8-hydroxyquinoline) catalyzed by laccase from the white rot fungus *Trametes pubescens* (strain CBS 696.94). The synthesis of conjugated oligomers such as poly (8-hydroxyquinoline-hydroquinone), poly (8-hydroxyquinoline-1,5-hydroxynaphthalene) and poly (8-hydroxyquinoline-4-phenylphenol) using horseradish peroxidase has been reported by Liu et al. [9], and poly (8-hydroxyquinoline-5,7-dimethylene) has been synthesized non-enzymatically [10]. The application of the polymers comprising 8-hydroxyquinoline as a building block

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was investigated by Xie et al. [4]. Poly (8-hydroxyquinoline-5,7-dimethylene) was shown to possess chelation properties which suggests the potential application of these polymers as cytotoxic agents [10–12].

To further explore the properties of the 8-hydroxyquinoline-derived polymer produced in the present study, an investigation was conducted into the activity of product as an antioxidant, comparing it with the monomer starting material. 8-Hydroxyquinoline itself is an antioxidant and a metal chelator which has been investigated by others for its beneficial activity, for instance in protecting monocytes against copper ion-induced lipid peroxidation [12,13].

2. Experimental

2.1. Materials

8-Hydroxyquinoline was obtained from Sigma.

2.2. Growth of *Trametes pubescens* for laccase production

T. pubescens (CBS 696.94) was grown on agar plates containing 50 g/l malt extract agar, supplemented with a laccase inducer [1% phenol mixture (phenol: 82.8 mM, *p*-cresol: 24.99 mM, *m*-cresol: 25.8 mM, *o*-cresol: 77.03 mM); see Ref. [14]]. *T. pubescens* mycelial blocks were aseptically inoculated on the plates and incubated at 28 °C for 6 days. *T. pubescens* was also cultured in *Trametes* Defined Medium (TDM) liquid medium containing 10 g glucose, 10 g bacteriological peptone, and 2 g KH_2PO_4 per litre [15]. Portions of 200 ml of sterilised medium were inoculated with homogenised mycelial plug taken from a solid Petri dish preculture (see above). Cultures were incubated (in 1000 ml Erlenmeyer flasks) at 28 °C with agitation at 180 rpm, for 9 days. These cultures were used to inoculate two 4 l airlift bioreactors each containing TDM supplemented with 0.03% antifoam [14]. After 5 days of incubation the medium in the bioreactors was supplemented with 30 ml phenol inducing mixture (see above) and 1 g glucose (previously filter-sterilised). Subsequently, further portions of 30 ml phenol mixture and 1 g glucose were added to the medium daily. Extracellular laccase activity in the medium was assayed daily, from 6 days after inoculation (see below). After 13 days, the medium, containing laccase displaying activity of 2–3 U/ml, was harvested and stored at −20 or 4 °C until further use. Freeze-dried samples were stored at −20 °C until needed.

2.3. Enzyme activity assay

Laccase activity was determined with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate [16]. The assay mixture contained 0.33 ml of 5 mM ABTS, 2.5 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.17 ml aliquots of culture supernatant or enzyme solution. Oxidation of ABTS was monitored by following the increase in absorbance at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min at 25 °C.

2.4. Isolation of laccase

The laccase in the reaction medium was precipitated using either acetone or ammonium sulphate according to the method developed by Ryan et al. [14]. The laccase product obtained had a specific activity of 1.8 U/mg.

