Production of hydroxyl radicals by lichens via extracellular hydroquinone-driven redox cycling

By

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ABSTRACT

Hydroxyl radicals play crucial roles in biology, and these include the breakdown of lignocellulosic residues in the soil, which may allow species to live a partially saprotrophic lifestyle, and the breakdown of potentially toxic soil chemicals. The aim of this study was to conduct a survey to determine if the extracellular hydroxyl radical formation occurs in a range of lichenized ascomycetes. In this study, the production of hydroxyl radicals by lichen via an extracellular redox cycling process was examined. It was also tested whether lichen species could decolorize different synthetic dyes. There is evidence that free-living white and brown rot fungi can generate hydroxyl radicals using extracellular redox cycling. Results showed that given a quinone and chelated ferric ions, many lichens can readily produce hydroxyl radicals, and at the same time reduce ferric ion to ferrous ions. There were no significant differences in the rate of hydroxyl radical formation in non-Peltigeralean and Peltigeralean lichens. While in white rot fungi extracellular redox enzymes have been proposed to be involved in hydroxyl radical generation however, in lichens hydroxyl radical production is not correlated to the activities of laccase and peroxidase. Therefore, it seems likely that the radicals are produced by Fenton chemistry, by a mechanism like that proposed for brown rot fungi. In these fungi, a surface reductase reduces a quinone to a hydroquinone, which reacts directly with ferric ions non-enzymatically generating a quinone radical. This radical spontaneously re-forms a quinone, producing H_2O_2. The resulting ferrous ion and H_2O_2 can react together to give the ·OH radical.

Results indicate that lichens can decolorize a range synthetic dyes on their own, but when given quinones and chelated Fe decolorisation was greatly enhanced as this will facilitate the formation of hydroxyl radicals. RBBR was the best metabolized dye and *Usnea undulata* was the most effective lichen species from the list of dye and lichen tested. However, adding H_2O_2 concentration had little effect on dye decolorisation.

DECLARATION 1

The experimental work described in this study was carried out in the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg Campus, from 2015 to November 2016, under the supervision of Professor Richard P. Beckett.

The experiments and studies represent the original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

We the undersigned certify that the above statement is correct:

CEM

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PROFESSOR RICHARD P. BECKETT (SUPERVISOR)

DECLARATION 2 – PLAGIARISM

I, **CALVIN EDDINGTON MOYO**, student number: **210546195** declare that

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2. This thesis has not been submitted for any degree or examination in any other University.
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DECLARATION 3 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/ or include research presented in this thesis (include publications in preparation, in press and submitted and give details of the contributions of each author to the experimental work and writing.

Publication 1: **Moyo CE**, Beckett RP, Trifonova T, Minibayeva FV. 2016. Extracellular redox cycling and hydroxyl radical production occurs widely in lichenized Ascomycetes. *Fungal Biology***. (Submitted 28 October 2016).**

Publication 2: Moyo CE, Beckett RP. Dye decolorisation by lichens via redox cycling process**. (In preparation).**

Signed By……. CEM

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LIST OF ABBREVIATIONS

·OH	Hydroxyl radical
ABTS	2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
BQ	1, 4-benzoquinone
$CaCl_2$	Calcium chloride
DMBQ	2, 6-Dimethoxy-1, 4-benzoquinone
$FeCl_3$	Iron (III) chloride
H_2O_2	Hydrogen Peroxide
KDa	Kilo Dalton
MBQ	2-Methoxy-1, 4-benzoquinone
MD	2-methyl-1, 4-naphthoquinone (menadione)
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium chloride
$O_2\cdot^-$	Superoxide anion radical
RBBR	Remazol Brilliant Blue R
ROS	Reactive oxygen species
Rpm	Revolutions min^{-1}
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TRIS	2-Amino-2-(hydroxymethyl)-1, 3-propanediol

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CHAPTER 1 - GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Lichens

Lichen are an association of a fungus and a photosynthetic symbiont (Nash 1996; Russo et al. 2008). Moreover, Ahmadjian (1993) defined a lichen as "*An association between fungi, usually an ascomycete but in few cases a basidiomycete or deuteromycete, and one or more photosynthetic partners, generally green algae or cyanobacteria. In all lichens the fungus forms a thallus or lichenized stroma that may contain unique secondary compounds".* The definition comprises information on more recent discoveries about the relationship between lichen and fungi association. Recently Spribille et al. (2016) suggested that other microorganisms are involved in the lichen symbiosis such as basidiomycete yeasts found in the cortex of lichen thalli. Researchers show that lichens across six continents also contain basidiomycete yeasts, single-celled fungi that likely produce chemicals that help lichens ward off predators and repel microbes (Spribille et al. 2016). However, the association is not a simple structure of two or more species. The fungus produces the first layer (non-reproductive tissue) thallus while the second layer includes the photobionts. Lichens can be grouped according to their thallus type, i.e. fruticose, crustose and foliose (Hale 1969). The fungal partner cannot photosynthesize; therefore, they are often thought to depend on the alga partner to produces sugars. However, it has been suggested that lichen fungi may have the ability to breakdown dead or living organic material for food to supplement algal photosynthesis (Beckett et al. 2013). The association has been regarded as successful given the distribution of lichen species on earth which ranges from rocky, bare soils, moist and dry conditions (Ahmadjian 1993; Beckett et al. 2014). The precise nature of the symbiosis has been much debated, and there have been suggestions that the relationship between the species is more commensalistic or parasitic than mutualistic as the photosynthetic partner in nature can survive by its own, while fungal partner depends on the algae for survival (Ahmadjian 1993).

The Ascomycetes are the largest Class of lichen fungi, comprising 98 % of species, while 2 % belongs to the Basidiomycetes, Phycomycetes and Hyphomycetes Classes (Beckett et al. 2013). Approximately 20 % of all fungi are lichenized, and there are 13,500 species of lichens in the world (Beckett et al. 2013; Russo et al. 2008). Although lichens often represent a minor component of terrestrial ecosystems, they form the dominant plant life over large areas of the world. These include the vast areas of dryland crusts that occur in southern Africa, and the lichen-dominated vegetation of the Arctic and Antarctic regions. These habitats are characterized by severe abiotic stresses such as desiccation, temperature extremes and high

light intensities (Nash 1996; Russo et al. 2008). Lichens are often the first organisms to colonize bare rock or soil after disturbance, presumably because of their tolerance to stress and low demand for nutrients (Ahmadjian 1993; Nash 1996). There is also evidence that lichens contain unique secondary metabolites "lichen substances" and have morphological features which enables them to survive in stressful environmental conditions (Nash 1996). For this reason, lichens are often called "extremophiles" (Beckett et al. 2008, 2013). Destructive change in environmental conditions could result in the formation of reactive oxygen species (ROS) and the inhibition of photosynthetic apparatus. Often morphological traits are important to decrease the extent of these stresses, for example, the different forms of lichens (fruticose, crustose etc.) allow lichens to inhabit a variety of substrates and environments.

1.1.1 Are lichens autotrophs or saprophytes?

Organisms can be divided mainly into two classes depending on how they get their energy. Autotrophs are organism that can make their own food from photosynthesis (sunlight) or simple chemical reactions. Conversely, saprotrophs obtain their food from other organisms. It is currently unclear whether lichens should be classified as autotrophic or saprotrophs. However, it is believed that lichens normally have an autotrophic lifestyle, because the photobiont partner (algae or cyanobacteria) is photosynthetically active (Palmqvist 2000). Beckett et al. (2015) found evidence suggesting that lichens may be also able to live a saprotrophic lifestyle, as they contain redox enzymes such as laccases and peroxidases. Beckett et al. (2015) showed that during times of low photosynthesis these enzymes may be upregulated to provide additional carbon.

If lichens are saprotrophs then they should be able to breakdown lignocellulose; this may occur by direct action of enzymes such as cellulases, but also indirectly, for example by laccases and peroxidases working with "redox mediators" or by enzymes producing extracellular radicals through redox cycling.

1.2 Enzymes

There are biological catalysts that speed up chemical reaction processes without being consumed, there are usually proteins with secondary of tertiary structure. Each enzyme has an active site for catalyzing a reaction. Lichens contain redox enzymes, specifically laccase, tyrosinase, cellulase, and peroxidase which in free-living fungi participate in carbon turnover. These enzymes can work directly or indirectly by the production of ROS (Baldrian 2008; Beckett et al. 2013; de los Rios et al. 1997, Laufer 2006 a, b; Zvararzina and Zavarzin 2006).

1.2.1. Peroxidase

Peroxidase (EC 1. 11 .1. x) belong to a large family enzymes that are found in all living organisms (Beckett et al. 2013; Liers et al. 2011; Welinder 1992). Peroxidase have higher affinities and redox capabilities due the involvement of H_2O_2. The enzyme can catalyze one electron in oxidation of different compounds for example phenolics using H_2O_2 as an electron acceptor (Beckett et al. 2013; Welinder 1992). In fungi, there is convincing evidence that peroxidase is involved in carbon turnover and breakdown of xenobiotic compounds.

Basidiomycetes have Class II peroxidases such as manganese peroxidase, lignin peroxidase and versatile peroxidase. These enzymes are lignin degrading and plays a crucial role in wood decay (Kerem et al. 1999). In white rot fungi peroxidases, have been found to be involved in lignin and cellulose degradation, also the enzyme activity has a direct impact in the production of hydroxyl radicals (Gomez et al. 2009a, b).

Studies shows that Peltigeralean species contain active peroxidases and there is some evidence of the presence of peroxidases in non-Peltigeralean lichens (Beckett et al. 2013; Liers et al. 2011). While lichens contain peroxidases, they do not contain classical Class II peroxidases as in bacterial or fungal hence new class of peroxidase have been proposed (Liers et al. 2011).

1.2.2. Cellulases

Cellulases are catalyze cellulose hydrolysis enzymes, occurs in fungi, lichens, bacteria, and protozoans (Rabinovich et al. 2002; Yagüe, Estévez, 1990). There are different types of cellulases depend on type of reactions this include endocellulases (EC 3.2.1.4), cellobiases (EC 3.2.1.21), exocellulases (3.2.1.91), oxidative cellulaes and cellulose phosphorylases. The function of these cellulases include breakdown of internal bonds of cellulose and create new polysaccharide chain ends, hydrolyze products of exocellulases to monosaccharides and to generate radicals for lignin degradation mainly by oxidative cellulases enzyme using Fenton type reactions (Rabinovich et al. 2002).

In lichens cellulases have been studied since 1980's, commonly in epiphytic species such as *Evernia prunastri* (Guerrero et al. 1992; Yagüe, Estévez, 1990). *Evernia prunastri* experimentally has been found to "hydrolyze Avicel" (a microcrystalline cellulose substrate). The implication is that lichens must produce all the components involved in cellulose breakdown. Therefore, this could theoretically support a saprotrophic lifestyle which would helpful in times of low photosynthesis (Becket et al. 2013; Yagüe, Estévez, 1990).

1.2.3. Laccases

Laccases (EC 1.10.3.2), are polyphenol oxidases that contain copper (Laufer et al. 2006a). Laccase a widely-distributed enzyme, occurring in lower and higher plants (Mayer, Staples 2002; Laufer et al. 2006a). It was first discovered by Yoshida in 1883 from a Japanese lacquer tree, *Rhus vernicifera*. In fungi species, it was first demonstrated by Bertrand in 1896 (Baldrian 2006). Laccase belongs to the oxidative enzyme group and depends on oxygen for activation. Generally, laccase occur in flowering plants, fungi and a few bacteria species fungi and in free living ascomycetes (Lyons et al. 2003; Tetsch et al. 2005).

The activity of laccases in lichens has been first detected in Petigerales and Lecanorales species, however with low activities in order Lecanorales (Beckett et al. 2013). The activity of laccases in lichens can be stimulated by wounding and desiccation (Laufer et al. 2006b), suggesting that they may be involved in defense against pathogen attack or grazing (Beckett et al. 2015; Laufer et al. 2006b).

