CHAPTER 5

DISCUSSION

5.1 Antioxidant production from *L. squarrosulus* mycelia grown on soya bean, maize and rice supplemented with different concentration of nitrogen sources and methanolic extract of mycelia from liquid fermentation

Solid substrate fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water and is a very efficient technology for the production of bioactive compounds from microorganism. SSF processes generally employ a natural raw material as carbon and energy source. In this study, soya bean (*Soja max*), maize (*Zea mays*) and rice (*Oryza sativa*) were chosen as the solid substrate for the growth of *Lentinus squarrosulus* because they are among the most important cereals produced in the world, and maize has been recorded as an outstanding feed for livestock which offers high energy, low amount of fiber and high digestibility (Peter, 2003). Industrially, fermented soya bean, maize and rice also produce a great number of products such as starch, oil, alcohols, acetaldehyde, acetone, glycerol, acetic, citric and lactic acids, which are usually obtained by the wet-milling, extraction and fermentation processes (Bushra *et al*., 2007). Nowadays, mycelial fermentation on maize, soya bean and rice products are generally used to produce nutraceutical ingredients or functional food to provide a balanced diet.

Antioxidant supplements contain compounds that could act independently or synergistically to reduce disease and may have a potential in preventing chronic diseases (Miller *et al*., 2002). In this study, antioxidant capacity of all the methanolic

and dichloromethane extracts extracted from solid substrate fermentation of *L. squarrosulus* (soya bean, rice and maize) and liquid fermentation (GYMP) was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay to determine the total free scavenging activity. The DPPH reaction was very stable at room temperature, producing reliable values in repeated tests, because DPPH which contains an odd electron, gives a strong absorption at 515nm in visible spectrophotometer (Lhami *et al*., 2004). The decrease in absorbance of DPPH is caused by reaction between antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation (Mital *et al*., 2008). In this study, the absorbance was measured at 0, 1, 2 minutes and every 15 minutes interval until it reached a steady state. According to William *et al.* (1995), the IC_{50} value at 30 minutes did not show any difference for compounds that reacted rapidly with the DPPH radicals. However for the slower kinetic behaviour, the IC_{50} at 30 minutes would be inaccurate because the reaction would still be progressing. Therefore, analysing the IC_{50} value at the steady state is recommended (William *et.al.*, 1995).

The reaction kinetics for all extracts used in DPPH radical reactions were measured against time. The time required for the different formulations of *L. squarrosulus* extracts in this study to reach the steady state ranged from 15 to 75 minutes. Mau *et al*. (2005) explained in his study that antioxidants from mushrooms exhibited different reaction kinetics. The reaction kinetics was divided into three types which depend on the nature of the antioxidant used. For the rapid kinetic behaviour, the compounds will react rapidly with the DPPH radicals and reach a steady state in less than 1 minute. The second kinetic behaviour is intermediate where the compounds reach a steady state after 5 to 30 minutes, and for the slower kinetics, the compounds take about 1 to 6 hours to reach a steady state. The reaction graphs were obtained in hyperbolic curves (William *et*

al., 1995). Most of the *L. squarrosulus* extracts obtained by solid substrate fermentation can be categorized as an intermediate to slow kinetic behaviour, except for ascorbic acid which exhibited a rapid kinetic behaviour with only 2 minutes to reach a steady state.

The antioxidant activity is expressed by IC_{50} value, which is defined as the effective concentration of substrate that causes 50% loss of the DPPH activity (Molyneux, 2004). According to Mau *et al*. (2007), fermented soybeans in human diet might supply a new alternative for health protection to help humans reduce oxidative damage. This was proved when 5.0mg/ml of cold water extract of fermented soybeans gave 70.5% of antioxidant activity which compared favourably with a 1.0mg/ml extract from unfermented soya bean with 55% antioxidant activity, and ascorbic acid with 64% at 20mg/ml. The antioxidant capacity of fermented substrate in this study showed maize as the best substrate for growth of *L. squarrosulus* mycelia and antioxidant production with IC_{50} at 20.2mg/ml compared to soya bean and rice with 96.2mg/ml and 37.0mg/ml respectively. It is also supported by Daker *et al*., (2008) which showed maize fermented with *Marasmiellus* sp. as the best antioxidant extract compared to unfermented maize with 4.33mg/ml and 8.93mg/ml respectively (Daker *et al*., 2008). In this study, at 0.04% nitrogen concentration of methanolic unfermented maize, soya bean and rice extracts exhibited lower DPPH scavenging activity compared to fermented maize, soya bean and rice with higher IC_{50} 53.8mg/ml, 116.3mg/ml and 119.1mg/ml respectively. Similarly, *L. squarrosulus* was able to convert compounds in maize, soya bean and rice into secondary metabolites, including antioxidants (Daker *et al*., 2008).

