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Filamentous fungi as a source of natural antioxidants

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ABSTRACT

Ten species of filamentous fungi grown in submerged flask cultures were investigated for antioxidant capacity. Effective antioxidant activity was demonstrated in terms of β–carotene/linoleic acid bleaching, radical scavenging, reduction of metal ions and chelating abilities against ferrous ions. Different extraction methods affected antioxidant activities through their effect on biologically active compounds produced in fungal mycelia. The methanolic extract of each fungus was typically more effective in antioxidant properties. Phenolic content was established in the range of 0.44–9.33 mg/g, flavonoid contents were in the range of 0.02–3.90 mg/g and condensed tannin contents were in the range of 1.77–18.83 mg/g. Total phenol content of each extract was attributed to overall antioxidant capacity ($r \geqslant 0.883$–1.000). Submerged cultivation of *Grifola frondosa*, *Monascus purpureus*, *Pleurotus* spp., *Lentinula edodes* and *Trametes versicolor* proved to be an effective method for the production of natural antioxidants.

1. Introduction

For thousands of years fungi have been recognised as nutritious, highly palatable functional foods in many societies and are now accepted as a valuable source for the development of medicines and nutraceuticals (Chang & Buswell, 1996; Wasser, 2002). Pharmacological and medicinal studies of fungi have shown that the Basidiomycete and Ascomycete divisions are an immense source of biologically active components, yet less than ten percent of all species have been described and even less have been tested for therapeutic significance (Blackwell, 2011; Lindequist, Niedermeyer, & Julich, 2005). Extensive epidemiology studies have demonstrated a variety of natural foods to be sources of multiple antioxidants which are strongly associated with reduced disease risk (Ferreira, Barros, & Abreu, 2009; Liu, 2004).

In the present study, nine Basidiomycetes from the genera; Grifola, Pleurotus, Lentinula and Trametes, in addition to one Ascomycete from the genera Monascus were cultivated in submerged flask cultures. *Grifola frondosa*, *Lentinula edodes*, *Pleurotus ostreatus*, *Monascus purpureus*, *Pleurotus citrinopileatus*, *Pleurotus eryngii*, *Pleurotus salmoneo-stramineus* and *Trametes versicolor* species were investigated based on the lack of information on antioxidant compound production in submerged liquid and the potential of each strain from a natural product perspective (Asatiani, Elisashvili, Songulashvili, Reznick, & Wasser, 2010; Elisashvili, 2012).

The Basidiomycetes chosen in this study have long been recognised as edible or therapeutic fungi (Chang, 1996). For years, research has focused on the antioxidant activity of the fungal fruiting body, as this stage of fungal growth is widely accepted as a nutritional food source. With current research demonstrating that extracts of fungi possess important biological activities, there is increasing interest in the antioxidant potential of fungi at various stages of their development. In addition, there has been little research focused on antioxidant production by submerged cultures of medicinal fungi. Accordingly, the objective of this study was to evaluate the antioxidant properties from the mycelia of several species of filamentous fungi. This incorporated analysis of total antioxidant capacity in terms of β-carotene bleaching activity, reducing power, CUPRAC, radical scavenging ability and chelating effects on ferrous ions.

Polyphenolic compounds of higher fungi have been demonstrated *in vitro* to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Molecules, such as simple phenolic acids, phenylpropanoids and flavonoids, as well as the highly polymerised molecules; lignin, melanin and tannins, accumulate naturally as end products from

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the shikimate and acetate pathways, with flavonoids representing the most common and widely distributed sub-group with antioxidant activity (Bravo, 1998; Ferreira et al., 2009; Liu, 2004). There are over 8000 naturally occurring phenolic compounds currently known (Balasundram, Sundram, & Samman, 2006). In this study, the antioxidant production of the main bioactive antioxidant components in the crude mycelial, hot water and methanolic extracts were determined. The data obtained from the present investigation provides a comprehensive description of the antioxidant capacity of each of the selected fungi following growth in submerged culture.

2. Material and methods

2.1. Materials and preparation of extracts

Nine Basidiomycetes from the genera; Grifola, Pleurotus, Lentinula and Trametes, in addition to one Ascomycota from the genera Monascus, were chosen for investigation. *G. frondosa*, *P. ostreatus* (*OYRM*1) and *L. edodes* were maintained by Alltech (Bioscience Centre, Dunboyne, Co. Meath, Ireland). *P. ostreatus* 32783 was supplied by ATCC (American Type Culture Collection, VA, USA). *M. purpureus* 1604, *P. ostreatus* 1833, *P. citrinopileatus* 5341, *Pleurotus eryngii* 9619, *Pleurotus salmoneo-stramineus* 5338 and *T. versicolor* 3086 were supplied by DSMZ GmbH (Braunschweig, Germany). Fungal mycelium was grown by submerged liquid fermentation (SLF) in 500 ml Erlenmeyer flasks. Mycelial biomass was collected and freeze-dried using an Alpha 1–4 LD plus freeze drying unit (Sigma, Osterode am Harz, Germany). A fine powder was collected (1.0 mm (No. 10) mesh) and the dried extract (crude extract) was further extracted using hot water or methanol extraction processes for comparison of antioxidant activity.