1.2.4. Quinones reductases

Quinones are conjugated and oxidized directives of aromatic compounds. Quinones are said to be made from reactive aromatic species such as phenols and catechol's. They belong to a class of chemicals that occur naturally in bacteria, fungi and plants (Monks, Jones 2002). Quinones at a large scale are used in the production of hydrogen peroxide. Many industrial and natural dyes are derivatives of quinone compounds However, in medicine quinones have anti-tumoral activity, and can have antimicrobial and antiparastic functions (Stepanenko et al. 1997).

There are specific quinones in organisms.e.g.1, 2-Benzoquinone, 1, 4-Benzoquinone, 1, 4-Naphthoquinone and 9, 10-Anthraquinone.

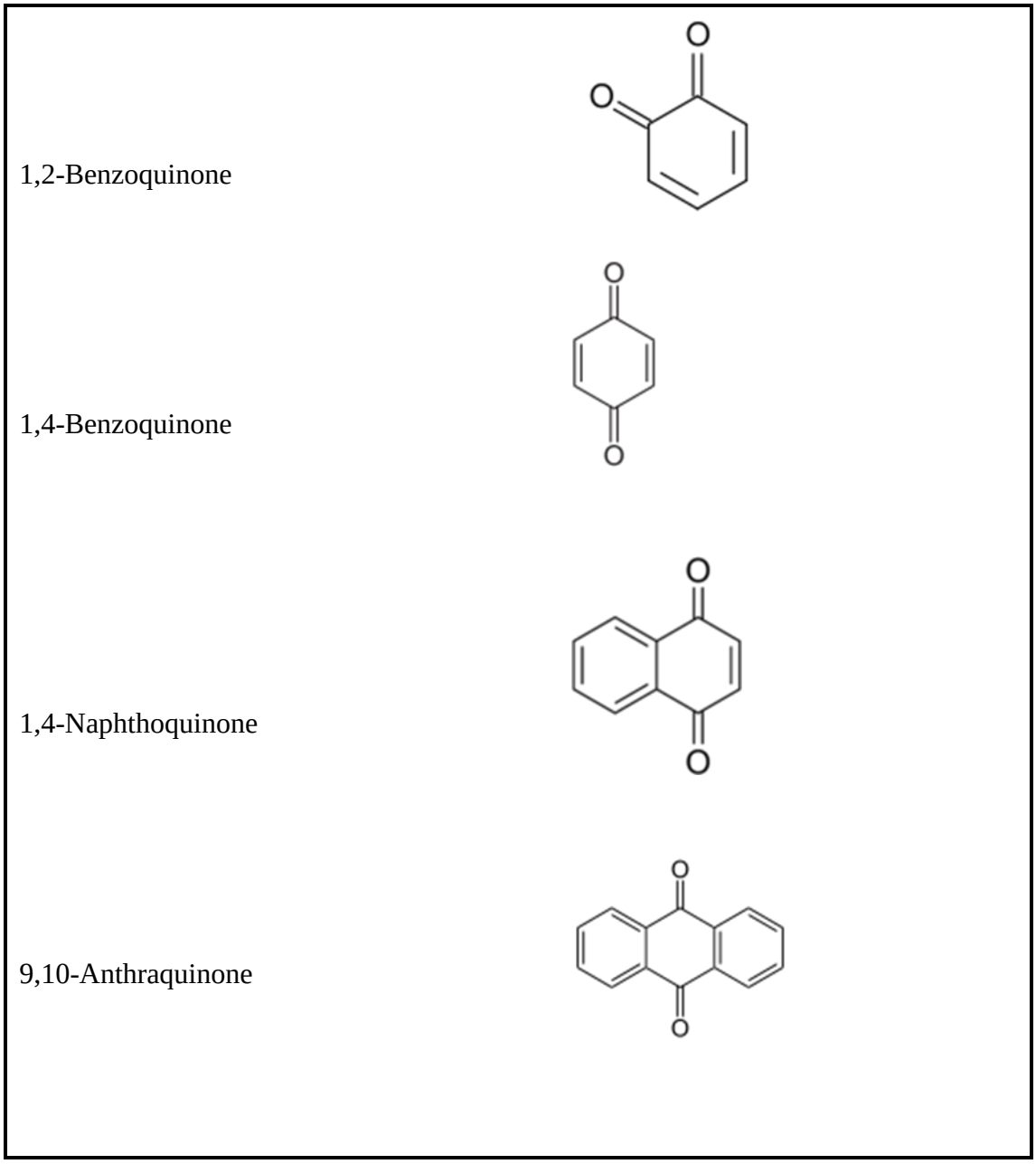


Figure 1.1. Structure of quinones found in plants species

Recently there was evidence quinones may be involved in extracellular redox cycling in lichens (Beckett et al. 2015), although the naturally occurring quinones in lichens are unknown. Recently Zavarzina et al. (2017) discovered that lichens secrete a range of soluble phenols, with most phenol being *p*-hydroxybenzoic, syringic, salycilic and vanillic acids. They also secret a range of enzymes and there is a possibility that the enzymes will synthesize the quinones needed for redox cycling from the phenolics. In other organisms, it has been shown that syringic acid can be metabolized by laccases into 2.6-dimethoxy-1.4-benzoquinone (Koschorreck et al. 2008). Some evidence for the existence of quinone reductases enzymes in

lichens comes from bands that produce superoxide “in gel” when supplied with NADH (Beckett et al. 2015; Liers et al. 2011).

1.2.5. Possible roles of quinones reductases, laccases, peroxidases and cellulases in lichens

The detailed biological roles of laccases, peroxidases, quinones reductases and cellulases are still unknown, however it seems certain that each enzyme plays more than one role in lichen biology. The roles of these enzymes in lichen mainly are pathogen defence, protect against quinone induced damage, abiotic stress tolerance and possibly to facilitate a saprophytic lifestyle (Beckett et al. 2013; Tan, Berridge 2010). As mentioned earlier these enzymes can metabolize a rage of organic or inorganic compounds. Furthermore, they can work indirectly by generating ROS.

1.3. Reactive oxygen species

Reactive oxygen species (ROS) are compounds that are chemically reactive molecules which contain oxygen molecule. Successive reduction of oxygen molecules lead to formation of different group of ROS. The formation of ROS species in plants is almost always stimulated by stress (Kranner et al. 2008). Example of the different types of ROS species are peroxides, superoxide, singlet oxygen and hydroxyl radical.

Peroxides are very reactive molecules; therefore, they occur in nature only in limited forms for example H_2O_2. H_2O_2 can be regarded as a signaling compound in plants and is used for protection especially against pathogens. Superoxide is usually produced in large quantities by the mitochondria of most organisms, and used by white blood cells to kill pathogenic microorganisms. Furthermore, these radicals can function as mitogens (Aruoma et al. 1989).

Oxygen radical are biologically toxic and can cause oxidative stress if produced in excess (Aruoma et al. 1989). However, antioxidants such as superoxide dismutases (SOD), catalase and peroxidase facilitate the control of oxygen radicals and their products. There is strong evidence of superoxide production by lichen species (Beckett et al. 2003).

Hydroxyl radical (·OH) is a lethal and most aggressive non-specific oxidant in biological systems, it is a short lived radical (10^{-9s}) (Wood 1988; Hayyan 2016). Fungi produce this aggressive oxidant for lignocellulose breakdown or xenobiotic compounds degradation especially degradation of wood and it is believed to play a key role in the saprophytic lifestyle in fungi (Hammel et al. 2002).

Hydroxyl radical is very aggressive; therefore, the production of the radical must be close to their targets due to diffusion limitations, and at some distance from the hyphae to prevent injury to the plant. In plants the production of ·OH can occur in a cell wall, plasma membrane and intracellularly (Richards et al. 2015). In fungi, there are different pathways that have been discussed in the production of ·OH. These pathways involve the reduction of Fe and O_2 by secreted hydroquinone's or by cellobiose dehydrogenase enzymes (Hammel et al. 2002). It is suggested that the production of ·OH in white rot fungi and brown fungi is mostly via the Fenton reaction mechanism (Gomez et al. 2009a, b).

1.3.1. Fenton chemistry

Fenton chemistry is the reaction between peroxides e.g. H_2O_2 and active metal ions such as iron (Fe). Fenton chemistry can produce hydroxyl radicals or higher oxidation states of Fe (Fenton 1894; Winterbourn 1995). The reaction mainly occurs in acid environments where it spontaneously occurs to form ROS, mostly hydroxyl radicals (Fenton 1894; Hayyan 2016). The products of this reaction capable of oxidizing different organic compounds thereby causing biological damage (Winterbourn 1995). Generally, in Fenton reaction chemistry the first step is the reduction of naturally occurring oxygen molecules in cells which will produce superoxide and the dismutation of superoxide then produces H_2O_2. However, the reduction of oxygen is very complex in non-aqueous or aqueous solution (Hayyan 2016). The H_2O_2 may partially be reduced to hydroxyl radical or H_2O. In the presence of transition metals such as (Fe and Mg), H_2O_2 can react with Fe^{2+} to form ·OH radicals. In the process Fe^{3+} is reduced to Fe^{2+} (Kang et al. 2002).

Equation 1 $O_2 + e \rightarrow O_2^-$

Equation 2 $2H + O_2^- + O_2^- \rightarrow H_2O_2 + O_2$

Equation 3 $H_2O_2 \rightarrow HO + OH$

Equation 4 $H_2O_2 + Fe^{3+} \rightarrow \cdot OH + OH\cdot + Fe^{2+}$

Equation 5 $Fe^{2+} + H_2O_2 \rightarrow \cdot OH$

The above equation (1-5) shows a mechanism of Fenton reaction process. All the reactions can occur as reverse reactions (Fenton 1894).

1.3.2. Production of ROS by white and brown rot fungi

Interesting findings by Cowling (1961) experimental suggested that enzymes which have been said to be responsible for wood decay were too large to penetrate undamaged wood cell wall,

hence the research focused on non-enzymatic action and the roles of ROS in lignin degradation. It has since been discovered that the production of ROS, especially of the hydroxyl radical was involved in wood decay (Arantes, Milagres 2006a; Goodell et al. 1997; Hammel et al. 2002). Koenigs (1974a, b) provided a detailed comparison between the effects of Fenton chemistry and brown rot fungi on wood decomposition and proposed that during early stages hydroxyl radicals are produced by brown rot fungi. Later it was realized that the early stages of decay of both lignin and cellulose is facilitated by the production of highly aggressive hydroxyl (·OH) radicals generated by Fenton chemistry (Arantes et al. 2014).

1.4. Models for extracellular redox cycling in fungi

In free-living fungi, various models have been proposed to explain how free-living fungi can produce extracellular ROS e.g. ·OH.

In the first model, it is suggested that redox enzymes oxidize hydroquinone's to semiquinone radicals, which then spontaneously form quinones, producing superoxide and hydroxyl radicals by Fenton-type reactions (Gomez et al. 2009a, b). It has been proposed that chelated Fe^{3+} reacts with hydroquinone's producing Fe^{2+} and semiquinone radicals. These radicals then spontaneously form quinones, reducing O_2 to $^{\cdot}OOH$ in the process; the $^{\cdot}OOH$ radical dismutates to H_2O_2. H_2O_2 and Fe^{2+} react together to give hydroxyl radicals, while hydroquinone's are regenerated from the quinones using a reductase on the surface of this hyphae (Kerem et al. 1999). The whole process has been called "hydroquinone-redox cycling", and an essential aspect of this mechanism is that hydroxyl radical production can occur at a distance from the fungal hyphae. Hydroquinones are possibly regenerated from quinones by a membrane bound quinone reductase.

