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Production of Antioxidants by *Marasmiellus* sp. via Solid Substrate Fermentation

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Abstract: This study was aimed to evaluate the antioxidant properties of methanol extracts of fermented substrates optimised for antioxidant production by *Marasmiellus* sp. KUM 50061 mycelial biomass. Extract of fermented maize supplemented with (w/w) malt extract 4%, yeast extract 4% and rice bran 4% exhibited the highest 1,1diphenyl-2-picrylhydrazyl radical scavenging ability. The effective concentration of extract to scavenge 50% radicals was 1.875 mg mL^{-1}. This formulation was chosen as the optimum substrate for antioxidant production by *Marasmiellus* sp. KUM 50061 mycelial biomass. The thiobarbituric acid reactive substance (TBARS) assay showed that the effective concentration to inhibit lipid peroxidation of buffered egg yolk by 50% was 6.00 mg mL^{-1}. Total phenolics amounted to 31.41±1.56 mg GAE g^{-1} extract as measured by the Folin-Ciocalteau method.

Key words: 1,1diphenyl-2-picrylhydrazyl, lipid peroxidation, thiobarbituric acid reactive substance, radical scavenging, total phenolic content

INTRODUCTION

Oxidation, though essential to living organisms, if uncontrolled, could result in cell death and tissue damage and trigger many diseases, including, atherosclerosis, cancer, diabetes, rheumatoid arthritis and degenerative processes associated with aging (Halliwell and Gutteridge, 1984; Packer and Weber, 2001). Free radicals, such as superoxide (O_2^-), Nitric Oxide (NO), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and alkyl peroxy radicals, are unstable and can initiate toxic oxidation reactions and cause peroxidative disintegration of cells. These contribute to tissue damage and have been implicated in several diseases (Halliwell and Gutteridge, 1985). Superoxide, hydrogen peroxide and hydroxyl radicals are Reactive Oxygen Species (ROS) which are harmful because they can cause membrane lipid peroxidation, decrease membrane fluidity and attack DNA leading to mutation and cancer (Cerutti, 1994; Halliwell, 1997; Pietta, 2000). Antioxidants are needed to reduce or eliminate the toxic effects of ROS. However, the human body does not synthesize an excess of antioxidants and free radical damage is not completely prevented. Compounds with the ability to inhibit reactive oxygen species are important in therapeutic intervention to reduce the generation or effects of ROS. Therapeutic intervention includes treatment with antioxidant. Preventive intervention for free radical-mediated diseases calls for potent scavenger of ROS (Ames *et al*., 1995).

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In this study, the yield of bioactive compounds by Solid Substrate Fermentation (SSF) of agricultural products by mushroom was studied. The solid substrates were selected based on cost of products of natural/organic origin, availability and continuous supply to ensure bulk production of biomass with medicinal properties within a short time and low cost. The mycelia of *Marasmiellus* sp. (KUM 50061) as a source of antioxidant was selected because fungi exist in this state for most of their life. This particular mushroom was chosen, because Daker *et al*. (2008) showed that antioxidants from *Marasmiellus* sp. KUM 50061 grown on maize was efficient in inhibiting lipid peroxidation in cooking oil upon heating. In addition, it possessed antifungal activity against *Candida albicans* and antimicrobial activity against *Bacillus cereus*, *B. subtilis* and *Proteus vulgaris* (Pavalamalar *et al*., 2003; Noorlidah *et al*., 2005). The objectives of this study were to formulate the solid substrate for maximum antioxidant production by *Marasmiellus* sp. (KUM 50061) mycelial biomass and to determine the antioxidant properties of the extracts based on its ability to scavenge free radicals, inhibit lipid peroxidation and the total phenolics content.

MATERIALS AND METHODS

Maintenance and Inoculum Preparation of *Marasmiellus* sp. KUM 50061 for Solid Substrate Fermentation (SSF)

An axenic culture of *Marasmiellus* sp. (KUM 50061) was obtained by tissue culture of the fruit bodies collected from Endau-Rompin, Johore National Park, Malaysia. The mycelia were maintained at 4°C on Malt Extract Agar (MEA) slants and deposited at the Mycology Laboratory, Institute of Biological Sciences, Faculty of Science, University Malaya. Mycelial inoculum for SSF was prepared by inoculating centrally one 7 mm diameter plug taken from the outer rim of mycelial colony onto Glucose-Yeast-Malt-Peptone (GYMP) agar plates (6.0 g glucose, 3.2 g yeast extract, 3.2 g malt extract, 3.2 g peptone, 7.0 g agar, 0.4 g $MgSO_4.7H_2O$, 0.4 g NH_4Cl, 0.4 g KH_2PO_4 and 0.4 g K_2HPO_4 dissolved in 400 mL distilled water). The plates were incubated at 25°C for 5 days.

