Bioconversion of Agricultural Products for Quality Improvement

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List of Abbreviations

ABTS	2, 2'-azino-bis-3-ethylbenzothiazoline- 6-sulphonic acid diammonium salt
AF	Aflatoxin
ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
FRAP	Ferric reducing antioxidant powder
G. lucidum	*Ganoderma lucidum*
GRAS	Generally regarded as safe
IDF	Insoluble dietary fiber
IVPD	*In vitro* protein digestibility
LDL	Low density lipoprotein
P. nebrodensis	*Pleurotus nebrodensis*
P. sajor-caju	*Pleurotus sajor-caju*
PA	Phytic acid
PDA	Potato dextrose agar
SDF	Soluble dietary fiber
SSF	Solid-state fermentation
SMF	Submerged fermentation
TDF	Total dietary fiber
TEAC	Trolox equivalent antioxidant capacity
TPTZ	Tripyridyltriazine
Trolox	6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid

Abstract

Bioconversion refers to the conversion of a compound from one form to another by the actions of microorganisms. Fungi are commonly used for such purpose. Fermentation is a typical example of bioconversion process. In general, bioconversion is a value added process that may improve the nutritional quality of the substrates.

In this project, the mycelia of *Pleurotus sajor-caju*, *Pleurotus nebrodensis* and *Ganoderma lucidum* were tested for their bioconversion abilities. A total of fifteen types of seeds were chosen as substrates for bioconversion studies, it including eleven legumes and four cereals. The legume included adzuki beans (*Vigna angularis*), black soybean (*Glycine max* (L.) Merr.), chickpea (*Cicer arietinum* L.), hakmeitau bean (*Vigna sinensis*), lentil (*Lensculinaris esculenta*), mung bean (*Vigna radiata* L.), navy bean (*Phaseolus vulgaris*), pinto bean (*Phaseolus vulgaris*), red kidney bean (*Phaseolus vulgaris*), soybean (*Glycine max* (L.) Merr.) and split peas (*Lensculinaris esculenta*). The cereals were included barley (*Hordeum vulgare* L.), buckwheat (*Fagopyrum esculentum*), millet (*Staria glauca* (L.) Beauv.) and oat (*Avena sativa* L.). Based on the results of screening studies, the seeds of buckwheat, mung bean, oat, pinto bean, red kidney bean and hakmeitau bean were selected for further studies as they supported the growth of the fungi the best. The qualities of the fermentation products after bioconversion were compared with that of the corresponding initial substrates.

It was found that the fermented products were free from aflatoxin contamination. The nutritional values of the fermented products were better than the seed substrates, since the protein content and protein digestibility of the products were improved, and the antinutrient phytic acid level was also reduced. In addition, the antioxidant activities of most fermented products were higher than the

corresponding unfermented substrates. Dietary antioxidants are associated with reduced risks of cancer, cardiovascular disease, diabetes, and chronic diseases. Therefore these fermented products have a potential to become a functional food.

Among the three of fungi investigated, the growth of *P. sajor-caju* mycelia on most substrates was better than that of *P. nebrodensis* and *G. lucidum*. *P. sajor-caju* was the only fungal species that can lower the lipid content of the fermented products. It had the highest phytic acid reduction ability. In addition, the *P. sajor-caju* fermented products had the highest protein content and better *in vitro* protein digestibility. Therefore *P. sajor-caju* had better bioconversion ability than *P. nebrodensis* and *G. lucidum*.

摘　要

生物轉化是解作利用微生物將混合物由一種形態或結構轉化成另一種形態或結構，而菌類便是經常使用在這個範籌上。生物轉化過程的其中一個最常見例子便是發酵作用。 一般而言，生物轉化是一個價值提升的過程，透過生物轉化能提高轉化原料的營養價值。

這項研究的目的爲測試鳳尾菇、百靈菇及靈芝的菌絲體之生物轉化能力。 其中有十五種種子會用作轉化能力測試的培養基，當中包括十一種豆類及四種穀類。豆類的種子有紅豆、 黑豆、雞心豆、扁豆、綠豆、菜豆、紅花豆、紅腰豆、黃豆、馬豆及黑眉豆。而穀類的種子有大麥、蕎麥、小米、燕麥。從篩選之結果來看，三種菌絲體生長在蕎麥、綠豆、燕麥、紅花豆、紅腰豆及黑豇豆之生較其他種子爲佳，因此對這五款種子作進一步研究，來對比發酵製品經生物轉化後及農作物原料的品質分別。