The supplementation of carbon substrate alone in SSF is not enough to optimize the growth of *L. squarrosulus* mycelia. Addition of nitrogen sources is also required to enhance the growth capabilities and increase the production of antioxidants (Schmit,

2002). Nitrogen sources also help in synthesizing amino acids, chitin, nucleic acids, vitamins and protein in the substrates. The nitrogen sources used in this study were peptone, malt extract and yeast extract. Based on the results, substrate added with peptone gave the best antioxidant scavenging activity compared to yeast extract and malt extract. The IC_{50} of methanolic extract for fermented maize supplemented with 2.0% peptone exhibited the lowest IC_{50} value of 20.7mg/ml compared to yeast extract and malt extract with IC_{50} of 21.3mg/ml and 22.0mg/ml respectively. Rice supplemented with peptone also showed the highest antioxidant activity with IC_{50} value 20.2mg/ml. Lloyd *et al*. (2000) showed that even though malt extract and yeast extract are rich in carbohydrate and protein, peptone itself was high in amino acid which is readily suitable for microbial growth.

The extraction of antioxidants in this study was done using methanol and dichloromethane (DCM). Methanol extracts gave better radical scavenging activity compared to DCM. Oscar *et al*. (2007) showed that the IC_{50} values of methanolic extract for plant *A. coelophylla* were better at 41.1mg/l followed by dichloromethane extract of *Atrichoseris platyphilla* (112.0mg/l). Methanolic extract of *Citrus macroptera* also showed the best IC_{50} value compared to dichloromethane extracts with IC_{50} of 242.8µg/ml and 255.8µg/ml respectively (Oscar *et al*., 2007). In this study, the IC_{50} values of methanolic extract from solid substrate fermentation of *L. squarrosulus* were in the range of 22.2-96.2mg/ml (soya bean), 20.2-22.8mg/ml (maize) and 20.2-147.1mg/ml (rice). The IC_{50} values were lower compared to the IC_{50} values obtained from dichloromethane extracts ranging from 25.1-69.4mg/ml for soya bean, 20.2-89.3mg/ml for maize and 69.4-151.5mg/ml for rice. This was also supported by Baros *et al*., (2007) who showed that the scavenging effects of wild mushroom methanolic extracts on DPPH radicals increased with the increase in concentration and were

excellent for *Leucopaxillus giganteus* (100% at 5mg/ml), even higher than the scavenging effects of BHA (96% at 3.6mg/ml), a-tocopherol (95% at 8.6mg/ml), *Sarcodon imbricatus* (80% at 5mg/ml) and moderate for *Agaricus arvensis* (68.3% at 5mg/ml). This study showed that dichloromethane extracts of *L squarrosulus* exhibited lower antioxidant activity compared to methanolic extracts. Thus, it can be concluded that methanol is the best solvent to extract antioxidant compounds due to their polarity and good solubility of many antioxidative components (Bushra *et al*., 2007).

The selected antioxidant extract obtained by solid substrate fermentation (maize supplemented with 0.04% peptone) was compared with antioxidant extract obtained from liquid fermentation. Liquid fermentation was also considered as an economical and practical method for biomass production in developed countries (Auld and Morin, 1995). Liquid cultures are homogenous, which makes them easier to control, maintain and monitor (Jackson, 1997). Liquid cultures are generally easier to operate aseptically compared to solid media and they are also usually relatively easy to recover using centrifugation or filtration methods (Jackson, 1997), which make them generally more efficient than most other harvest techniques used in solid-substrate productions (Auld and Morin, 1995). However, in this study, the IC_{50} value of antioxidant from solid substrate fermentation exhibited better antioxidant activity than antioxidants from liquid fermentation of *L. squarrosulus* with IC_{50} value of 23.5mg/ml and 26.9mg/ml respectively. Pastrana (2005) did a **c**omparative study between solid substrate fermentation (SSF) and submerged liquid fermentation (SLF) and resulted in a high yield for SSF. It provides sufficient nutrient for the growth of mycelia and gives a similar environment to the natural habitat of mushroom.