Chemicals

Food grade grains and beans selected as potential carbon sources were purchased from a local supermarket. Malt extract from Oxoid, yeast extract from Merck and rice bran from a local rice processing mill were also purchased. The selenium-rich substrates tested were oat bran (Country Choice) and wheat germ (Kretschmer Original Toasted). Methanol (analytical grade) was from Mallinckrodt Chemicals, USA. L-ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH•), ferrous sulphate ($FeSO_4$), 2.0 N Folin-Ciocalteau's phenol reagent and Gallic acid (3,4,5-trihydrobenzoic acid) were obtained from Sigma-Aldrich. Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were from AppliChem, Darmstadt. DL-Catechin and sodium carbonate (Na_2CO_3) were purchased from Sarsynthèse and R and M Chemicals, UK, respectively.

Substrate Formulation for Antioxidant Production by *Marasmiellus* sp. KUM 50061 via SSF

Selection of Grains for Antioxidant Production via SSF

Approximately 250 g of grains and beans were washed with filtered tap water and then soaked for 4-24 h until the substrates softened. Lentil, rice and wheat were soaked for 4 h, while chickpea, soya bean and maize were soaked for 7, 8.5 and 24 h, respectively. The substrates were then rinsed with filtered tap water, drained and 50 g were distributed into each of several 250 mL Erlenmeyer flasks. The flasks were covered with non-absorbent cotton plugs and aluminium foil and autoclaved for 20 min at 121°C. Fifty seven millimeter diameter plugs of a five-day old *Marasmiellus* sp. KUM 50061 inoculum were inoculated into the cooled substrates and then incubated at 25°C for 14 days. Substrates without the mycelial plugs served as the control. For each substrate evaluated, five flasks were set-up.

Effect of Nitrogen Supplementation on SSF by *Marasmiellus* sp. KUM 50061

The best grain that supported the growth of *Marasmiellus* sp. KUM 50061 mycelium, with the highest antioxidant activity, was further supplemented with malt extract, yeast extract and rice bran as nitrogen sources for SSF. Each flask of 50 g of grains was supplied with two sets of supplementation (w/w); containing: (1) a mixture of malt extract 2%, yeast extract 2%, rice bran 6% and (2) malt extract 4%, yeast extract 4%, rice bran 4%. The ratio of supplementation was chosen based on preliminary investigation (unpublished data). Un-inoculated substrates served as the control. Fourteen-day old mycelial biomass was harvested for antioxidant assay.

Effect of Selenium-Rich Substrate on SSF by *Marasmiellus* sp. KUM 50061

The best grain and combination of nitrogen sources that yielded the highest antioxidant activity after SSF by *Marasmiellus* sp. KUM 50061 mycelia was further supplemented with selenium-rich substrates. The preparation, sterilization and inoculation of the formulation were as described above. Four replicate flasks containing the best grain and combination of nitrogen sources were supplemented with (w/w) 1% oat bran or 1% wheat germ. The un-inoculated substrates served as the control.

Extraction of Total Solubles

The mycelial biomass after 14 days incubation was broken up and approximately 250 mL methanol was added into each Erlenmeyer flasks. The mixture was shaken for 48 h in a rotary shaker incubator at 150 rpm at room temperature. The mixture was filtered through Whatman No. 1 filter paper. The methanol filtrate was dried using a Büchi Rotavapor R-114 (Switzerland) to yield total solubles as crude extracts. The extracts were further freeze-dried to remove traces of water. Antioxidant analyses were carried out not later than 24 h after storage at −20°C.

Scavenging Effect on 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radicals

The extracts obtained from fermented substrates, as well as the extracts of the unfermented substrates, which served as the control, were analysed for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals scavenging ability (Brand-Williams *et al*., 1995). Stock solutions of each extracts at concentration of 50 mg mL^{-1} were prepared by dissolving 0.5 g of dried extract in 10 mL methanol. However, the stock solutions for extracts of unfermented soya bean, lentil and chickpea were prepared at much higher concentrations, i.e., 80, 150 and 500 mg mL^{-1}, respectively. An aliquot of 3.9 mL methanol solution containing 0.06 mM DPPH• was added to 0.1 mL of methanol diluted crude extracts. The solution was mixed vigorously and absorbance was then measured at 515 nm with methanol as the blank. The decrease in absorbance was recorded at 0, 1, 2 min and every 15 min until the reaction reached steady state. This condition means that the absorbance taken every 15 min subsequently, are no longer significantly different, determined by one-way Analysis of Variance (ANOVA). Antioxidant activity was compared with L-ascorbic acid (0.1-1.0 mM) as a positive standard. All determinations were performed in triplicates. The scavenging activity on DPPH• was expressed as percentage radical scavenging calculated as follows:

$$\text{Radical scavenging effect (\%)} = \frac{A_0 - A_s}{A_0} \times 100$$

where, A_0 refers to the absorbance of 0.06 mM DPPH• methanol solution only whereas, A_s is the absorbance of the reaction mixture.