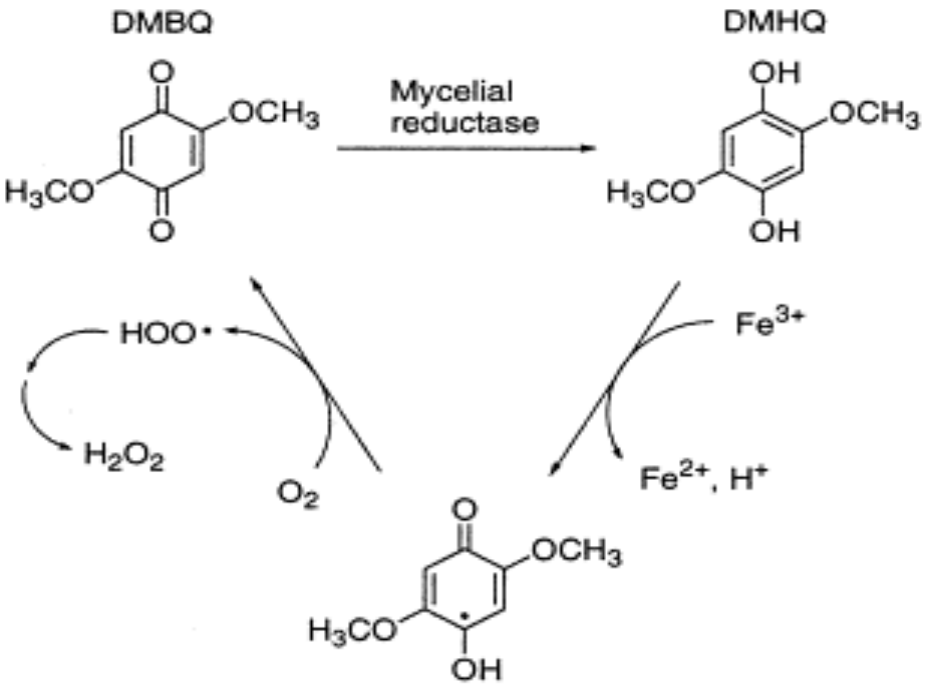


Figure 1.2. Simplified mechanism for extracellular Fe^{3+} reduction and H_2O_2 production in brown rot fungi (*Gloeophyllum trabeum*). Taken from Kerem et al. (1999).

The above diagram shows a series of reaction for the reduction of Fe^{3+} and H_2O_2 production which promote hydroxyl radical production in brown fungi explained in first model.

In the second model, it is suggested that surface quinone reductases reduce quinones to hydroquinone's that react directly with Fe^{3+} non-enzymatically generating a quinone radical and Fe^{2+} (Arantes et al. 2014). The quinone redox cycling in white rot fungi is stimulated by active cell-bound enzyme (quinone reductases) which will reduce the quinones to hydroquinone's. In the presence of enzymes such as laccase or peroxidase which assist in oxidation, hydroquinone reduced to a quinone radical. Quinone radical then reduce Fe^{3+} to Fe^{2+}. The resulting Fe^{2+} ion and H_2O_2 can react together to give the hydroxyl radical (Kerem et al. 1999). The quinone radical spontaneously re-forms to quinone to complete the cycle (Gomez et al. 2009a).

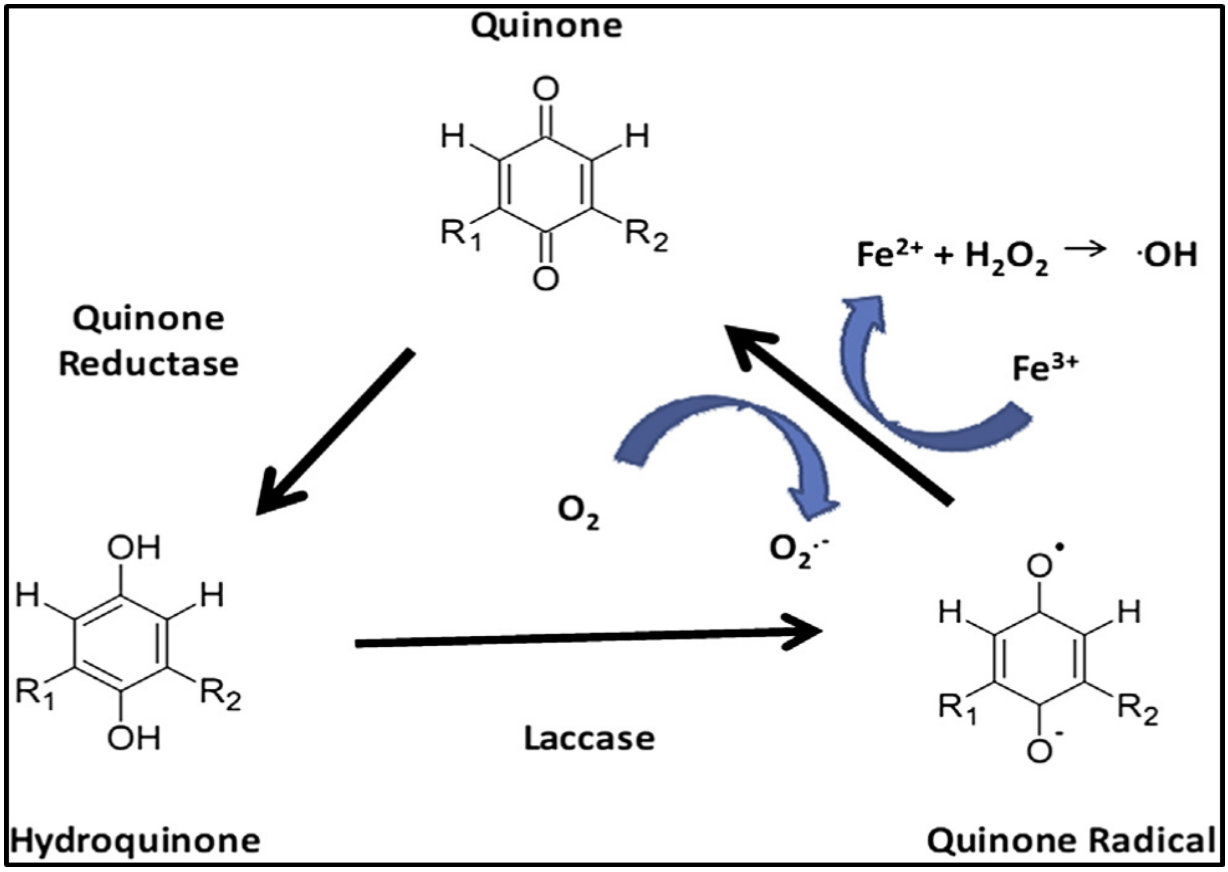


Figure 1.3. Simplified model for how extracellular quinone redox cycling can generate hydroxyl radicals. The quinone reductase may either be membrane bound or intracellular. The H_2O_2 required for ·OH production could be derived from spontaneous dismutation of $O_2^{\cdot-}$, or from other enzymes. (Beckett et al. 2013).

The above diagram shows how extracellular quinone redox cycling can generate hydroxyl radicals explained in second model.

It is not known if either of these two models are responsible for hydroxyl radical production by lichens. However, it is clear that if it occurs such redox cycling may play a key role in carbon cycling, particularly in lichen-dominated high stress environments.

However, it is worth noting that other mechanisms have been proposed to explain how hydroxyl radicals may be produced. There is evidence that phenolic compounds in brown rot fungi also act as iron chelators and produce electrons for Fe reduction which promote the formation of hydroxyl radicals (Cohen et al. 2004). Some researchers suggest that brown rot fungi accumulate oxalic acid and produce cellobiose oxidase that can reduce Fe^{3+} to Fe^{2+} and the oxalate will then donate electron with Fe^{2+}. Fe^{2+} will react with O_2 to form the H_2O_2 required for ·OH production (Kerem et at. 1999).

1.4.1. Possible roles of hydroxyl radicals in Lichen biology

ROS play a variety of roles in fungal biology such as signaling, the control of differentiation and pathogen defense (Kim 2014). As discussed above, in many fungi, a key role of ROS is to participate in the breakdown of lignocellulosic residues, facilitating a saprotrophic lifestyle (Baldrian et al. 2008; Hammel et al. 2002).

The presence of hydroxyl radicals in cells may initiate lignin demethylation, demethoxylation, aromatic ring hydroxylation, oxidation of the resulting catechol groups, and side-chain oxidation in plants (Goodell 2003). It has been discovered that cellulose breakdown in fungi can be facilitated by hydroxyl radical production (Martinez et al. 2009). Organisms capable of extracellular redox cycling can therefore cause significant lignin and cellulose breakdown.

1.5. Applied applications of hydroxyl radicals

Research on hydroxyl radicals has gained momentum because of their involvement in many hydroxylation and biochemical reactions. Hydroxyl radicals are strong oxidizing agents therefore can be used in decontamination polluted water by toxic substances. Hydroxyl radicals can also breakdown macromolecules such as carbohydrates, amino acids, lipids and nucleic acids thus making toxic compounds. Hydroxyl radicals are directly involved in xenobiotic compounds breakdown such as pesticides, synthetic dyes or herbicides (Alexander 1999).

Xenobiotic compounds are foreign chemicals that are introduced in the system in different ways. Many of these compounds are pollutants and each compound will present its own problem of how to remove and some are very difficult to degrade thereby causing risk to health or environment. Microorganisms are believed to have the capacity to decompose most of the xenobiotics (Alexander 1999). However not all compounds are degradable by all microorganisms. Hydroxyl radicals can facilitate the oxidation of micro plastics (Singh, Sharma 2008).

1.5.1. Dye decolorisation

Water pollution can result from rapid industrial development especially of the textile, leather, food and agro industries (Chequer et al. 2013; Robinson et al. 2001). Specifically, in the dyeing of textiles approximately 90 % of the total wastewater contains a mixture of different pollutants such as surfactants, acids or bases, heavy metals, salts, suspended solids and synthetic dyes (Bafana et al. 2011; Banat et al. 1996; Juang et al. 1996). There are more than 10 000 different synthetic dyes used in industries and c. 7×10^5 tons of synthetic dyes produced worldwide per

year (Robinson et al. 2001; Ogugbue, Sawidis 2011). Azo dyes are the most commonly used class of dye in the textile industry because of their high fixing quality, durability against microbial degradation and resistance to breakdown by light (Poots, McKay 1976; Robinson et al. 2001). Synthetic dyes structures are more complex, which makes them difficult to break down to compounds that are less harmful to living organisms (Chequer et al. 2013; Laufer 2012). Some fungi can decolorize synthetic dyes via redox cycling process (Junghanns et al. 2008; Lucas et al. 2008; Kaushik, Malik 2009). Laufer (2012) showed that lichens from Peltigerineae and non-Peltigerineae suborders can decolorize a range of synthetic dyes and the degree of decolorisation depend on the type of dye used.

1.6. Aims and Objectives of the study

The main aim of the work described here was to conduct a survey in a range of lichens species for their ability to generate hydroxyl radicals. An aim was to investigate the mechanism of hydroxyl radical production in lichens. The activity of the redox enzymes laccase and peroxidase was determined to test whether their activity is related to the rate of hydroxyl radical production. It was hypothesized that species with high redox enzymes activity may carry out direct lignocellulose breakdown using these enzymes, while other species may rely on hydroxyl radicals. In other words, it was predicted that there may be an inverse relationship between the activity of redox enzyme and the ability of a lichen to produce hydroxyl radicals. In addition, the ability of lichens to decolorize synthetic dyes through redox cycling process was tested.

CHAPTER 2- METHODS AND MATERIALS

2.1. Plant material and chemicals

The lichen species examined in this study and their collection localities are listed in Table 2.1. Specimens were collected from the field dry after which they were thoroughly cleaned to remove debris from the thalli. The lichen material was kept refrigerated for a maximum of four weeks before use. All chemicals used were purchased from Sigma-Aldrich (St Louis, USA) and were of the highest commercially available purities.

2.2. Preparation of lichens fragments and discs

For each species, a large quantity of material was collected, the healthiest thalli chosen for experimentation and discs (6 mm) or 1 cm strips randomly selected. "Fully hydrated" was defined at no further increase in fresh mass following incubation on wet non-cellulosic cloth; this was achieved within 24 h. Lichen material was rehydrated for 24 to 48 h in a LABCON Growth Chamber at 10 ^{0}C in the dark before use.