Hot water extraction was performed on a Sineo MD-S10 microwave digester (Elementec Ltd., Summerhill, Co. Meath, Ireland). Dry mycelial biomass (0.5 g) was accurately weighed into the digester cylinders. After the addition of 10 ml deionised water, the mixture was heated at 100 °C for 20 min under reflux and allowed to cool for 20 min. After this time, the filtrate was separated from the mycelial biomass by vacuum filtering using No. 42 (×2) Whatman filter paper. The process was repeated twice. The combined filtrate was then freeze-dried and the dry weight recorded. For the methanol extract, freeze-dried mycelial biomass (5 g) was accurately weighed into a 100 ml sterilin and shaken overnight (18–24 h) with 50 ml of methanol at room temperature. The extract was then filtered through Whatman paper No. 1 (×2) by vacuum filtration. The residue was then resuspended in another 50 ml of methanol. The process was repeated twice. The combined methanol filtrate was transferred to a pre-weighted sterilin and the solvent was evaporated by applying a constant flow of nitrogen. Residual methanol was removed by vacuum pressure at 50 °C overnight in an oven and the dry weight recorded. Both dried extracts were used directly for analysis of antioxidant components or redissolved in deionised water or methanol, respectively to a concentration of 100 mg/ml and then diluted to 50, 20, 10, 5, 1, 0.1 and 0.01 mg/ml for further uses.

2.2. Total antioxidant activity determination by β-carotene bleaching assay

Evaluation of antioxidants was performed using a method developed by Miller (1971). An aliquot (1 ml) of crystallized β-carotene (0.2 mg/ml in chloroform) was dispensed into a round-bottom flask containing 25 μl of purified linoleic acid and 200 mg of TWEEN 20 emulsifier. The chloroform was removed using a Heidolph laborota 4000 rotary evaporator and 50 ml of oxygenated, distilled water (shaken at 500 rpm for 30 min) was added to the flask and shaken vigorously. Aliquots (200 μl) of each extract (1 mg/ml) were added to 2.5 ml β-carotene/linoleic acid emulsion by pipetting into a series of spectrophotometer tubes with caps. A zero reading was taken at λ_{470nm} immediately after the addition of the emulsion to the antioxidant solution. Samples were capped and subjected to oxidation by placing in an oven for 3 h at 50 °C. Antioxidant activity was calculated as follows: $AA\% = 100 \times [1 - (A_0 - A_t/A_{00} - A_{0t})]$ where, A_0 is the absorbance at the beginning of the incubation with the extract, A_t is the absorbance after 3 h with the extract. A_{00} = is the absorbance at the beginning of the incubation without extract and A_{0t} is the absorbance after 3 h without the extract. Samples were read against a blank containing the emulsion minus β-carotene, i.e. 20 mg linoleic acid with 200 mg TWEEN mixed with 50 ml saturated H_2O (30 min). BHT and α-tocopherol (1 mg/ml) were tested as comparative positive controls.

2.3. $ABTS^+$ radical scavenging activity

Radical scavenging activity was determined according to the method first reported by Miller, Riceevans, Davies, Gopinathan, and Milner (1993) with slight modifications. ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was dissolved in water to give a 7 mM concentration stock solution. ABTS radical cation ($ABTS^+$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate 1:1 (v/v) and left in the dark at room temperature for 12–16 h before use. $ABTS^+$ solution was diluted in 95% ethanol to an absorbance between 0.7 and 0.75 at λ_{734nm}. The photometric assay was conducted with 180 μl of the ABTS reagent and 20 μl of the test samples. The optical density was measured at time zero. The radical scavenging activity of the fungal extracts was calculated using the following equation: $E = [(A_o - A_t)/A_o] \times 100$, where, A_0 the absorbance of the negative control and A_t is the absorbance of the samples. Radical scavenging activity was expressed as the concentration that scavenged 50% of the $ABTS^+$ radicals (EC_{50}). All determinations were carried out in triplicate. Trolox was used as a positive control. The results are normalised and expressed as EC_{50} values (mg extract per ml) for comparison. Effectiveness of antioxidant properties is inversely correlated with EC_{50} value.

2.4. Ferric reducing antioxidant power (FRAP)

Reducing power was determined according to the method of Oyaizu (1986), with slight modifications. Fungal extracts (200 μl) were mixed with 0.5 ml of 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of 1% (w/v) potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min. After the addition of 0.5 ml of 10% (w/v) trichloroacetic (TCA), the mixture was centrifuged at 1000 rpm for 10 min using a Heraeus multifuge (DJB labcare Ltd., Buckinghamshire, UK). An aliquot of supernatant (0.5 ml) was mixed with 0.5 ml of deionised water and 0.1 ml of 0.1% (w/v) ferric chloride. Absorbance was measured at λ_{700nm} against a blank. Higher absorbance indicated greater reducing power. Tests were carried out in triplicate and the results are expressed as mean values ± standard deviations. Reducing power was expressed as the concentration (mg/ml) where the reducing power reached 0.5 (EC_{50}). BHT and α-tocopherol were used as positive controls and deionised water was used a negative control. The results are normalised and expressed as EC_{50} values (mg extract per ml) for comparison. Effectiveness of antioxidant properties is inversely correlated with EC_{50} value.