Inhibition of Lipid Peroxidation of Buffered Egg Yolk by Extracts Obtained via SSF on Optimised Substrate

Inhibition of lipid peroxidation induced by Fe^{2+} was estimated by the thiobarbituric acid reaction method of Kuppusamy *et al*. (2002). Extracts of fermented optimum substrate and unfermented

substrate were assayed for lipid peroxidation. A stock solution of 360 mg mL^{-1} was prepared and then diluted to final extract concentrations of 0.1-30 mg mL^{-1}. The reaction mixture for inducing lipid peroxidation contained 1 mL of 25 g L^{-1} buffered egg yolk emulsified with 0.1 M phosphate buffer (pH 7.4), 100 μL of extract in solution and 100 μL of 1000 μM Fe^{2+}. The reaction mixture was incubated at 37°C for 1 h, after which, it was treated with freshly prepared 0.5 mL 15% trichloroacetic acid and 1.0 mL 1% thiobarbituric acid. The reaction tubes were kept in a boiling water bath for 10 min. Upon cooling, the tubes were centrifuged at 3500 rpm for 10 min to remove precipitated protein. The formation of Thiobarbituric Acid Reactive Substances (TBARS) complex was measured by removing 100 μL of supernatant and reading its absorbance at 532 nm, using a Power Wave X340 microplate reader (Bio-Tek Instruments, Inc.). The control was buffered egg with Fe^{2+} only. DL-Catechin within the range of 5-500 μg mL^{-1} was used as the standard. Each assay was carried out in triplicates. The percentage inhibition ratio was calculated from the following equation:

$$\text{Inhibition (\%)} = \frac{X_0 - X_s}{X_0} \times 100$$

where, X_0 refers to the absorbance of the control and X_s is the absorbance of the sample.

The percentage of lipid peroxidation inhibition was plotted against extract concentration in order to determine the concentration required to achieve 50% inhibition of phospholipids oxidation.

Total Phenolics Assay

Solutions were prepared in distilled water at room temperature. Only the extracts from fermented optimum substrate and unfermented substrate were assayed. Solutions of extracts were prepared at a concentration of 0.1 mg mL^{-1}. The method was a modification of that reported by Singleton and Rossi (1965). Two hundred and fifty microlitres of 10% Folin-Ciocalteau reagent were added to 250 μL of dried extract in 1.5 mL disposable plastic cuvettes and then mixed. After 3 min, 500 μL of 10% Na_2CO_3 was added to the mixture. After standing for an hour in the dark, the intensity of the blue colour was measured at 750 nm. A standard calibration curve was constructed using gallic acid with concentrations 1-15 μg mL^{-1}. Blank consisting of distilled water plus reagents without the extract samples was prepared. The absorbance values were converted to total phenolics content calculated on the basis of the gallic acid calibration curve and expressed as Gallic Acid Equivalents (GAE) in mg g^{-1} extract. Three replicates were prepared for each concentration of extracts.

Statistical Analysis

Data were recorded as Mean±SD and analysed by SPSS (version 12 for Windows 2000). One-way Analysis of Variance (ANOVA) and Tukey multiple comparisons were carried out to test for any significant differences between the means; the mean values of antioxidant activities between two extracts or two treatments were analyzed by independent-samples t-test. A $p<0.05$ were considered statistically significant. All analysis were performed in triplicates except for the assay on inhibition of lipid peroxidation in buffered egg yolk by extract obtained via SSF on optimised substrate formulation, which was done in duplicates.

RESULTS AND DISCUSSION

Substrate Formulation for Antioxidant Production by *Marasmiellus* sp. via SSF

Selection of Grains for Antioxidant Production via SSF

Antioxidant activity was defined as the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50% (EC_{50}; unit = mg extract mL^{-1} methanol). The lower the EC_{50} is, the

higher the antioxidant power. In contrast to other methods whereby the EC_{50} was determined after 30 min of reaction time, the antiradical activities in this study were analysed at the steady state. Steady state was considered to be achieved when the decrease in absorbance values were no longer significant, compared by one-way Analysis of Variance (ANOVA). Figure 1 showed that all extracts of fermented grains and beans except rice exhibited higher DPPH• scavenging activities compared to unfermented grains and beans. This indicates that during SSF, mycelia of *Marasmiellus* sp. were able to convert compounds present in chickpea, lentil, maize, soya bean and wheat into secondary metabolites, including antioxidants. The EC_{50} values of chickpea, maize, lentil and soyabean fermented with *Marasmiellus* sp. were significantly ($p<0.05$) lowered compared to unfermented form. Fermented maize exhibited the lowest EC_{50} value of 4.333 mg mL^{-1} followed by unfermented maize with EC_{50} of 8.933 mg mL^{-1} as shown in Fig. 1. However, the activity was much less than that of ascorbic acid (EC_{50} = 0.2 mM or 200 μM, equivalent to 0.035 or 35 μg mL^{-1} (Fig. 2) but comparable with mycelia of *Ganoderma tsugae* and *Grifola frondosa*, with EC_{50} values of 4.28 and 4.95 mg mL^{-1}, respectively (Mau *et al*., 2004). The EC_{50} values of methanol extracts from *Agaricus blazei*, *Antrodia camphorata*