2.3. Measurement of hydroxyl radical formation

Hydroxyl radical production was estimated by measuring deoxyribose oxidation rates (Gómez et al. 2009a). Unless indicated otherwise lichen material used was hydrated for 5 d in the dark at 12^{o}C on non-cellulosic cloth. Four replicates each containing an equivalent of 0.2 g dry mass were then shaken (50 rpm) in 20 ml of 20 mM phosphate buffer (pH 5) containing 0.5 mM DMBQ, 0.1 mM Fe^{3+} as $FeCl_3$, 0.6 mM oxalic acid and 2.8 mM deoxyribose. Samples (990 µl) were taken at the start and 1, 2 and 3 h, subsequently with the addition of 10 µl of 50 % H_3PO_4 as a stop solution and the reactants were frozen further analysis. The samples were later thawed and 50 µl of the sample was mixed with 250 µl of 2.5% trichloroacetic acid, 250 µl of 1% thiobarbituric acid in 50 mM NaOH, and 450 µl of distilled water, volume made up to 1 ml. Samples were then heated in water at 100 ^{o}C for 10 min. Absorption was measured at 532 nm after cooling at room temperature. Readings were converted to MDA equivalents (ε_{532} = 0.156 µM cm^{-1}, Devasagayam et al. (2003)). Solutions lacking lichen material but otherwise treated in the same way were used as blanks.

In preliminary experiments, it was confirmed that a range of lichens can reduce quinones to hydroquinones in the presence of chelated iron (Fe) by reversed-phase high performance liquid chromatography (data not shown). Varying the pH of the solution from 4 to 5.5 had little effect

on the rate of hydroxyl radical production, and we therefore used pH 5 throughout (data not shown). Production rates were very low when either the quinone or chelated Fe^{3+} were omitted from the incubation solution.

2.3.1. The effect of Mn^{2+}, H_2O_2 and anisaldehyde on hydroxyl radical production in lichens

As hydroxyl formation in white-rot fungi can be stimulated by anisaldehyde and Mn^{2+} (Gomez et al. 2009b). A range of concentration was tested for their effects on deoxyribose oxidation in *Usnea undulata*. The material was treated the same way as mentioned above (section 2.3) The concentration used were 0, 20, 100, 250, 500 µM for Mn^{2+}, anisaldehyde at 2.3 mM, 23 mM, 100 mM, 230 mM and for H_2O_2 was 0, 0.1, 1.0, 10 mM. Absorbance readings were taken at 532 nm and the rate was calculated using the co-efficient of 0.156 $\mu M^{-1}cm^{-1}$. Four replicates, each containing an equivalent of 0.2 g dry mass of lichen material, were used for each treatment.

2.3.2. Effect of hydration time on hydroxyl radical formation in *Cladonia mitis*

The effect of rehydration on hydroxyl radical formation in *C. mitis* was tested. The material was air-dried, and stored refrigerated for several weeks until experimentation. Lichens were hydrated for 1 h, hydroxyl radical formation measured as described earlier, and the remaining material was stored moist at 10 ^{0}C in darkness on wet non-cellulosic cloth. Hydroxyl radical formation was measured at intervals for up to 15 d in four replicates, each comprising the equivalent of 0.3 g dry mass.

2.3.3. The effect of different quinones on hydroxyl radical production in *Usnea undulata*

U. undulata was used to study the effect of quinone on hydroxyl radical formation. The lichen material was treated as above (section 2.3), with four replicates used per each treatment. The quinones tested in this experiment were 2, 6-Dimethoxy-1, 4-benzoquinone (DMBQ), 2-methyl-1, 4-naphthoquinone (menadione (MD)) and 2-Methoxy-1, 4-benzoquinone (MBQ) at the concentration of 1.2 mM in 20 ml.

2.4. Measurement of enzyme activities

The activity of laccase and peroxidase were measured in a range of lichen material treated in the same way as for hydroxyl radical determination (section 2.3). Enzymes were assayed by grinding *c*. 0.5 g dry mass of lichen material in 10 ml of 50 mM phosphate buffer (pH 7). Four replicates of extracts were centrifuged at 5000 *x g* for 20 min at 4 ^{0}C, and the supernatant was then used for enzyme analyses. Laccase activity was estimated by following the oxidation of

0.3 mM 2,2-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS; Sigma) at 420 nm (ε_{420} = 36 mM^{-1} cm^{-1}) in 50 mM sodium acetate buffer pH (4.5) (Eggert et al. 1995). Peroxidase activity was estimated as the stimulation of the rate of ABTS oxidation following the addition of 0.1 mM H_2O_2 (Liers et al. 2011).

2.5. Reduction of Fe^{3+} to Fe^{2+}

Reduction of Fe^{3+} ions to Fe^{2+} ions was measured in a range of lichen material using the method of Harvey et al. (1955). Briefly, the equivalent of 0.2 g dry mass of lichens was shaken at 50 rpm for 70 min, with the components required for hydroxyl radical production. After every 10 min intervals for 70 min, 0.5 ml of the incubation solution was taken and mixed with 2.5 ml of 0.3% 1, 10-phenanthroline solution. Absorption of Fe^{2+} concentrations was measured at 510 nm. The results were calculated using the co-efficient (ε = 12.1 μM^{-1} cm^{-1} Barr et al. (1992)).

2.6. Gel Electrophoresis

SDS-PAGE was used to determine the molecular masses and number of isoforms capable of making superoxide (O_2^-) "NADH-oxidase" from *Lobaria retigera.* SDS-PAGE was performed using Hoefer Mini-Vertical Electrophoresis System (Hoefer Scientific Instruments). The separating gel used were 12% and 15%. Using a mortar and pestle 1 g hydrated material was ground in 20 ml of 50 mM phosphate buffer (pH 7) solution for approx. 10 min. The extracts were then centrifuged at 5000 *x g*, 4 ^{0}C for 20 min, followed by centrifugation at 1000 *x g*, 4 ^{0}C for 5 min of the supernatant. The supernatant was transferred into a dialysis tube, tied in both ends and placed in a plastic container with sugar for 24 h. The concentrate solution was then transferred to Eppendorf tubes, centrifuged for 5 min at 3000 *x g*, 4 ^{0}C. With reverse dialysis, the extracts were placed in Eppendorf lids separated by dialysis tube and left to float in buffer solution overnight in a cold-room. The extracts were then centrifuged and placed in ice for further analysis. Three replicates were used for the dialysis process with initial volume of 10 ml unconcentrated solution. The loading buffer was prepared with 4.5 g TRIS, 21.6 g Glycine and 1.5 g SDS. The sample, standards and native enzyme were then loaded in the gel. The electrophoresis was typically run for 15 min at 120 V and then at 150 V for a further 2.5 h.

NAD (P) H oxidoreductase activity was visualised by incubating the gel in 50 mM sodium phosphate buffer (pH 7.4) with 10 % glycerol containing 0.1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min. The gels were then incubated at room temperature in the same buffer containing 0.4

mM NADH and staining with 0.5 mM nitroblue tetrazolium chloride (NBT) in 20 ml for 30 min to 12 h in the dark and then scanned (Lopez-Huertas et al. 1999).

To visualise peroxidase activity, the gels were washed in 20 ml of 10 % glycerol, 0.25 M sodium acetate buffer pH 4.5 for 5 – 10 min. After 5 min 20 mM of *o*-dianisidine was then added and 20 mM H_2O_2 (24.6 µl from 50 % stock solution/20 ml). Laccases were visualised without the addition of H_2O_2. Blue bands started to appear just after few minutes and the gels were scanned.

2.7. Decolourization of dyes

The potential of different species of lichens to decolourize different type of synthetic dyes was studied. Table 2.2 shows the dyes used and their properties. The degree of the decolourization process was measured spectrophotometrically after 0, 3, 6, 24 and 48 h. The oxidation process described by Gómez et al. 2009a was used, with deoxyribose replaced with 0.01% dye concentration. The lichen material was rehydrated for at least 24 h before testing. Four replicates each containing the *c.* 0.2 g dry mass or 10 discs were then shaken at room temperature in 20 ml of 20 mM phosphate buffer (pH 5) containing 0.5 mM DMBQ, 0.1 mM Fe^{+3} as $FeCl_3$, 0.6 mM oxalic acid and 0.01 % dye concentration and the controls were without redox chemicals.

Samples (1 ml) were taken at 0, 3, 6, 24 and 48 h. Absorption was measured at the respective wavelength of the dye used (Table 2.2). Solutions lacking lichen material but otherwise treated in the same way were used as blanks. Decolourization of each dye was then expressed in percentage of dye decolourization against time. Formula-:

Percentage of dye decolourization = (Initial absorbance − final absorbance)/Initial absorbance × 100

In the experiments four lichen species were used (*Usnea undulata*, *Heterodermia speciosa*, *Ramalina celastri and Parmelia cetrarioides*). In the preliminary experiments the results showed that *Usnea undulata* proved most effective and was therefore selected as a model species to be used for the rest of the dye decolorisation experiments

2.7.1. Effect of H_2O_2 on dye decolourization

U. undulata was treated as mentioned above in (section 2.7) with the addition of 0, 0.1, 1.0 and 10 mM concentration of H_2O_2 and 0.01 % concentration of RBBR synthetic dye. Absorbance

readings were measure at different time intervals 0, 3, 6, 24 and 48 h. Degree of decolourisation was then expressed as percentage of dye decolourisation as a function of time.

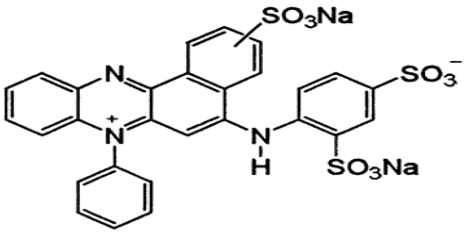
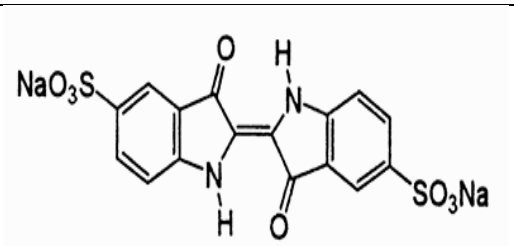
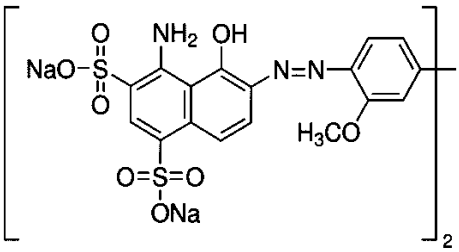
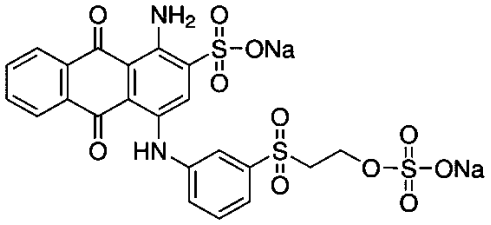
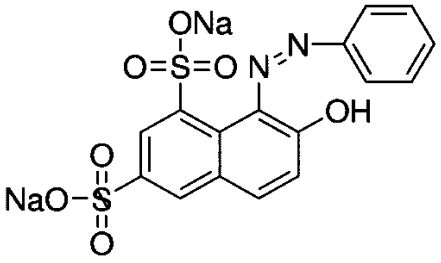
2.8. Statistical analyses

All measurements were done using 4 replicates per each treatment unless indicated otherwise. Using IBM SPSS Statistics V22.0, correlation and regression analysis was performed to calculate the rates of hydroxyl radical production. Where applicable the significant difference between the mean (replicates) was measured at value ($P < 0.05$).

Table 2.1. Lichen species used in the study and their collection sites.

Species	**Collection locality**
Lichens from suborder Peltigerineae	
Leptogium saturninum (Dicks.) Nyl	Fort Nottingham, RSA
Lobaria pulmonaria (L.) Hoffm.	Porsgrunn, Norway
Lobaria retigera (Bory.) Trevis	Monks Cowl, RSA
Peltigera canina (L.) Willd.	Kazan, Russia
Peltigera malacea (Ach.) Funck	Kazan, Russia
Pseudocyphellaria aurata (Ach.) Vain	Fort Nottingham, RSA
Pseudocyphellaria gilva (Ach.) Malme	Fort Nottingham, RSA
Sticta cf. limbata (Sm.) Ach.	Fort Nottingham, RSA
Sticta cf. sublimbata (J. Steiner) Swinscow & Krog	Fort Nottingham, RSA
Lichens from suborder Non-Peltigerineae	
Cladonia cervicornis (Ach.) Flotow	Porsgrunn, Norway
Cladonia mitis Sandst.	Kazan, Russia
Cladonia rangiferina (L.) Nyl.	Kazan, Russia
Evernia prunastri (L.) Ach.	Porsgrunn, Norway
Heterodermia speciosa (Wulfen) Trevis.	Fort Nottingham, RSA
Parmelia cetrarioides (Duby) Nyl.	Scottsville, RSA
Ramalina celastri (Sprengel) Krog & Swinscow	Fort Nottingham, RSA
Ramalina farinacea (L.) Ach.	Porsgrunn, Norway
Usnea undulata Stirton	Fort Nottingham, RSA

Table 2.2. The absorption spectra and type of synthetic dyes used in the experiment.