2.5. Bioconversion of 8-hydroxyquinoline using laccase

A typical reaction mixture contained 0.25 g 8-hydroxyquinoline dissolved in 1 ml glacial acetic acid, 3 U laccase (prepared as described by Ryan et al. [14]) in 5 ml sodium acetate buffer (0.1 M pH 5.0), 2.5 ml acetone and 22.5 ml sodium acetate buffer (0.1 M pH 5.0). Reaction vials were covered with foil and incubated at 30 °C in the dark with shaking at 180 rpm. Control vials contained no enzyme. Polymerised product was recovered by filtration using No. 1 Whatman filter paper on a buchner funnel. The polymerised product was washed with 3 × 10 ml portions of acetone and then air-dried. To study the effect of reaction time on the molecular weight of the polymerised product, the reaction was repeated as described above, and 3 ml samples were withdrawn from the reaction mixture after 24, 48 and 72 h. In each case, the polymerised product was recovered as described above and its molecular weight was determined by MALDI-TOF spectrometry. The activity of laccase in the filtrate of each sample was also determined. In order to determine the effect of temperature on 8-hydroxyquinoline conversion by the laccase, reactions were conducted at temperatures of 20, 30, 40, 50 and 65 °C, while other conditions remained the same. Samples were analysed using high performance liquid chromatography (HPLC, as described below) after 24 h reaction time. To study the effect of an acidic pH on the laccase, 1.5 U of laccase was dissolved in 31 ml of sodium acetate buffer (0.1 M pH 3.5) and the activity of laccase was determined after 24 h for a period of 5 days.

2.6. Analysis of the polymeric product

The conversion of 8-hydroxyquinoline by laccase was monitored using HPLC (Merck La Chrom). The mobile phase was methanol–acetic acid–water (60:2:40) with a flow rate 1 ml/min, and using a C18 Waters (250 mm × 4.6 nm) reverse phase column and UV detection at 240 nm. Peaks were analysed using HPLC Manager, Merck Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of reaction sample with that of a control.

MALDI-TOF mass spectrometry was used to determine the molecular weight of the compound. MALDI-TOF mass spectra were obtained using a Perseptive Biosystems DE-PRO MALDI mass spectrometer equipped with a TOF analyser operated in positive ion mode. Sample aliquots (1 ml) in 50% chloroform were mixed with 1 ml of 2,5 dihydroxybenzoic acid matrix and applied to the gold sample plate (analyses performed as a service in the Department of Molecular and Cellular Biology, University of Cape Town).

For infrared (IR) spectroscopic analysis, a 1 mg sample was dissolved in dichloromethane and analysed for functional

groups using a Perkin Elmer Spectrum One FT-IR spectrometer.

2.7. Antioxidant activity of the polymeric product using the DPPH free radical method

The method used to determine the antioxidant activity was a modified version of the methods of Siddhuraju and Becker [17] and Sánchez-Moreno et al. [18]. A stock solution 400 mg/l of poly (8-hydroxyquinoline) was prepared in the mixture of 1,4-dioxane and methanol (3:7, v/v). An aliquot of this solution (100 μl) of different concentrations (400, 267, 200 and 100 mg/l) was added to 3.9 ml DPPH solution (6×10^{-5} M in methanol). The decrease in the absorbance at 515 nm was monitored using a Unicam UV–vis spectrophotometer, until the reaction reached the steady state. A control experiment was conducted using the same reaction mixture in the absence of poly (8-hydroxyquinoline) to confirm that the dioxane did not affect the DPPH assay. For comparison, the same experiment was conducted using 8-hydroxyquinoline to determine the antioxidant activity of the monomeric starting material.

The percentage of remaining DPPH was calculated as follows:

$$\%\,\text{DPPH remaining} = \frac{[\text{DPPH}^\bullet]_t}{[\text{DPPH}^\bullet]_{t=0}} \times 100$$

where $[\text{DPPH}^\bullet]_t$ was the concentration of $\text{DPPH}^\bullet$ at the time of steady state and $[\text{DPPH}^\bullet]_{t=0}$ was the concentration of $\text{DPPH}^\bullet$ at time zero. The concentration of the antioxidant [poly (8-hydroxyquinoline)] required to decrease the initial DPPH concentration by 50% (the EC_{50}) was determined graphically [18]. The antiradical efficiency (AE) is defined as $AE = 1/EC_{50} \times T_{EC_{50}}$ where $T_{EC_{50}}$ was the time needed to reach a steady state when using an initial concentration of antioxidant equal to the EC_{50} concentration [18]. The experiment was performed in duplicate.