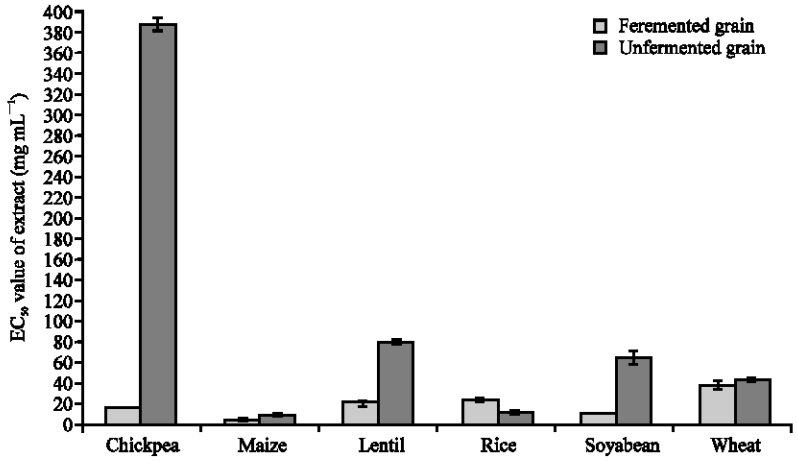

Fig. 1: Concentration of extracts of fermented and unfermented grains necessary to decrease the initial amount of DPPH• by 50% (EC_{50} value, unit = mg mL^{-1}). Values are Means±SD, n = 3. Average values of fermented grains/beans compared to unfermented grains/beans with asterisk indicates significant at p = 0.05 using t-test

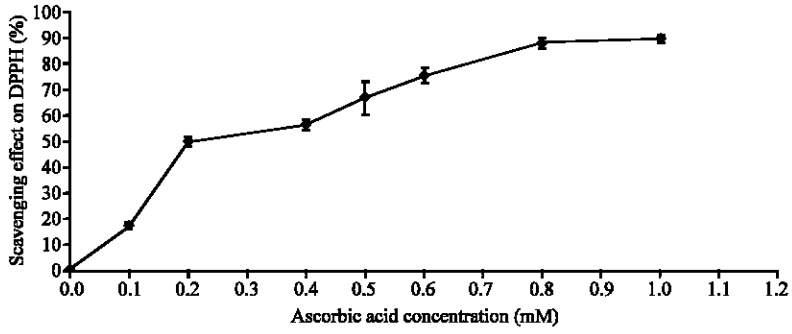

Fig. 2: DPPH· scavenging activity of ascorbic acid, used as the positive standard. Values are Means±SD, n = 3

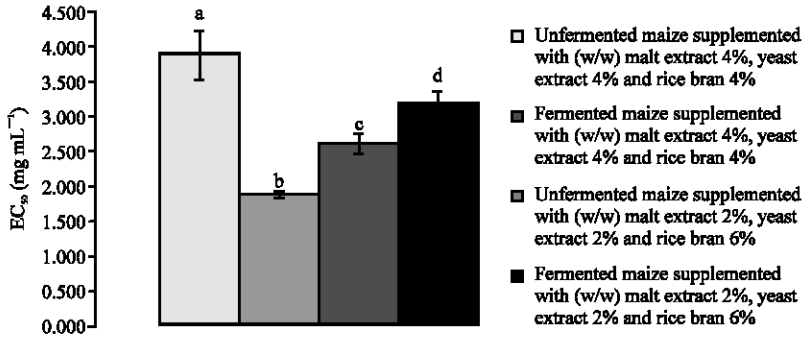

Fig. 3: DPPH· scavenging activity of extracts of unfermented and fermented maize supplemented with nitrogen sources. Values are Means±SD, n = 3. Average values with different letter(s) indicates significant at p = 0.05 by t-test

and *Agrocybe cylindracea* were 1.55, 2.26 and 3.95 mg mL^{-1}, respectively (Huang, 2000; Tsai, 2002). According to Song *et al*. (2003), the EC_{50} value of *Phellinus linteus* was 0.022 mg mL^{-1}. In comparison, the water extract from *Lentinula edodes* had a 55.4% inhibition at 6 mg mL^{-1} (Cheung *et al*., 2003) while the methanol extract from *Pleurotus ostreatus* scavenged DPPH• by 81.8% at 6.4 mg mL^{-1} (Lin, 1999). Maize was chosen for supplementation with nitrogen sources and selenium-rich substrate for optimisation of antioxidant production by *Marasmiellus* sp.

Effect of Nitrogen Supplementation on SSF by *Marasmiellus* sp.