Name of Dye	**Chemical class**	**Absorption (nm)**	**Chemical Structure**
Acid Red 103	quinone-imine	505 nm	
Acid Blue 74	Indigoid	610 nm	
Chicago Sky Blue 6B	Diazo	620 nm	
Remazol Brilliant Blue R	Anthraquinone	595 nm	
Orange G	Phenylazo	480 nm	

CHAPTER 3 - EXTRACELLULAR REDOX CYCLING AND HYDROXYL RADICAL PRODUCTION IN LICHENIZED ASCOMYCETES

3.1 Introduction

Free-living fungi can produce reactive oxygen species (ROS) using extracellular redox cycling (Cohen et al. 2004; Kerem et al. 1999; Gomez et al. 2009a, b; Krueger et al. 2015, 2016). ROS play a variety of roles in fungal biology as discussed in the Introduction. In many fungi, a key role of ROS is to break down lignocellulosic residues, thus facilitating a saprotrophic lifestyle (Baldrian 2008; Hammel et al. 2002). In the early stages of the decay of compact higher plant tissues, conventional enzymes such as peroxidases and laccases are too large to penetrate these tissues. The breakdown of both lignin and cellulose requires the production of highly aggressive hydroxyl (·OH) radicals probably generated by "Fenton" chemistry, essentially by reactions between H_2O_2 and Fe^{2+} (Arantes, Goodell 2014; Guillen et al. 1997). The hydroxyl radical is the most aggressive non-specific oxidant in biological systems; it has a very short half-life (10^{-9} s). Therefore, it is a dilemma that radicals must be produced near their targets due to diffusion limitations, and on the other hand, at some distance from hyphae to prevent injury to the fungus. For the Fenton reaction to occur, ferric (Fe^{3+}) ions must be solubilized from plant material and reduced to ferrous ions (Fe^{2+}); in addition, H_2O_2 must be available. A consequence of the Fenton reaction is that lignin is rearranged in such a way that it facilitates access of hydrolytic enzymes to polysaccharides for further degradation.

The main aim of the work here was to survey a range of lichens for their ability to generate hydroxyl radicals. The ability of lichens to reduce Fe^{3+} to Fe^{2+} was tested and further investigated the mechanism of radical production by attempting to correlate rates of production to the activities of laccases and peroxidases. Also, to determine whether rates of hydroxyl radical formation are stimulated by Mn^{2+}, anisaldehyde and redox enzymes as found in white-rot fungi by Gomez et al. (2009b).

3.2. Results

The time courses of hydroxyl radical production for selected species are presented in Figure 3.1. Rates of production were almost constant for at least 3 h of incubation in the hydroxyl radical assay solution. The addition of a range of concentrations of anisaldehyde and Mn^{2+} (0.2 units' ml^{-1}) had no effect on hydroxyl radical production (data not shown). However, oxidation

of deoxyribose was greatly inhibited by the presence of thiourea or sorbitol in the incubation medium (Table 3.1). In all species, Fe^{3+} was reduced to Fe^{2+} during the assays; Fe^{2+} concentrations typically reached stable values after 10 to 20 min (Figure 3.2). Detailed kinetics varied between species. Moist storage for 15 d had little effect on the rates of hydroxyl radical formation (Figure 3.3). DMPQ was by far the most effective quinone for promoting hydroxyl radical formation (Figure 3.4), although some radicals were formed when menadione was used.

Table 3.2 presents a survey of rates of hydroxyl radical formation, and laccase and peroxidase activities in a range of lichens. Rates of hydroxyl radical production varied considerably between species, ranging from 0.2 to 1.4 μmol g^{-1} dry mass h^{-1}, but on average were similar comparing Peltigeralean with non-Peltigeralean species. Similarly, following hydration for 5 d laccase and peroxidase activities did not differ greatly between the two groups. No significant correlation existed between the rates of hydroxyl radical production and laccase activity, peroxidase activity or the sum of these two redox enzymes (Figure 3.5).

Figure 5.6 presents the gel electrophoresis results of crude extracts from *Lobaria retigera*. The molecular mass of laccases was 250 kDa, while that for superoxide were 60 (unclear), 75, 100 and 150 kDa, though there was unknown band that exceeded 250 kDa. No bands were visualized when 12 % gel was used for superoxide. Standard (1) was for 15 % gel and standard (2) for 12 % gel.

3.3. Discussion

Extracellular hydroxyl radical production, driven by hydroquinone redox cycling occurs in a wide range of lichenized Ascomycetes (Table 3.2). Although the rates varied between species, they appear to be similar in both Peltigeralean and non-Peltigeralean lichens. Hydroxyl radical production is not directly correlated to the activities of the redox enzymes laccase and peroxidase (Figure 3.5), and is not stimulated by exogenous addition of peroxidase. While intuitively these enzymes would be expected to play a role in extracellular redox cycling, this study does not indicate how they may be involved. Taken together, results presented here suggest that the mechanism of hydroxyl radical formation in lichens may be analogous to that of brown rot fungi.

3.3.1 Evidence for redox cycling

As discussed in the Introduction, in the field lichens produce or have access to the quinones, oxalate, and Fe^{3+} needed for redox cycling. Our preliminary data (not shown) indicate that intact lichens incubated in the presence of quinones can readily reduce them to hydroquinone's.

Furthermore, this study reveals that during cycling Fe^{3+} is reduced to Fe^{2+} (Figure 3.3). Reduction of first, quinones to hydroquinone's, and second, Fe^{3+} to Fe^{2+}, are important requirements of all models proposed for hydroxyl radical formation by fungi (Arantes, Goodell 2014). The specificity of our assay is supported by the ability of scavengers of hydroxyl radicals (thiourea and sorbitol) to effectively prevent the oxidation of deoxyribose (Table 3.1). More work is needed to characterize the quinone reductases that regenerate hydroquinone's. As rates of hydroxyl radical formation immediately after rehydration and following prolonged moist storage are almost constant (Figure 3.3) it seems likely that these enzymes are constitutively expressed, as suggested for the quinone reductases of free-living Ascomycetes (Cohen et al. 2002; Krueger et al. 2016). Taken together, results presented strongly suggest that redox cycling occurs widely in lichenized Ascomycetes.

3.3.2. Mechanism of redox cycling in lichens

As outlined in the Introduction, in the brown rot fungi hydroxyl radicals are produced by the Fenton reaction, where H_2O_2 reacts with Fe^{2+}, producing hydroxyl radicals and Fe^{3+}. The H_2O_2 is produced from the auto-oxidation of hydroquinone's to quinones via a semi-quinone radical, while in the same reaction Fe^{2+} is produced by the reduction of Fe^{3+}. Continuous hydroxyl radical production occurs by "redox cycling" as hydroquinone's are regenerated by quinone reductases on the surface of the hyphae (Arantes, Goodell 2014). While it has been suggested that in white rot fungi laccases and peroxidases assist in the oxidation of hydroquinone's to quinones (Gomez et al. 2009a, b), there are indications that redox cycling in the two groups of fungi may differ more fundamentally. For example, in general white-rot fungi secrete far less quinones to the apoplast than brown-rot fungi (Arantes, Goodell 2014), but possess a greater diversity and activity of redox enzymes that may more effectively reduce any quinones that are secreted (Floudas et al. 2012). The present study found no correlation between rates of hydroxyl radical production and thallus laccase or peroxidase activity (Table 3.2, Figure 3.4). This is consistent with our earlier findings that increasing laccase and peroxidase activity in *Usnea undulata* by storing material hydrated or treating it with effectors such as xylidine did not increase rates of hydroxyl radical formation (Beckett et al. 2015). Furthermore, our attempts to stimulate redox cycling by the exogenous applications of peroxidases were unsuccessful (data not shown), unlike the stimulation observed in white rot fungi by the addition of redox enzymes (Gomez et al. 2009a). Similarly, Mn^{+2} and anisaldehyde, which strongly stimulate hydroxyl formation in white rot fungi (Gomez et al. 2009a), have no effect in lichens. Taken together, our results suggest that hydroxyl radical production in lichens

probably occurs by mechanisms that resemble more closely those proposed for brown than white-rot fungi.

3.3.3. Naturally occurring components of redox cycling

The naturally-produced quinones used by lichens to carry out redox cycling are unknown. In the field, rates of hydroxyl radical formation are undoubtedly lower than those reported here, as lichens will depend on naturally available chelated Fe^{3+} and quinones. The most effective quinone for promoting radical formation in *U. undulata* was DMBQ (Figure 3.4), although menadione allowed lichens to produce some radicals. How lichen synthesise extracellular quinones is also unclear, although recently Zavarzina et al. (2017) showed that all lichens tested secrete a range of soluble phenols, with the most common being *p*-hydroxybenzoic, syringic, salicylic and vanillic acids. Lichens also secrete a range of redox enzymes such as peroxidases, laccases and tyrosinases (Beckett et al. 2013). Potentially, these enzymes can metabolize the secreted phenols to supply the quinones needed for redox cycling. For example, some bacteria use laccases to metabolize syringic acid into 2, 6-dimethoxy-1, 4-benzoquinone (Koschorreck et al. 2008). However, further work is needed to understand the synthesis and identity of the naturally occurring quinones used by lichens to form radicals.

3.3.4. Gel electrophoresis

Several high molecular mass bands could be visualised in *L. retigera* that could make O_2^- from NADH, a characteristic of quinone reductases (Galkin and Brandt 2005). Attempts to visualize peroxidases, another enzyme capable of producing O_2^- from NADH were unsuccessful, although lichen peroxidases normally have an active mass of c. 80 kDa, which does not correspond to the superoxide producing bands visualised here (Mika et al. 2004; Liers et al. 2011). The only redox enzyme visualised was laccases, with a molecular mass of *c*. 250 kDa, like values reported for other lichen laccases (Figure 3.6) (Beckett et al. 2013).

3.3.5. Roles of hydroxyl radical production

What roles do hydroxyl radical production pay in lichen biology? One important role is likely to be the facilitation of saprotrophic activities to provide additional carbon for the mycobiont during periods of low photosynthate supply or intensive growth. This is analogous to the role suggested for ROS production by free-living fungi (Hammel et al. 2002; Baldrian 2008). Lichens produce hydroxyl radicals at a variety of rates (Table 3.2), and interestingly, many fruticose and pendulose species display relatively high rates of hydroxyl radical formation. If a major role for radical production is to break down lignocellulosic residues, intuitively foliose

species would be expected to display the highest activities, as these lichens have a large proportion of their thallus in contact with their substratum. However, it may be that pendulous species are more nutrient stressed than foliose species, as they depend on rainfall and stemflow for nutrients. Rain and stemflow can be rich in nutrient-containing organic molecules (Eaton et al. 1973; Jickells et al. 2013), and hydroxyl radicals may break these compounds down, making elements such as nitrogen and phosphorus available for uptake. Quite possibly, lichens decompose organic matter to derive nutrients rather than carbon, as has recently been proposed for ectomycorrhizal fungi (Lindahl, Tunlid, 2015). Other potential uses for hydroxyl radicals include control of differentiation and pathogen defence (Kim 2014). Moreover, aggressive radicals such as hydroxyl radicals are likely to degrade harmful soil pollutants such as phenols, dyes and even micro-plastics (Gomez et al. 2009b; Krueger et al. 2015) (see Chapter 4).

Table 3.1. The effect of hydroxyl radical scavengers' thiourea and sorbitol on hydroxyl radical formation in *Cladonia mitis*. Figures are given ± 1 s.d., n = 4.