3. Results and discussion

Extracellular laccase was produced by culture of *Trametes pubescens* in an airlift reactor [14] and partially purified by ammonium sulphate precipitation. The laccase, with an activity of 1.8 U/mg, was used in bioconversion experiments in which the enzyme catalyzed the transformation of 8-hydroxyquinoline in an acetate buffer medium containing 8% acetone to yield a polymeric product. It is suggested that the laccase oxidized 8-hydroxyquinoline to form aromatic radicals, which in turn combined to form a polymeric product which precipitated spontaneously from solution due to low solubility. The polymeric product was not soluble in water or the sodium acetate buffer used for the reactions, and it was only poorly soluble in methanol, but completely soluble in 1, 4-dioxane.

It is well-recognised that the mechanism of action of laccase involves radical-forming steps which can result in the formation of carbon–carbon or carbon–oxygen linked polymeric products ([6] and references therein). However, relatively few reports have included confirmed structural characterisation of products of such reactions. Oxidation of 8-hydroxyquinoline by laccase resulted into a mixture of polymeric products as depicted by the MALDI-TOF spectrum (Fig. 1) where the peak distribution reflects the quantity of various oligomers present in a solution. Tetramer, decamer, and hexamer products gave the greatest signal heights, therefore representing the most abundant oligomers in the sample. Structures also detected varied from dimers (289 *m/z*), trimers (431 *m/z*) to a polymer (2149 *m/z*) with 15 monomers of 8-hydroxyquinoline (145 *m/z*) indicating the highest degree of polymerisation. The mass difference between the peaks representing various oligomeric structures was 143 *m/z* which is the mass of a monomer (145-2H), with loss of 2 hydrogens being attributable to linking with another monomer. The peak average molecular weight or number average molecular weight M_n was found to be 789 *m/z* as calculated

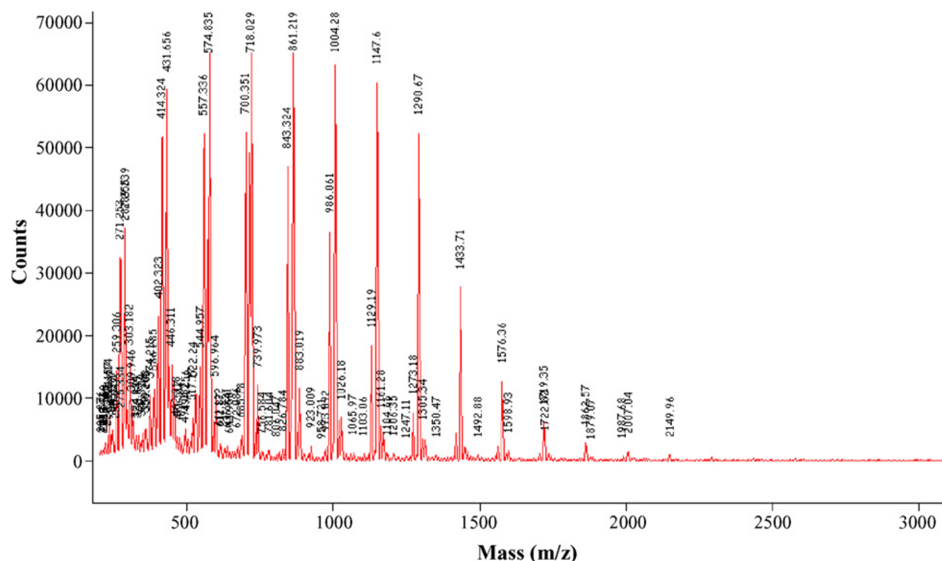

Fig. 1. MALDI-TOF mass spectrum of poly (8-hydroxyquinoline) obtained by laccase oxidation of 8-hydroxyquinoline in acetone/sodium acetate buffer.