The nitrogen sources utilized in this study were yeast extract, malt extract and rice bran. Certain fungi can only utilize organic nitrogen sources. Frequently, an organic nitrogen source is superior. Nitrogen is required for synthesizing amino acids, chitin, nucleic acids, proteins, purines, pyrimidines and vitamins. Maize supplemented with (w/w) malt extract 4%, yeast extract 4% and rice bran yielded optimum antioxidant production (significant at p = 0.05) by *Marasmiellus* sp. via SSF compared to maize supplemented with (w/w) malt extract 2%, yeast extract 2% and rice bran 6% (as shown in Fig. 3). Thus, the former was selected for further experiments on the effect of supplementation with selenium-rich substrate. The higher combined amount of nitrogen sources available for growth, at 12% resulted in higher biomass yield with better antioxidant activity, compared to 10%. Gern *et al*. (2008) conducted a study on alternative medium composition for production of *Pleurotus ostreatus* biomass by supplementation of wheat extract with different sources and concentrations of organic nitrogen; i.e., yeast extract or corn steep liquor. It was found that the organic nitrogen sources has a significant effect on the maximum concentration of biomass and that the substitution of yeast extract by corn steep liquor provides better results. Increasing the organic nitrogen concentration had a significant positive effect on the biomass concentration but when ammonium sulphate as an inorganic nitrogen source was employed in the absence of an organic nitrogen source, a decrease of all biomass parameters was observed. In a study carried out by Fasidi and Olorunmaiye (1994) on *Pleurotus tuber-regium*, among the nitrogen compounds tested, yeast extract supported the greatest growth. Fasidi (1996) explored the potential of agricultural wastes for vegetative growth and fructification of *Volvariella esculenta*. He concluded that rice straw (10 g) extended the diameter of the mycelia the greatest. Meanwhile, rice bran (10%) produced the highest mycelial density, probably because the oils and vitamins present in rice bran stimulate yield. Jonathan and Fasidi (2001) tested a series of nitrogen sources on growth of *Psathyrella atroumbonata* and concluded that yeast extract (2 g L^{-1}) was the most utilizable, whereas significant growth was supported by malt extract (2 g L^{-1}) and L-tryptophan (0.1% N). This may be attributed to the carbohydrate and protein component of those compounds.

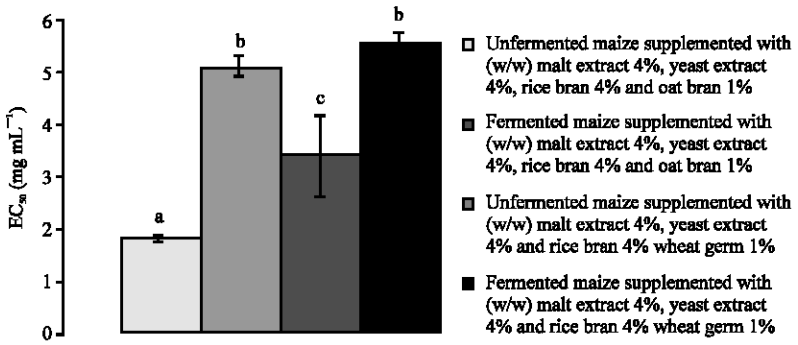

Fig. 4: DPPH· scavenging activity of extracts of unfermented and fermented maize supplemented with nitrogen sources and selenium-rich substrates. Values are Means±SD, n = 3. Average values with different letters indicates significant at p = 0.05 by t-test

Effect of Selenium-Rich Substrate on SSF by *Marasmiellus* sp.

This study attempted to utilize oat bran and wheat germ cereal as selenium-rich substrates to develop selenium-enriched *Marasmiellus* sp. mycelial biomass by solid substrate fermentation of an optimised substrate that can enhance antioxidative activities. Based on the results in Fig. 4, scavenging activity was still detected but none of the extracts from fermented maize supplemented with nitrogen and selenium-rich substrate produced improved EC_{50} values. In fact, unfermented substrates showed better EC_{50} values than fermented substrates. This could be attributed to the absence or lack of enzymes in *Marasmiellus* sp. mycelia to utilize the complex selenomethionine or any other organic selenium forms present in oat bran and wheat germ. None of the combinations in this section was selected for further experiments and the idea of selenium supplementation was abandoned. Further tests on lipid peroxidation and determination of total phenolics content were carried out with extracts of unfermented and fermented maize supplemented with (w/w) malt extract 4%, yeast extract 4% and rice bran 4% because the extracts exhibited the best antioxidant properties as determined by the DPPH• scavenging method.