Scavenger	**Concentration**	**Rate of ·OH radical production (μmol g^{-1} dry mass h^{-1})**
Thiourea	**0**	0.61 ± 0.05
	1	0.56 ± 0.04
	2	0.44 ± 0.03
	5	0.14 ± 0.04
	10	0.00 ± 0.00
Sorbitol	**0**	0.73 ±0.02
	1	0.56 ± 0.05
	10	0.21 ± 0.06
	100	0.05 ± 0.02

Table 3.2. The rates of hydroxyl radical production, laccase activity and peroxidase activity in a range of lichen species.

Species	Collection locality	Rate of ·OH radical production (µmol g^{-1} dry mass h^{-1})	Laccase activity (units g^{-1} dry mass)	Peroxidase activity (units g^{-1} dry mass)
Peltigerlean species				
Lobaria pulmonaria (L.) Hoffm.	Porsgrunn, Norway	0.15 ± 0.01	1.20 ± 0.12	1.26 ± 0.19
Peltigera malacea (Ach.) Funck	Kazan, Russia	0.17 ± 0.09	1.05 ± 0.13	0.97 ± 0.14
Loberia retigera (Bory.) Trevis	Fort Nottingham, RSA	0.90 ± 0.34	3.20 ± 0.20	0.00 ± 0.00
Sticta limbata (Sm.) Ach.	Fort Nottingham, RSA	0.60 ± 0.24	0.50 ± 0.30	0.00 ± 0.00
*Pseudocypheria gilva (*Ach.) Malme	Fort Nottingham, RSA	1.43 ± 0.19	0.82 ± 0.16	2.10 ± 0.33
Leptogium saturninum (Dicks.) Nyl	Fort Nottingham, RSA	0.75 ± 0.45	0.00 ± 0.00	2.18 ± 0.58
Mean		**0.67**	**1.13**	**1.09**
Non-Peltigeralean species				
Cladonia cervicornis (Ach.) Flotow	Porsgrunn, Norway	0.88 ± 0.07	0.37 ± 0.07	2.03 ± 0.55
Cladonia rangiferina (L.) Nyl.	Kazan, Russia	0.44 ± 0.07	0.54 ± 0.02	0.30 ± 0.02
Cladonia mitis Sandst.	Kazan, Russia	0.52 ± 0.04	5.76 ± 0.12	0.22 ± 0.03
Evernia prunastri (L.) Ach.	Porsgrunn, Norway	0.45 ± 0.01	0.55 ± 0.05	0.06 ± 0.03
Heterodermia speciosa (Wulfen) Trevis.	Fort Nottingham, RSA	0.66 ± 0.12	0.05 ± 0.10	7.14 ± 4.30
Parmelia cetrarioides (Duby) Nyl.	Scottsville, RSA	0.40 ± 0.08	0.63 ± 0.02	2.39 ± 0.05
Ramalina celastri (Sprengel) Krog & Swinscow	Fort Nottingham, RSA	0.55 ± 0.19	0.45 ± 0.13	0.61 ± 0.22
Ramalina farinacea (L.) Ach.	Porsgrunn, Norway	0.81 ± 0.17	0.00 ± 0.00	0.00 ± 0.00
Usnea undulata Stirton	Fort Nottingham, RSA	0.42 ± 0.11	3.14 ± 0.84	1.84 ± 1.20
Mean		**0.57**	**1.28**	**1.62**

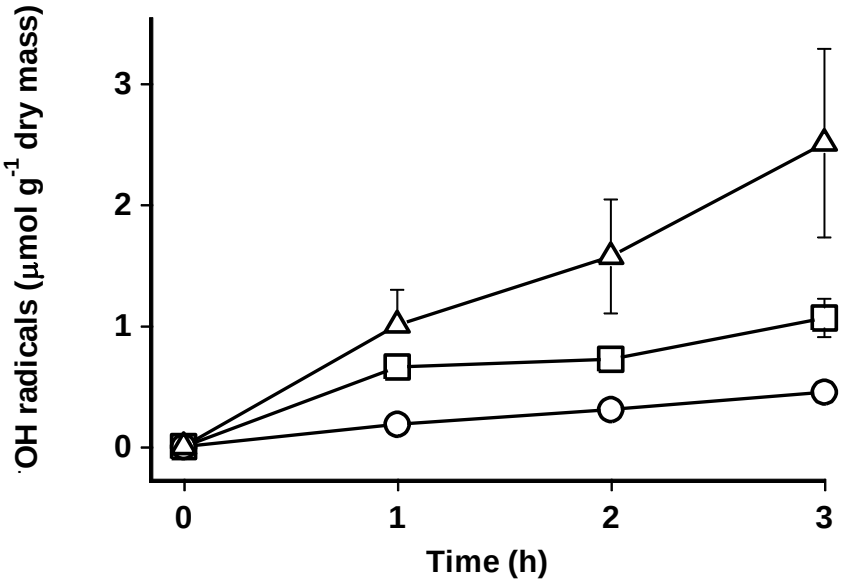


Figure 3.1. Hydroxyl radical formation as a function of time in *Lobaria pulmonaria* (circles), *Usnea undulata* (squares) and *Ramalina farinacea* (triangles). Values are given ± 1 s.d., n= 4.

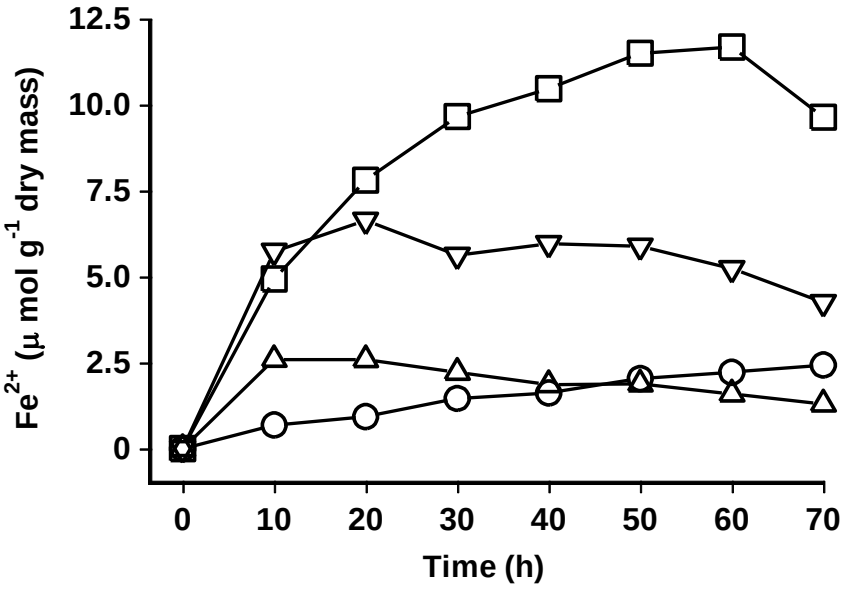


Figure 3.2. Reduction of Fe^{3+} (original concentration was 100 µM) to Fe^{2+} in *Heterodermia speciosa* (circles), *Usnea undulata* (squares), *Peltigera canina* (upwards pointing triangles) and *Evernia prunastri* (downwards pointing triangles). Values are means of four replicates.

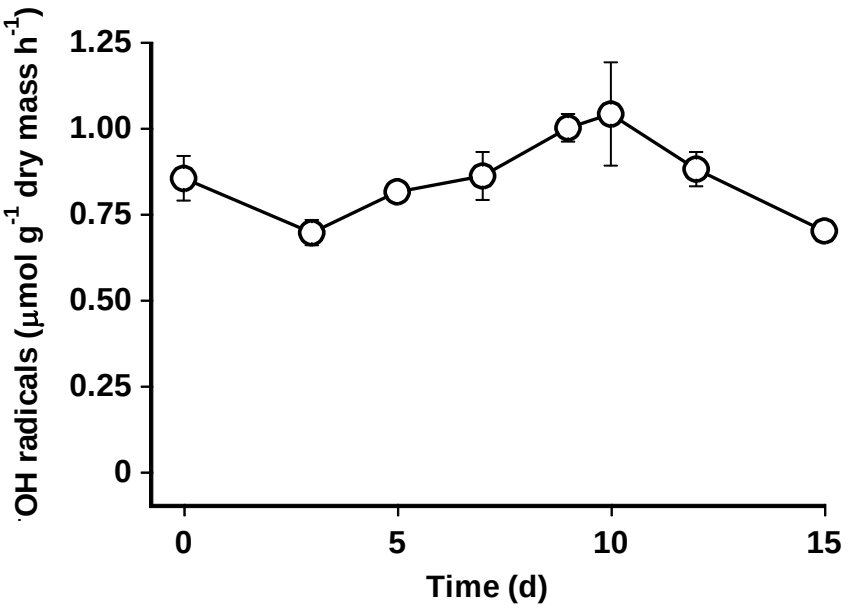


Figure 3.3. The effect of rapid rehydration (1 h, indicated as 0 d) followed by moist storage on hydroxyl radical formation in *Cladonia mitis*. Values are given ± 1 s.d., n= 4.

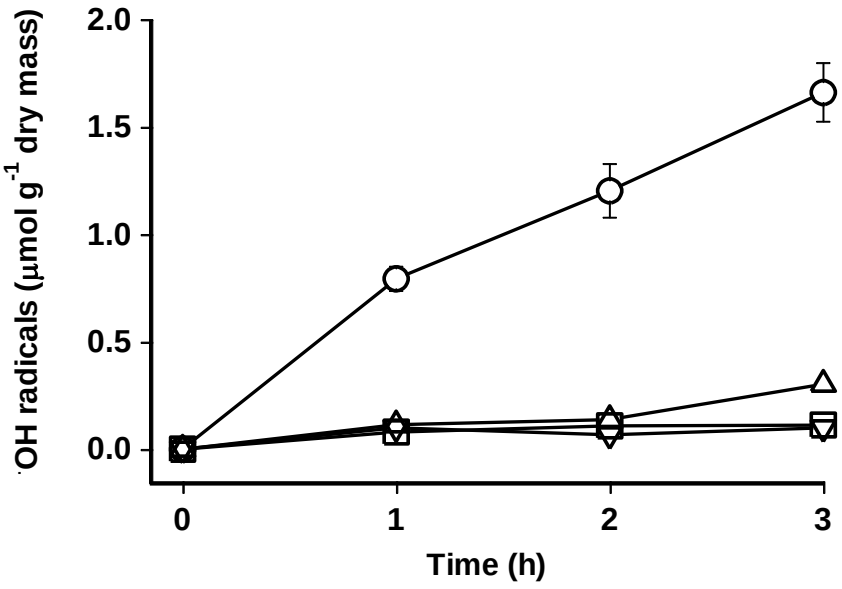


Figure 3.4. The rate of hydroxyl production in *Usnea undulata* in the presence of effect of various quinones. Control (no quinone added), downwards pointing triangle; DMBQ, circles; menadione, upwards pointing circles; 1, 4-benzoquinone, squares. Values are given ± 1 s.d., n= 3.

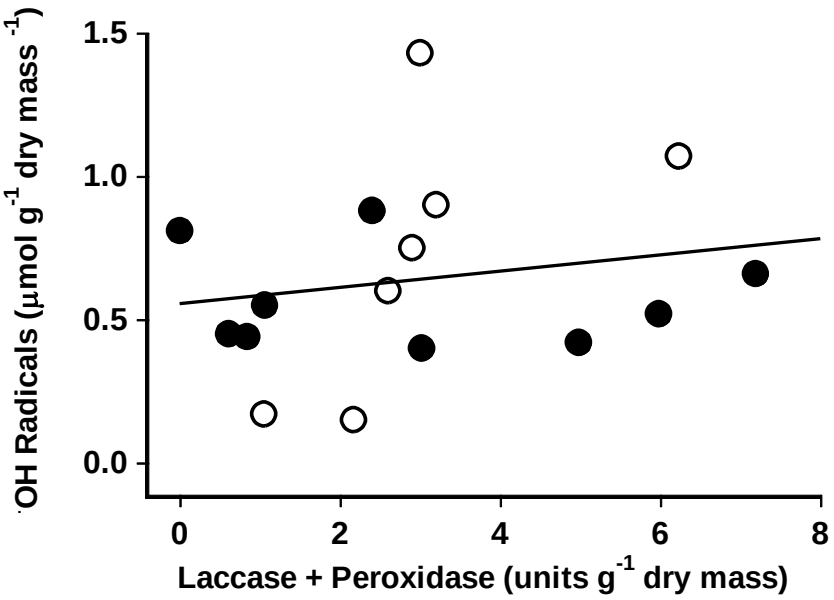


Figure 3.5. Absence of a correlation ($P > 0.05$, R-value = 0.23) between the rate of hydroxyl radical formation and the combined activities of laccase and peroxidases in Peltigeralean (open symbols) and non-Peltigeralean (closed symbols) lichens.