using the formula:

$$M_n = \frac{\text{total weight}\,(N_i M_i)}{\text{number of polymers}\,(N_i)}$$

where N_i is the number of polymer molecules with mass M_i [19]. The type of linkages of monomers cannot be conclusively determined from the MALDI-TOF spectrum provided, since, for example, four monomers constituting a tetramer (574 *m/z*) could be joined by either C–O or C–C linkage with concomittant lost of a total of six hydrogen atoms. Furthermore, the spectrum gives no information on the regiochemistry of these linkages. In this study, further characterisation by NMR of the product was complex and largely unhelpful, due to the complexity of polymerisation resulting in a mixture of oligomeric and polymeric structures which were not found to be separable, in common with a similar phenomenon reported by Reed et al. [20]. Furthermore, the complications associated with the interpretation of the NMR spectrum can be attributed to the fact that positions of monomer additions may differ among oligomers of the same degree of polymerisation [20,21]. Further characterisation of the poly (8-hydroxyquinone) product was attempted by infrared spectroscopy to identify the functional groups present. The infrared spectrum, measured in the region of 4000–400 cm^{-1}, showed the presence of a peak at the region of 3686.37 cm^{-1} due to the hydroxyl group (O-H) vibrations in the compound. The absence of this peak would suggest that the monomers are linked by C–O bonds. Other bonds observed were C–N at 1274.91, C–H at 3004.90, C–C at 1605.68 and C–O at 1252.99 cm^{-1} region. These IR results were also similar to those obtained by Krishnakumar and Ramasamy [22] who reported the vibrational spectra of isoquinoline and 8-hydroxyquinoline; this supports our conclusion that the product obtained was a polymer of 8-hydroxyquinoline. The structure of the product was thus confirmed to comprise 8-hydroxyquinoline monomers linked by C–C or C–O bonds to form the polymeric structures shown in Fig. 2. A similar result was obtained by Ikeda et al. [23] who reported that C–C coupling occurred during the laccase-catalyzed polymerisation of 2,6-dimethyl-1,4-phenylene oxide under acidic conditions.

In an investigation of the time course of the reaction, the polymeric product was isolated from the reaction mixture after periods of 24, 48 and 72 h, and the laccase activity in the reaction mixture was monitored during this time. The laccase activity was found to decrease steadily and the amount of polymeric product being produced in each time period decreased correspondingly as the activity of laccase in the reaction mixture decreased (Fig. 3). The highest amount of product (85 mg per 30 ml reaction volume, initially containing 3 U laccase) was obtained within 24 h, correlating with laccase activity being relatively high over this period; a further 20 mg of the polymeric product was obtained from the same reaction mixture after the reaction had run for 48 h. The reaction was continued until the activity of the laccase was zero and no further product was obtained. The total yield of the polymeric product after 72 h was 50% (mass of the product/mass of the starting material × 100). Upon addition of 2 U of fresh laccase to the same reaction mixture, a further 9 mg product was obtained within a further 24 h, confirming that the formation of polymeric product was dependent on the presence of active laccase in the reaction medium although this additional yield of product, obtained after the second dose of laccase, represents only 10% of the 100 mg theoretically possible. Approximately half of the original laccase activity was lost within the first 24 h of reaction, and while this loss of the enzyme activity may be attributable to the acidic conditions, both of these effects may also be due to accumulating polymeric product.

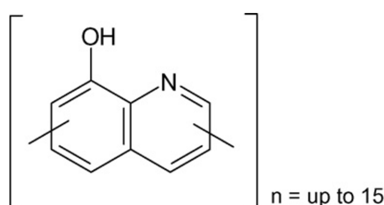

Fig. 2. Proposed structure of the poly (8-hydroxyquinoline) product.

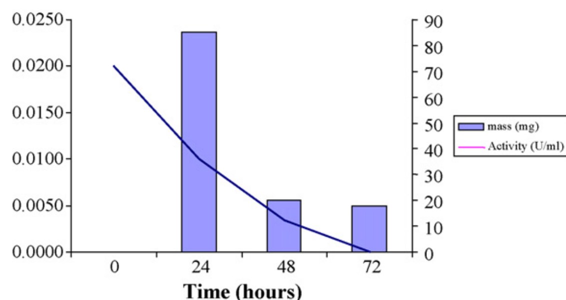

Fig. 3. Activity of laccase in the reaction mixture with 8-hydroxyquinoline in acetone–sodium acetate buffer, showing the relationship between laccase activity (left side axis and line graph) and the amount of polymeric product produced (right side axis and bars).