Inhibition of Lipid Peroxidation of Buffered Egg Yolk by Extracts Obtained via SSF on Optimised Substrate

The assay was carried out to determine the ability of the extracts to inhibit peroxidation of phospholipids present in egg yolk, thus assess the potential of the dried extracts as a source of natural antioxidants. At lower concentrations of extracts, the lipid peroxidation inhibition was marginal but significant (p=0.05) inhibitory response was evident as the concentrations increased. The percentage lipid peroxidation inhibition by extract of fermented substrate was always higher than those of extract of unfermented substrate. However at concentrations of 20 and 30 mg mL^{-1}, the inhibition were comparable (Fig. 5). According to Shahidi and Wanasundara (1992), the effect of antioxidant concentration on autoxidation rates depends on antioxidant structure, nature of sample being oxidized and oxidation conditions. Phenolic antioxidants often lose their activity at high concentrations. Table 1 shows the EC_{50} value (mg mL^{-1}) of extracts of unfermented and fermented maize supplemented with nitrogen sources, as determined by the lipid peroxidation assay using buffered egg yolk. Although, the extract of fermented substrate exhibited a better EC_{50} value of 6.00 mg mL^{-1} than that of the extract of unfermented substrate extract at 8.25 mg mL^{-1}, this difference was insignificant. TBARS levels were progressively suppressed by the addition of increasing amounts of the catechin. With an EC_{50} of 225 μg mL^{-1} (0.225 mg mL^{-1}), catechin was more effective than either extracts at all concentrations tested (Fig. 6). This indicated a lower inhibitory activity of lipid peroxidation by both extracts relative to catechin.

Table 1: Concentration of extracts of unfermented maize and maize fermented by *Marasmiellus* sp. KUM 5006 mycelia via solid substrate fermentation that inhibited lipid peroxidation by 50% in buffered egg yolk determined by the thiobarbituric acid reaction method

Extract	Unfermented maize supplemented with (w/w) malt extract 4%, yeast extract 4% and rice bran 4%	Fermented maize supplemented with(w/w) malt extract 4%, yeast extract 4% and rice bran 4%
EC_{50} (mg mL^{-1})	8.25±0.35	6.00±0.71
Mean±SD	(n = 3)	(n = 2)

Average values are insignificant at p = 0.05

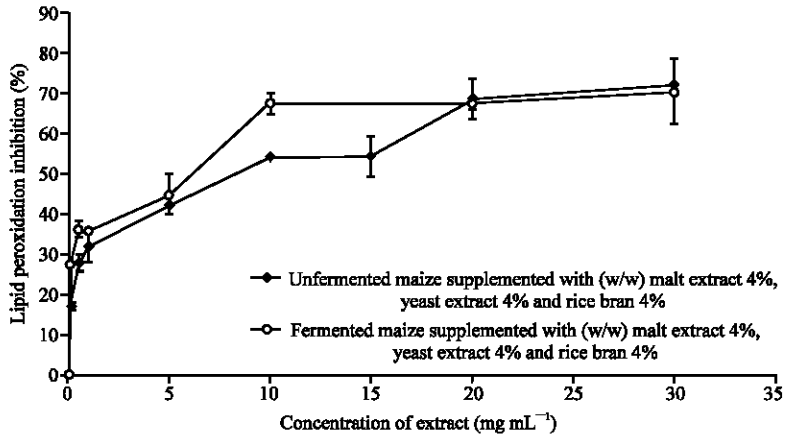

Fig. 5: Lipid peroxidation inhibition abilities of extracts of unfermented and fermented optimised substrate for antioxidant production by *Marasmiellus* sp. KUM 50061 via solid substrate fermentation, determined by the thiobarbituric acid assay on buffered egg yolk. Values are Means±SD, n = 3 for unfermented substrate, n = 2 for fermented substrate

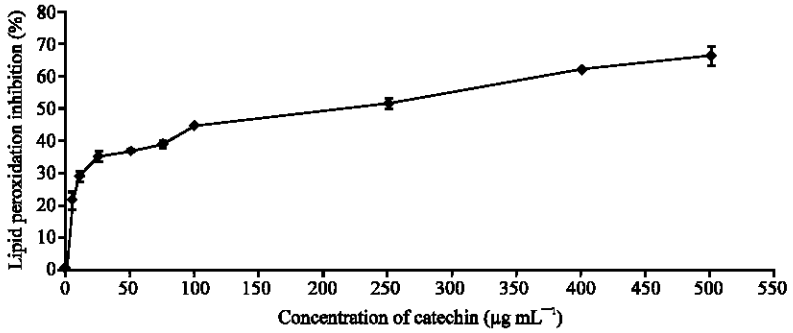

Fig. 6: Inhibition of lipid peroxidation in buffered egg yolk by catechin, used as the standard in the thiobarbituric acid assay. Values are Means±SD, n = 3

Total Phenolics Assay

The Folin-Ciocalteau method was used to measure the total phenolics content in the extracts of unfermented and fermented maize supplemented with (w/w) malt extract 4%, yeast extract 4% and rice bran 4%. The total phenolics content expressed as milligram Gallic Acid Equivalents (GAE) per gram extract are shown in Fig. 7. At 31.41 mg GAE g^{-1}, the total phenolics content of extract of fermented substrate was significantly (p = 0.05) lower compared to extract of unfermented substrate at 37.79 mg GAE g^{-1}. The reduction was probably because fermentation by *Marasmiellus* sp. KUM 50061 had