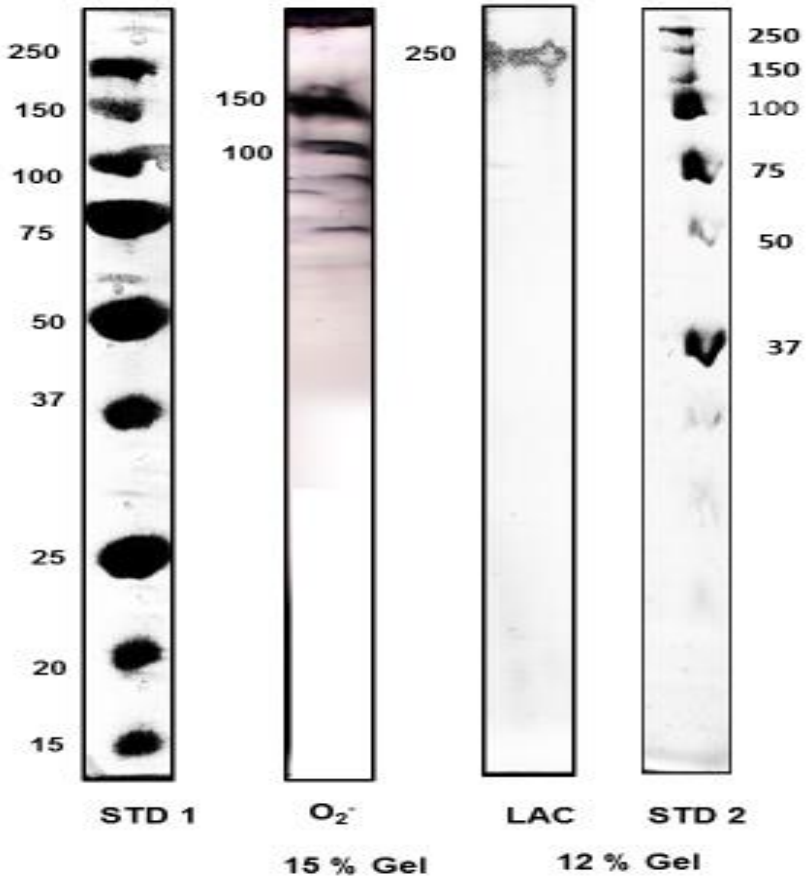


Figure 3.6. SDS-PAGE of crude extracts from *Lobaria retigera*. Production of O_2^- bands was visualised by a mixture of NBT and NADH, laccase activity by *o*-dianisidine in the absence of H_2O_2. Molecular masses are indicated in kDa.

CHAPTER 4 - DYE DECOLORISATION BY LICHENS VIA REDOX CYCLING PROCESS

4.1. Introduction

There is convincing evidence that fungi species can decolorize synthetic dyes. Dye decolorisation can occur by various mechanisms, including direct metabolism by enzymes, but also indirectly via redox mediators or through the production of hydroxyl radicals (Goodell et al. 2004; Junghanns et al. 2008; Lucas et al. 2008; Kaushik, Malik 2009). Studies have shown that artificially produced hydroxyl radicals can be effective in decolorizing dyes (Glaze et al. 1987). Goodell et al. (2004) also discovered that hydroxyl radicals derived from the Fenton reaction can degrade synthetic dyes. Fungi can certainly produce the hydroxyl radicals via different mechanism of extracellular redox cycling, and have been successfully used to decolorize synthetic dyes (Gomez et al. 2009a, b; Lucas et al. 2008). Results presented in Chapter 3 suggest that lichens given quinones and chelated (Fe) ions can readily produce hydroxyl radicals; therefore, it was hypothesized that they can be used in Advanced Oxidation Processes (AOP) for dye decolorisation. According to some preliminary experiments with lichens, *Usnea undulata* species shows evidence of an ability to carry out dye decolorisation (data not published) (Laufer 2012).

The use of "biological treatment" provides a better alternative to conventional techniques currently being used, because it is more environmentally friendly. Using plants to "phytoremediae" pollutants is gaining increase acceptance (Kamat 2014). In addition to being more environmentally friendly, phytoremediation is cheaper and easier to manage compared to physio-chemical techniques (Kamat 2014).

The main aim of work described in this chapter was to determine if lichens can breakdown synthetic dyes compounds via extracellular redox cycling, and if adding H_2O_2 has any effect on dye decolorisation. Theoretically, schemes of extracellular redox cycling based on the Fenton reaction predicted that, adding H_2O_2 will stimulate the formation of hydroxyl radicals. The earlier work of Laufer (2012) found that all lichens tested can decolorize a range of dyes at slow rates on their own. It was predicted, that the rate of decolorisation should be greatly enhanced if hydroxyl radical production occurs.

4.2. Results

4.2.1. Decolorisation of synthetic dye by lichen species

Dye decolourisation was monitored with *Usnea undulata*, *Heterodermia speciose*, *Ramalina celastri*, *and Parmelia cetrarioides* for 48 h. A control treatment was included comprising flasks without lichen material but with dye and redox chemicals. Incubation lichens with the dye RBBR always caused some decolorisation (Figure 4.1 A). However, adding redox chemicals greatly increased the rate of dye decolorisation and the final proportion of dye decolorized (Figure 4.1 B). In the presence or absence of redox chemicals *U. undulata* was the most effective species at dye decolorisation.

Figure 4.2 shows decolorisation by *U. undulata* of a variety of dyes in the presence of redox chemicals. After 48 h of incubation RBBR dye was the most decolorized dye (76 %), followed by Acid Red 103 (63 %), Chicago Sky Blue 6 B (31 %) and Orange G (29%) decolorized.

Decolorisation of RBBR can occur when H_2O_2 is added to with redox chemicals in the absence of lichens (Figure 4.3 A), but the extent and rate of decolorisation was always less than that observed with lichens. Furthermore, adding H_2O_2 together with lichens in the presence of redox chemicals did not increase the rate of decolorisation compared with lichens in the absence of H_2O_2 (Figure 4.3 B, compare with Figure 4.1 B). There was no correlation between the rate of hydroxyl radicals' formation of individual species and the percentage dye decolorisation of RBBR (data not shown).

4.3. Discussion

Results presented here show that the selected lichens can decolorize a range of synthetic dyes on their own at a slow rate, presumably by direct metabolism by redox enzymes. However, addition of quinones and chelated (Fe) ions greatly enhances the degree of decolorisation. As discussed in Chapter 3, quinones and chelated (Fe) ions facilitate the generation of hydroxyl radical in lichens, which probably promotes dye degradation. The presence of redox chemicals allows lichens to breakdown a variety of synthetic dyes; while RBBR was the most effectively metabolized dye, *U. undulata* can also decolorize Acid Red, Chicago Sky Blue 6 B and Orange G dye, although the latter two dyes are more resistant to decolorisation. In the tested lichen species, the rate of hydroxyl radical generation (Table 3.2) and percentage decolorisation of RBBR were not correlated (data not shown), although lichen species used in this study were specifically chosen because they produce hydroxyl radicals at high rates.

4.3.1. The effect of H_2O_2 on RBBR dye decolorisation

Hydrogen peroxide is one the main source of hydroxyl radical formation in Fenton chemistry (Fenton 1894; Winterbourn 1995). Therefore, theoretically, adding H_2O_2 should stimulate the production of hydroxyl radicals, and therefore promote dye decolorisation. However, in the present study addition of H_2O_2 had little effect in stimulating the process of dye decolorisation. This may be because, adding H_2O_2 may have damaged the surface quinone reductases enzymes that are indirectly involved in the production hydroxyl radicals, effectively inhibiting redox cycling process. Results confirm that adding H_2O_2 to Fenton chemicals (in the absence of lichens) can stimulate the breakdown of RBBR, although the rate is slow except when the highest concentration of H_2O_2 (10 mM) was used (Figure 4.3 A). Presumably lichens promote dye decolorisation because redox cycling continuously regenerates quinones and Fe^{3+} ions.

4.3.2. The effect of dye structures decolorisation

Different type of dyes respond differently to decolorisation process (Figure 4.2) RBBR has the highest rate of decolorisation percentage, while Orange G the lowest after 48 h of incubation in the presence of redox chemicals and *U. undulata*. Other studies reported that RBBR is strongly resistant in decolorisation when incubated simply with laccase containing leachates, because of its structure (Robinson et al. 2001). However, this dye can be easily decolorized in the presence of redox chemicals (Soares et al. 2001).

Results presented here show that a range of lichens can decolorize RBBR in the presence of redox chemicals. Other dyes are less well decolorized by lichens (Figure 4.2), presumably because they have different structures.

4.3.3. The absence of a correlation between dye decolorisation and rate of hydroxyl radical formation

No correlation existed between the rates of decolorisation of the dye RBBR in various lichen species (Figure 4.1 B) with rates of hydroxyl radical formation (Table 3.2). This was surprising, assuming that hydroxyl radical formation is presumably the main driver for decolorisation. However, the species tested here were all chosen because they have high rates of hydroxyl radical formation, and therefore were all expected to be effective at decolorizing dyes. The involvement of hydroxyl radicals in dye decolorisation should be tested in future studies using lichens (e.g. *Peltigera*) with low rates of radical formation. The prediction would be that such species would be relatively ineffective at decolorizing dyes.

NOTE: In the following Figures, error bars were less than the size of the symbols.

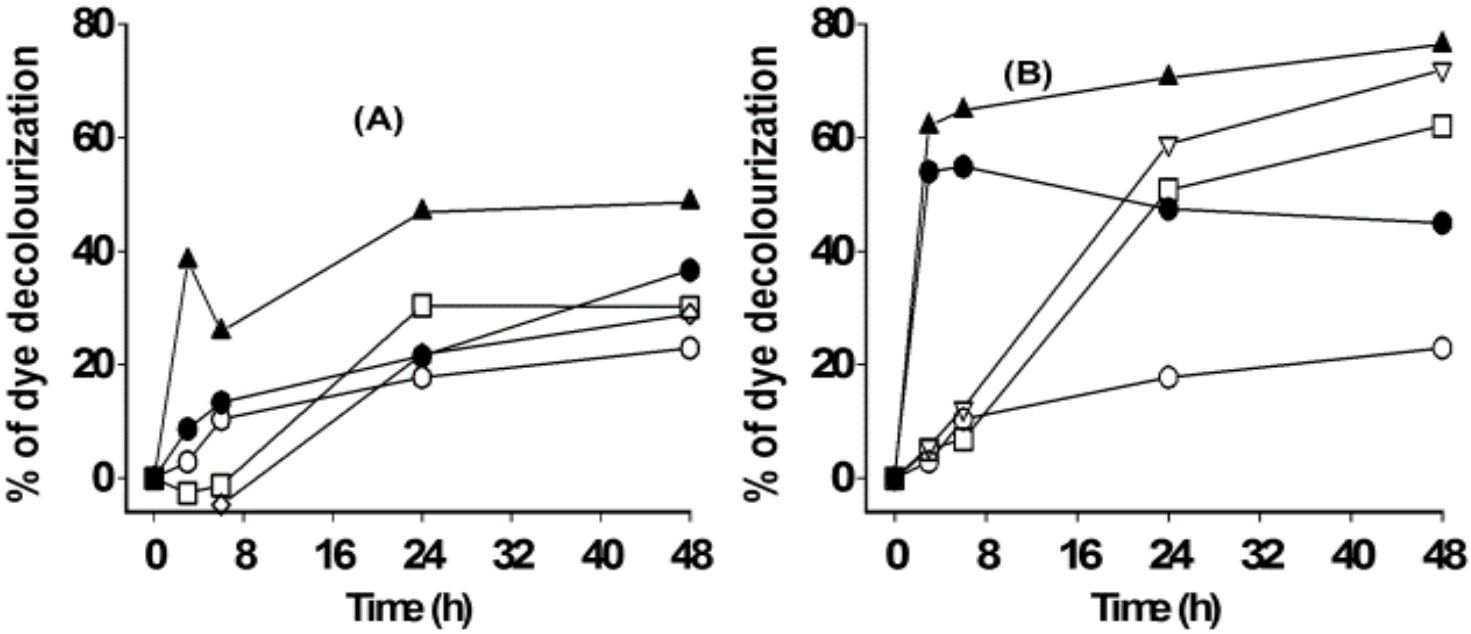


Figure 4.1. The percentages of RBBR dye decolorisation in different lichen species (**A**) without redox chemicals, (**B**) with redox chemicals. Control (No Lichen) (open circles), *U. undulata* (closed tringles pointing upwards), *P. cetrarioides*, (open squares); *R. celastri* (open triangles pointing downwards), and *H. speciosa* (closed circles).