5.2 Inhibition of lipid peroxidation by *L. squarrosulus* antioxidant extract

Lipid and fat oxidation in food is basically called lipid peroxidation and has more negative effects than the positive ones. Hydroperoxides, the primary products of lipid oxidation, may form dark-coloured, possibly toxic products or decompose to yield rancid off-flavour compounds. This problem has become a great concern in the food industry, because it leads to the development of rancidity (undesirable and toxic product). Thus, the oxidative deterioration of fats and oils in food is prevented by synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylated hydroxyquinone (TBHQ). Although they are efficient in preventing oxidation, these synthetic compounds are reported to have carcinogenic effect to human. Cheung and Cheung (2005) considered lipid peroxidation as a major cause of food deterioration and said that oxidative modification of low-density lipoproteins (LDLs) can be inhibited by antioxidants.

In this study, egg yolk and cooking oil were used in the lipid peroxidation assay to determine the ability of the optimized formulation extract to inhibit peroxidation of phospholipids present in egg yolk or cooking oil after heating. The fermented maize supplemented with 0.04% peptone and liquid fermentation of *Lentinus squarrosulus* was used as a source of natural antioxidant. Inhibition of lipid peroxidation of egg yolk was analysed by using TBARS assay. The results showed at 30% inhibition of lipid peroxidation, the *L. squarrosulus* fermented maize extract exhibited the highest inhibition activity with 0.63mg/ml compared to liquid fermentation of *L. squarrosulus* or unfermented maize with 0.71mg/ml and 1.03mg/ml respectively. This showed that extracts from fermented maize and liquid fermentation with *L. squarrosulus* were stronger inhibitors of lipid peroxidation, compared to extract from unfermented maize.

The lipid peroxidation assay using cooking oil was designed to investigate if the fermented maize and liquid fermentation of *L. squarrosulus* extracts were able to protect cooking oil from lipid peroxidation activity which usually occur during preparation of food or heating (cooking). After heating for 20 minutes, cooking oil undergoes lipid peroxidation by showing the highest absorbance value 0.286nm compared to other extracts. Heated oil supplemented with a lower concentration (1.0mg/ml) of liquid fermentation antioxidant extract of *L. squarrosulus* showed the best inhibition of lipid peroxidation activity with inhibition of 64.7% compared to fermented maize extract with 53.0% and unfermented maize extract with 51.2%. This showed that antioxidants from liquid fermentation of *L. squarrosulus* have a potential to be a good stabilizer of lipid rich food. However, when the concentration of extract was increased to 5.0mg/ml, oils supplemented with fermented maize extract and liquid fermentation extract showed no significant differences in their absorbance values of 64.8% and 66.0% respectively. Among the positive controls, quercetin dihydrate was the most effective positive control in protecting cooking oil from lipid peroxidation activity. Statistical analysis of lipid peroxidation of oil showed that by the addition of antioxidant extract the reaction was dosage-dependent. The absorbance values of fermented maize extracts were significantly different at different concentrations (5mg/ml and 1mg/ml). This suggests that *L. squarrosulus* can be a good potential source of natural antioxidants to replace chemicals such as BHA and catechin. This information is important, if the extracts are used in the industry as antioxidants, by adding a small amount of *L. squarrosulus* extract in the cooking oil, it can became a good inhibitor for peroxidation in food and contribute to cost-effectiveness.

The major pathway of lipid peroxidation explained by Lhami *et al*. (2004) contains a self-catalytic free radical chain reaction, i.e. it can be catalyzed by environmental factors such as light, oxygen, free radicals and metal ions. That is why lipid peroxidation can easily lead to a loss of functional properties and nutritional value. Currently, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertbutylhydroquinone (TBHQ) have been widely used for years in the food processing industries. However, some physical properties of BHT and BHA like their high volatility and instability at elevated temperatures have carcinogenic effects. That is why strict legislation on the use of synthetic food additives and consumer preferences has shifted the attention of manufacturers from synthetic to natural antioxidants (Lhami *et al*., 2004).