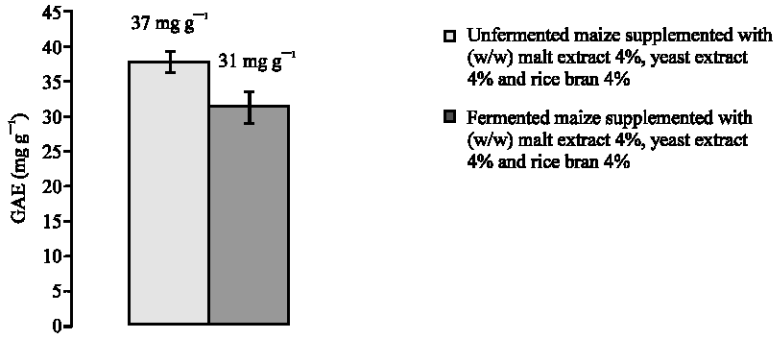

Fig. 7: Total phenolics content of extracts of unfermented and fermented optimised substrate for antioxidant production by *Marasmiellus* sp. KUM 50061 via solid substrate fermentation, determined by the Folin-Ciocalteau method. Values are means ± SD, n = 3. Average values are significant at P = 0.05 by t-test.

changed the phenolic compounds present in maize and/or nitrogen sources into other non-phenolic compounds. However, radical scavenging activity was still detected. In fact it was higher in the extract of fermented maize supplemented with nitrogen sources. It is not necessarily a single product, but a mixture of products that resist oxidative damage. Phenolic compounds are good inhibitors of lipid peroxidation but flavonoids, which are not only good inhibitors of lipid peroxidation but also scavengers of ROS and chelating agents of metal ions, may also be present (Halliwell, 1997). Other potential antioxidants include ascorbate, α-tocopherol, tocotrienols, carotenoids, flavonoids, phytates, thiol compounds, selenium and zinc.

Mau *et al*. (2002) reported that total phenols were the major naturally occurring antioxidant components in methanol extracts of medicinal mushrooms such as *Ganoderma lucidum*, *G. tsugae* and *Coriolus versicolor*. The total phenols content in *Ganoderma* species were 47.25-55.96 mg GAE g^{-1}, thus higher than *C. versicolor* at 23.28 mg GAE g^{-1}. This could account for the better antioxidant activity, reducing power and scavenging and chelating abilities of *Ganoderma*. In a study carried out by Yang *et al*. (2002) on commercial mushrooms, the highest content of total phenols was recorded in *Pleurotus ostreatus* (tree oyster mushroom) at 15.7 mg GAE g^{-1}. This was followed by *P. cystidiosus* (abalone mushroom), *Flammulina velutipes* (enokitake yellow strain), *Lentinula edodes* (shiitake strain Tainung 1), *F. velutipes* (enokitake white strain) and *L. edodes* (shiitake strain 271) at 10.24, 9.26, 9.11, 8.38 and 6.27 mg GAE g^{-1}, respectively. Mau *et al*. (2004) stated that methanol extracts of fruit bodies possessed higher total phenols than those from mycelia. Mycelia of *Agaricus blazei*, *Antrodia camphorata*, *Agrocybe cylindracea* and *G. tsugae* recorded total phenol contents of 19.8, 18.6, 23.5 and 24.0 mg g^{-1}, respectively. The total phenolics content of extract from the fermented optimised substrate in this study (31.41 mg GAE g^{-1} extract) was higher than the total phenolics content of fungal fruiting bodies or mycelia reported by other authors above, except for methanol extracts from *G. lucidum* and *G. tsugae*.

CONCLUSION

Secondary metabolites, many of which demonstrate biological activities and thus are of high value can be produced advantageously via SSF. An optimized medium needs to be defined before large-scale production or industrial application can be developed. Extract from fermented maize supplemented with (w/w) malt extract 4%, yeast extract 4% and rice bran 4% by *Marasmiellus* sp. mycelial biomass had enhanced radical scavenging ability with an EC_{50} value of 1.875 mg mL^{-1} determined by the

DPPH• method and EC_{50} value of 6.00 mg mL^{-1}, determined by the lipid peroxidation assay. Taking into consideration the antioxidant activity demonstrated in the DPPH• method and lipid peroxidation method, it may be suggested that the predominant antioxidant mechanism of extracts tested were by free radical scavenging, rather than inhibition of lipid oxidation. Phenolic compounds were detected at a concentration of 31.41 mg GAE g^{-1} extract. In the wake of increasing public interest in natural antioxidants, the use of the extract of fermented optimised substrate is suggested.

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REFERENCES

Ames, B.N., L.S. Gold and W.C. Willet, 1995. The causes and prevention of cancer. Proc. Natl. Acad. Sci. USA., 92: 5258-5265.

Brand-williams, W., M.E. Cuvelier and C. Berset, 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol., 28: 25-30.

Cerutti, P., 1994. Oxy-radicals and cancer. Lancet, 344: 862-863.