The effect of temperature on the bioconversion of 8-hydroxyquinoline by laccase was investigated; the optimal temperature was found to be 30 °C, and a yield of 76% was achieved after 24 h at this temperature (Fig. 4). This gives a clear indication that operation at higher temperature, with the objective of enhancing reaction rate, or at lower temperature to enhance protein stability, would not necessarily be advantageous in terms of product yield. We acknowledge that there is a need for optimisation of the reaction process and this work will be reported in due course.

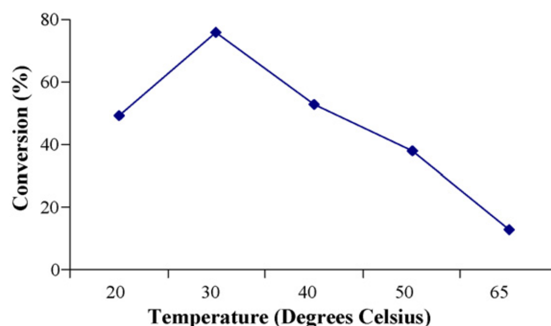

Fig. 4. Effect of temperature on the bioconversion of 8-hydroxyquinoline by laccase (monitored using HPLC).

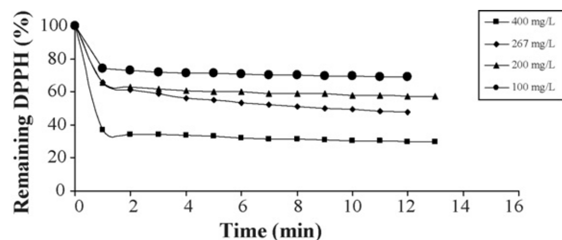

Fig. 5. Time courses of DPPH quenching reactions due to poly (8-hydroxyquinoline) antioxidant activity.

In view of the obvious potential of the poly (8-hydroxyquinoline) as an antioxidant, the antioxidant capacity of the product was evaluated by monitoring the quenching of the stable free radical DPPH [18]. Addition of the poly (8-hydroxyquinoline) solution to the purple coloured DPPH solution caused a colour change from purple to yellow, a positive indication of quenching of the free radical DPPH [24]. The percentage of DPPH remaining in the presence of poly (8-hydroxyquinoline) at different concentrations (400, 267, 200 and 100 mg/l) are shown in Fig. 5. When the same experiment was conducted with the unpolymerised 8-hydroxyquinoline monomer (400 mg/l) in place of the polymer, 7% of DPPH radical was quenched after 12 min as compared to 60% quenched by a polymer at the same concentration. Thus the antioxidant activity of the monomer was found to be lower by 53%. The percentages of the DPPH remaining at the steady state decreased with an increase in the concentration of the polymer, and the time required to reach the steady state varied between 1 and 3 min. This kinetic behaviour would be classified as rapid in terms of antioxidant activity relative to other known antioxidants (previously classified by Sanchez-Moreno et al. [18]). The concentration of the antioxidant [poly (8-hydroxyquinoline)] required to decrease the initial DPPH (EC_{50}) concentration by 50% was obtained by plotting the percentages of DPPH remaining against the concentration of the polymer per gram DPPH (Fig. 6). The EC_{50} of poly (8-hydroxyquinoline) was found to be 250 mg antioxidant/g DPPH. This value is comparable to the EC_{50} of DL-α-tocopherol which is 200 mg antioxidant/g DPPH, and is 10 times higher than that of gallic acid (26 mg antioxidant/g DPPH) as reported by Sánchez-Moreno et al. [18]. The antiradical efficiency (AE) of poly (8-hydroxyquinoline) was found to be 2×10^{-3} which is similar to that of gallic acid (2.62×10^{-3}) [18].