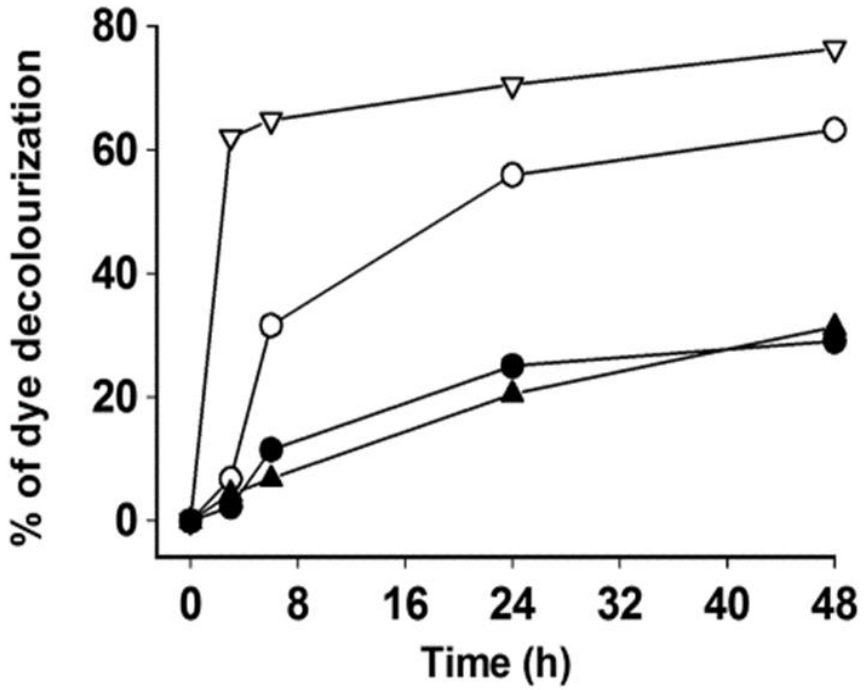


Figure 4.2. The percentage of dye decolorisation of different synthetic dyes, in the presence of *U. undulata* with the following redox chemicals: RBBR (open triangles pointing downwards), Acid Red (open circles); Orange G (closed circles) and Chicago Sky Blue 6B (closed triangles pointing upwards).

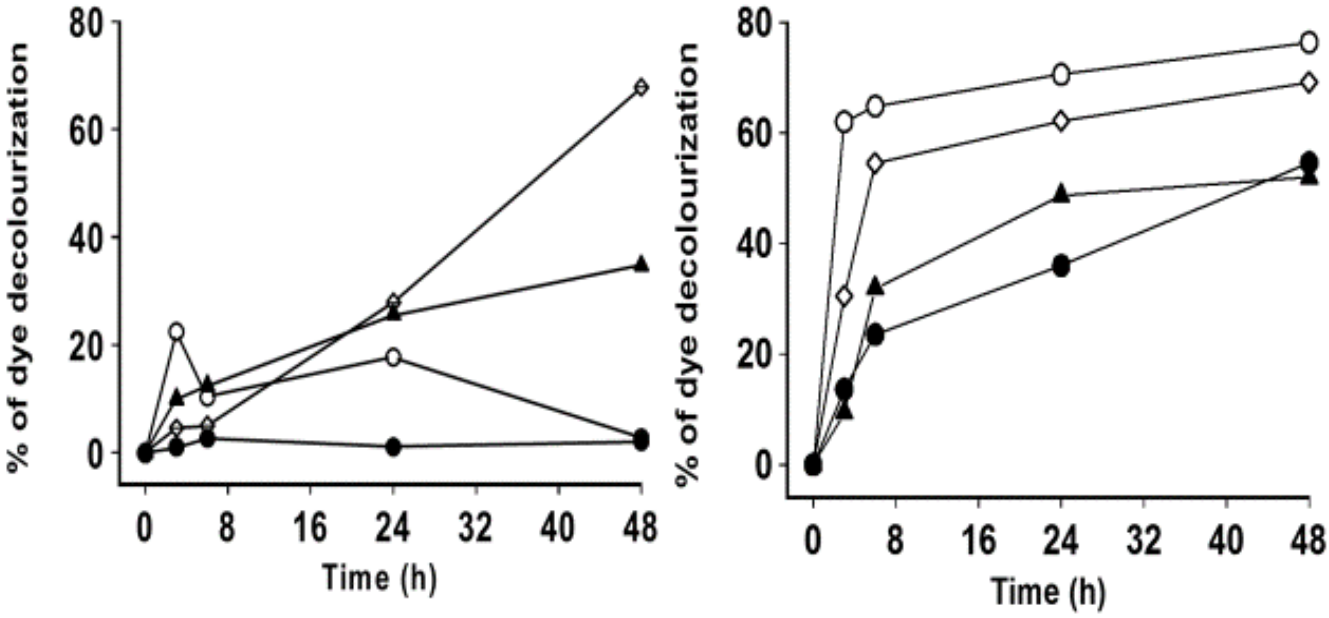


Figure 4.3. The effect of adding different concentration of H_2O_2 on RBBR decolorisation (**A**) in the absence of lichens and (**B**) in the presence of *U. undulata*, 0.1 mM H_2O_2 (closed circles), 1.0 mM H_2O_2 (closed triangles pointing upwards) and 10 mM H_2O_2 (open diamond).

NOTE: Controls: **(A)** No H_2O_2 or lichen (open circles) and **(B)** *U. undulata* with redox chemical (open circles)

CHAPTER 5 - GENERAL CONCLUSION

5.1. Hydroxyl radical production in lichens

The main conclusion of the work presented here is that hydroxyl radical generation by redox cycling is widespread in lichenized Ascomycetes. Results showed that when lichens are given chelated ions and quinones they can readily produce hydroxyl radicals. However, the rate of formation differs from one species to another. Hydroxyl radical formation was accompanied by the reduction of Fe^{+3} to Fe^{+2}. As discussed in (Chapter 1), in white rot fungi extracellular redox enzymes appear to be directly involved in the production of hydroxyl radicals. However, in this survey of hydroxyl radical production by lichens, the rate of formation does not correlate with the activity of laccases and peroxidases (Chapter 3). This is also supported by earlier studies, that an increase in laccase and peroxidase activity in *U. undulata* has no effect on rate of production of hydroxyl radicals (Beckett et al. 2015). Therefore, hydroxyl radicals in lichens are probably produced by the mechanism proposed to occur in brown rot fungi. This model suggests that surface quinone reductases reduce quinones to hydroquinones that react directly with Fe^{+3} non-enzymatically producing a quinone radical and Fe^{+2}. The reduction of both quinones and the presence of Fe^{+3} are important requirements for all mechanisms proposed for the production of hydroxyl radicals (Arantes, Goodell 2014).

In this study, adding H_2O_2 did not stimulate hydroxyl radical as predicted. Also the addition of a range of concentrations of Mn^{+2} and anisaldehyde had no effect on the generation of hydroxyl radicals as reported in brown-rot fungi (Gomez et al. 2009a, b). According to classic Fenton chemistry, theoretically the addition of H_2O_2 should stimulate the production of hydroxyl radicals, suggesting that there may be mechanisms involved in the generation of hydroxyl radicals in lichen in addition to simple Fenton chemistry reactions. Alternatively, H_2O_2 may have simply damaged the surface quinone reductases, effectively inhibiting redox cycling. Different quinones were tested in the study, and DMBQ was found to be the most effective quinone compared to other quinones tested. How lichens synthesize extracellular quinones in the field remains unclear, although recently lichens have shown to secrete a range of soluble phenols (Zavarzina et al. 2017). Therefore, enzymes such as peroxidases and laccases that occur in lichens may metabolize the secreted phenols to provide quinones for redox cycling process. Therefore these enzymes may indirectly contribute to hydroxyl radical production.

Taken into consideration of all the results presented in this thesis, it seems reasonable to conclude that the mechanisms involved resemble more closely those proposed for brown than white-rot fungi.

5.2. Possible roles of hydroxyl radicals in lichen biology

Hydroxyl radicals can initiate lignin breakdown, demethoxylation, aromatic ring hydroxylation, oxidation of the resulting catechol groups and side chain oxidation in different organisms (Goodell 2003). It has also been suggested that hydroxyl radicals may facilitate the breakdown of cellulose in fungi species (Martinez et al. 2009). Therefore, perhaps the most important role of hydroxyl radicals in lichens is to facilitate saprotrophic activities, to provide extra carbon during periods of low photosynthesis. Hydroxyl radicals can also be used for pathogen defence and control of differentiation process as has been suggested for free living fungi (Gomez et al. 2009a, b).

As discussed in the introduction, another potential role of this aggressive radical may be to degrade harmful pollutants such as phenols, synthetic dyes and micro-plastics (Krueger et al. 2015).

5.3. Decolorisation of synthetic dyes

The results on decolorisation experiments suggest that when lichens are given quinone and chelated Fe^{3+} they can readily decolorize different synthetic dyes. Lichens can decolorize dyes without extracellular redox cycling chemicals however, when added, the rate of decolorisation was much higher. Among the species tested, *U. undulata* was the most effective lichen and RBBR was best metabolized dye. The rate of dye decolorisation differs between the different dyes tested, probably as a result of their different structures and the orientation of their functional groups. It was predicted that addition H_2O_2 will stimulate the production of hydroxyl radicals thereby enhancing the dye decolorisation (chapter 4). However, the results showed that H_2O_2 inhibited decolorisation process as compared to decolorisation without the addition of H_2O_2. As discussed in (Chapter 4) addition of H_2O_2 might have simply damaged quinone reductases, thereby inhibiting extracellular redox cycling. Lichens are rare not in larger quantities; therefore, cannot be used commercially, but the enzymes can be engineered in the laboratory into fast growing free living fungi.

5.4. Future work

Future work should include identification of naturally occurring lichen quinones and characterizing the reductases responsible for the regeneration of hydroquinones from quinones. This will enable comparison of these enzymes with those present in brown-rot fungi (Cohen et al. 2004) and the similar quinone reductases that occur in free-living Ascomycetes (Espage et al. 2008). As mentioned earlier, the quinones used by lichens in the field to generate hydroxyl radicals remain unknown.

While the quinone used in assays here, DMBQ, can be produced by diverse brown-rot fungi (Korripally et al. 2011), there are no reports of DMBQ synthesis by Ascomycetes. However, lichens produce a diverse array of other quinones (Huneck, Yoshimura 1996), and other quinones can substitute for DMBQ (Figure 5). Quinones may be identified by analyzing lichen leachates. Further the roles of hydroxyl radical production in lichens need to be tested, for example, by testing their ability to break down lignocellulosic residues and pollutants. Possibly the enzymes involved in redox cycling could be engineered into fast growing free living fungi and used as a biological tool to treat waste water.

Additional future work, should investigate the degradation of a wide range of recalcitrant and toxic compounds by several lichen species using this mechanism. Also should investigate if a negative correction exists between hydrolytic enzymes such as cellulases and hydroxyl radical formation. Cellulase production and hydroxyl generation could be alternative methods for liberating carbon from cellulose substrates.

In summary, the results shown in the present study provide new information on the mechanisms used by lichen species in the formation of hydroxyl radicals. In addition, it also provides evidence on factors affecting hydroxyl radical production by quinone redox cycling, as well as the roles these radicals can play in lignin and organic degradation by these lichens.

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