Cheung, L.M., P.C.K. Cheung and V.E.C. Ooi, 2003. Antioxidant activity and total phenolics of edible mushroom extracts. Food Chem., 81: 249-255.

Daker, M., A. Noorlidah, S. Vikineswary, P.C. Goh and U.R. Kuppusamy, 2008. Antioxidant from *Marasmiellus* sp. as stabilizer of lipid-rich food. Food Chem., 107: 1092-1098.

Fasidi, I.O. and K.S. Olorunmaiye, 1994. Studies on the requirements for vegetative growth of *Pleurotus tuber-regium* (Fr.) singer, a Nigerian mushroom. Food Chem., 50: 397-401.

Fasidi, I.O., 1996. Studies on *Volvariella esculenta* (Mass) singer: Cultivation on agricultural wastes and proximate composition of stored mushrooms. Food Chem., 55: 161-163.

Gern, R.M.M., E. Wisbeck, J.R. Rampinelli, J.L. Ninow and S.A. Furlan, 2008. Alternative medium for production of *Pleurotus ostreatus biomass* and potential antitumor polysaccharides. Bioresource Technol., 99: 76-82.

Halliwell, B. and J.M. Gutteridge, 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J., 219: 1-14.

Halliwell, B. and J.M.C. Gutteridge, 1985. Free Radicals in Biology and Medicine. 1st Edn., Oxford University Press, New York, ISBN: 978-0-19-856869-8, pp: 139-189.

Halliwell, B., 1997. Antioxidants and human diseases: A general introduction. Nutr. Rev., 55: S44-S49.

Huang, L.C., 2000. Antioxidant properties and polysaccharide composition analysis of *Antrodia camphorata* and *Agaricus blazei*. Master's Thesis. National Chung-Hsing University, Taichung, Taiwan.

Jonathan, S.G. and I.O. Fasidi, 2001. Effect of carbon, nitrogen and mineral sources on growth of *Psathyerella atroumbonata* (Pegler), a Nigerian edible mushroom. Food Chem., 72: 479-483.

Kuppusamy, U.R., M. Indran and B.R.S. Balraj, 2002. Antioxidant effects of local fruits and vegetable extracts. J. Trop. Med. Plants, 3: 47-53.

Lin, H.C., 1999. Evaluation of taste quality and antioxidant properties of edible and medicinal mushrooms. Master's Thesis. National Chung-Hsing University, Taichung, Taiwan.

Mau, J.L., H.C. Lin and C.C. Chen, 2002. Antioxidant properties of several medicinal mushrooms. J. Agric. Food Chem., 50: 6072-6077.

Mau, J.L., C.N. Chang, S.J. Huang and C.C. Chen, 2004. Antioxidant properties of methanolic extracts from *Grifola frondosa*, Morchella esculenta and *Termitomyces albuminosus* mycelia. Food Chem., 87: 111-118.

Noorlidah, A., S. Vikineswary, R. Pavalamar, U.R. Kuppusamy, S.T. Yee and S. Rushita, 2005. Antioxidant and antimicrobial activities of mycelia of tropical mushrooms. Fifth International Conference on Mushroom Biology and Mushroom Products, April 8-12, Shanghai, China, pp: 159-159.

Packer, L. and S.U. Weber, 2001. The Role of Vitamin E in the Emerging Field of Nutraceuticals. In: Nutraceuticals in Health and Disease Prevention, Kramer, K., P.P. Hoppe and L. Packer (Eds.). Marcel Dekker, New York, pp: 27-43.

Pavalamar, R., A. Noorlidah, S. Vikineswary and U.R. Kuppusamy, 2003. Antioxidant properties of tropical mushroom mycelial extracts. International Seminar on Natural Products, October 13-16, University Malaya, Kuala Lumpur, Malaysia, pp: 75-75.

Pietta, P.G., 2000. Flavonoids as antioxidants. J. Nat. Prod., 63: 1035-1042.

Shahidi, F. and P.K.J.P.D. Wanasundara, 1992. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr., 32: 67-103.

Singleton, V.L. and J.A. Rossi, Jr, 1965. Colorimetry of total phenolics with phospshomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic., 16: 144-158.

Song, Y.S., S.H. Kim, J.H. Sa, C. Jin, C.J. Lim and E.H. Park, 2003. Anti-angiogenic, antioxidant and xanthine oxidase inhibition activities of the mushroom *Phellinus linteus*. J. Ethnopharm., 88: 113-116.

Tsai, S.Y., 2002. Antioxidant properties and their cytotoxic activities on tumor cells of *Ganoderma tsugae* and *Agrocybe cylindracea* and antimutagenic properties of *Agrocybe cylindracea*. Master's Thesis. National Chung-Hsing University, Taichung, Taiwan.

Yang, J.H., H.C. Lin and J.L. Mau, 2002. Antioxidant properties of several commercial mushrooms. Food Chem., 77: 229-235.