In a work done by Mau *et al*. (2002a) involving medicinal mushrooms reported that the antioxidant activity of methanolic *Dictyophora indusiata* extract had an excellent antioxidant activity, with 2.23% lipid peroxidation at 1.2mg/ml. At the same concentration, *Grifola frondosa*, *Hericeum erinaceus* and *Tricarpelema giganteum* showed 29.8%, 48.5% and 67.0% lipid peroxidation, respectively. Other study for antioxidant properties of freshly obtained commercial edible mushrooms consisting of *Lentinula edodes* (shiitake), *Flammulina velutipes* (enokitake), *Pleurotus cystidiosus* (abalone mushroom) and *Pleurotus ostreatus* (tree oyster mushroom) demonstrated the lipid peroxidation activity within 24.7–62.3% at 1.2mg/ml, and thus are classified as moderate to high peroxidation inhibitors. This result is comparable with BHA which showed 66.1% inhibition of lipid peroxidation at 10mg/ml (Yang *et al*., 2002). Cheung and Cheung (2005), stated that phenolic compound, proteins and amino acid found in mushrooms have been shown to have antioxidant activity in the inhibition of LDL oxidation. A recent study by Daker *et al.* (2008) showed, an antioxidant production

from mushroom as a potential source and can be applied as commercial food preservatives and synthetic products which will contribute to the production of natural antioxidant. As such, it is obvious that the prevention of lipid peroxidation in food is effective not only in the stability of the nutritional content but also the extension of the best-before date. Therefore, much attention has been paid to the natural antioxidants which are expected to prevent food and living systems from peroxidative damage (Yingming *et al*., 2007). As a conclusion, this experiment showed the exact ability of *L. squarrosulus* in protecting the oil from lipid peroxidation activity in the heated tested cooking oil.

5.3 Total phenolic content (TPC)

Phenolic acids are plant metabolites. They recently have become more important due to their potential protective role against oxidative damage diseases (coronary heart disease, stroke, and cancers). Phenolic compounds are essential for the growth and reproduction of plants, and are produced as a response to defending injured plants against pathogens (Ray, 2006). In this study, gallic acid was used as a standard of TPC assay. Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic antioxidant and also widely used in pharmaceutical industries for manufacture of trimethoprim and antibacterial agent. Gallic acid is also used in the enzymatic synthesis of gallic acid esters (e.g. propyl gallate), which is utilized mainly as a strong antioxidant in fats, oils, beverages, leather industry and in the pyrogallol manufacture (Garcia-Najera *et al*., 2002). The results showed that among the positive controls, ascorbic acid exhibited the highest content of phenolic at 931.86mgGAE/g followed by quercetin (628.04mgGAE/g) and BHA (194.24mgGAE/g). As for antioxidant extract, liquid fermentation of *L. squarrosulus* gave 31.39mgGAE/g extract followed by fermented maize and unfermented maize with

19.34mgGAE/g extract and 15.40mgGAE/g extract respectively. Research done by Ma (2004) stated that there is a correlation between phenolics and antioxidant activity in mushrooms.

Several mushrooms have various polyphenolic compounds, recognized as excellent antioxidants due to their ability to scavenge free radicals by single-electron transfer. Some common edible mushrooms, which are widely consumed in Asian culture as traditional foods and medicines, have currently been found to possess antioxidant activity, which is well correlated with the total phenolic content, total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities (Lillian *et al*., 2007). This is also consistent with the findings of many research groups who reported such positive correlation between total phenolic content and antioxidant activity (Bushra *et al*., 2007). Overall, we can say, natural phenolics exhibit high antioxidant activities. As a conclusion, fermented maize and liquid fermentation of *L. squarrosulus* extract contain phenolic compound which can contribute in antioxidant activity. Choi *et al*. (2007) reported that the total phenolic contents of alcohol extracts, from various species of mushroom, are correlated with anti-oxidant activity.