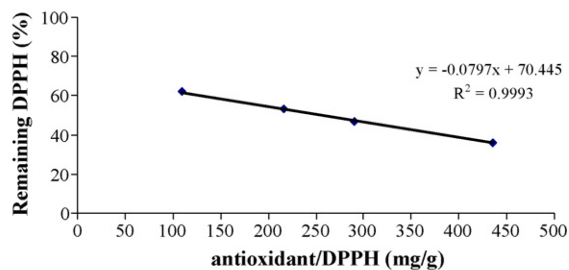

Fig. 6. Graph of DPPH remaining in solution as a function of poly (8-hydroxyquinoline) added per gram of DPPH, used to determine antioxidant efficiency AE.

The antioxidant capacity of any compound, as demonstrated by its ability to scavenge free radicals, is dependent on a combination of factors including its electronic structure and hence its ability to abstract or donate hydrogen atoms, the ionization potential of the reactive hydroxyl groups present in the molecule, and the potential for stabilisation of the resulting radical by charge delocalisation [25,26]. In aromatic polymeric structures such as lignin, free radical scavenging activity has been correlated with the molecular weight of the polymer and the extent of π-polyconjugated systems in the structure. It is suggested that antioxidant power is enhanced by the presence of extended π-polyconjugation systems as well as the presence of numerous specific groups such as phenolic hydroxyls and benzylic hydrogen moieties [26]. It is interesting to note also that the antioxidant power of kraft lignin fractions which are aromatic phenolic polymers of similar molecular weight (although lacking in hetero-nitrogen groups), have been reported to have similar antioxidant power, in the range 0.05 to 2×10^{-3} [27]. Correspondingly, in the present study, the scavenging activity of the poly (8-hydroxyquinoline) can be attributed to the increased additional conjugation of the radical product due to the extended aromatic structure which will contribute to π-polyconjugation and hence favour the antioxidant quenching reaction with free radical agents.

Uses of antioxidant products extend across a very broad range of applications, from medical products and nutraceuticals to industrial preservatives. The results obtained in this study suggest that poly (8-hydroxyquinoline) has potential for application as an antioxidant in a number of areas such as these. For example, the anti-bacterial properties of compounds with structures similar to poly (8-hydroxyquinoline), including lignins [13,26], melanins [27] and derivatives of 8-hydroxyquinoline itself [28] are attributed to their ability to inhibit free radical reaction processes. Similarly, aromatic antioxidants such as butylated hydroxytoluene (BHT) are well-known for their application as stabilisers in resins and plastics [29]. Our results indicate that the polymer has antioxidant properties superior to those of the monomer. Monomeric antioxidants (for example, BHT) are currently used in manufacture of materials such as plastics, but their preservative properties are known to be lost over time, largely as a result of physical loss, e.g., by leaching and evaporation [29]; it is likely that a less mobile antioxidant, such as the polymer reported here, would be less readily lost from the material and may thus enhance the stability of the material. Research to develop such applications are currently being conducted in our laboratory.

4. Conclusion

To our knowledge, this is the first report of the production of poly (8-hydroxyquinoline), using laccase from a *T. pubescens* strain and it is one of very few to suggest a structure for the product of a laccase-catalyzed polymerisation reaction. The polymerisation was catalyzed by the laccase in acetone–sodium acetate buffer at ambient temperature and pressure. This reaction, yielding an antioxidant polymer using a sustainable catalyst in an aqueous-based, low temperature system represents

a feasible environmentally benign alternative to conventional polymerisation methods. A loss of laccase activity during the production of this polymer was observed; however, this is not regarded as problematic because fungal laccases can be readily and cheaply produced. Thus, we envisage that, in an optimised process, fresh laccase could be fed into a reactor as necessary, until the desired level of conversion has been achieved. In order to explore the possible uses of the polymeric product, the DPPH antioxidant assay was performed to determine the antioxidant activity of poly (8-hydroxyquinoline). It was established that poly (8-hydroxyquinoline) can act as a potent antioxidant with an antiradical efficiency comparable with that of other compounds commonly used as antioxidants, while its polymeric nature may be an advantage in certain applications.

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