綜合而言，發酵製品並沒有受黃曲黴毒素的污染，而它的營養價值也比種子培養基優勝。因爲發酵製品的蛋白量及蛋白消化率也比種子原料的爲高，而且發酵製品的抗糙養因子植酸含量也降低了。再加上大部份發酵製品的抗氧化活性也比存放了十天未經發酵的種子培養基爲高，食用抗氧化物有可能降低患癌、管心病、糖尿病及慢性疾病，所以發酵製品有成爲功能食品的潛在能力。

就三種菌絲體的研究而言，鳳尾菇菌絲體生長在大多數培養基的狀況較百靈菇及靈芝爲佳。而且它是唯一能降低脂肪及比其餘兩種菌絲體更能降低植酸，再加上鳳尾菇菌絲體之發酵製品含有最高的蛋白量及能提升蛋白消化率。因此，鳳尾菇發酵製品的生物轉化是較百靈菇及靈芝強。

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Chapter One: Introduction

1.1 Bioconversion

Bioconversion refers to the conversion of a compound from one form to another by the actions of microorganisms. Usually, the conversion of organic materials, such as plant or animal wastes are converted into usable products or energy sources by biological processes or agents, such as fungi. Fermentation is a typical example of bioconversion process. In general, bioconversion is a value added process that may improve the nutritional quality of the substrates.

Fermentation of food is a value added process, which improve nutritive value as well as digestive qualities (Rodríguez *et al.*, 2004). It can divide into solid-state fermentation (SSF) and submerged fermentation (SMF). SSF is generally defined as the growth of the microorganisms on (moist) solid substrates in the absence or near-absence of free water (Pandey, 1994). SSF is one of the methods for mushroom cultivation (Soccol & Vandenberghe, 2003). The principle aim of composing in mushroom cultivation is to produce a selective growth medium, which renders successful mycelial colonization. Synthetic composts have been derived from every type of agro-industrial waste products and residues. Different cereal grains such as corn straw and wheat are used as carrier for fungal mycelium (Yang *et al.*, 2001; Bonatti *et al.*, 2004)

SSF is the most economical method for enrichment in microbial protein or increasing fiber digestibility. The use of mushroom to convert carbohydrates, lignocellulose and other industrial wastes into foodstuffs rich in protein is possible due to the following characteristics of mushroom (Pandey, 1994):

1. Mushrooms have fast growth rate
2. Their protein content is quite high varying from 35 to 60%
3. They can be grown in slurry or non solid substrate
4. Their nutritional values are as good as other conventional foods rich in protein.

1.2 Functional foods & quality improvement in fermentation

Recently, people have became more aware of the relationship between diet and disease, and believe that foods can be designed to help prevent various diseases (Al-Saqer *et al.*, 2004). Usually, the fermented products will have better nutritive value than the unfermented substrates and they have the potential to become functional foods. For example, a traditional Indonesian functional food called tempeh, it is a soybean fermentation product which is mainly produced by fungi of the genus *Rhizopus*. It is a compact and sliceable mass of boiled and dehulled soybeans covered and penetrated by a dense mycelium of the fungus. The tempeh fermentation by the *Rhizopus* mold binds the soybeans into a compact white cake. The fermentation process can be divided into 2 steps, a soaking phase which leads to a decrease in pH accompanied by growth of lactic acid bacteria and a second phase characterized by partial fungal digestion of soybean storage products (Heskamp & Barz, 1998).

Since tempeh is made from whole soybeans, it is rich in fibre and soy isoflavones. It is also a generous source of many nutrients such as calcium, B-vitamins and iron. From the USDA Nutrient Database for Standard Reference, the nutritional values of tempeh are including 19.0 % protein, 7.7% fat, 17 % carbohydrate, 4.8 % fiber and 1.4 % ash. The process of solid-state fermentation makes the soybeans softer, since enzymes produced by the mould predigests a large portion of the basic nutrients. The Rhizopus moulds produce an enzyme phytase, which breaks down phytates, thereby increasing the absorption of minerals such as zinc, iron and calcium. In addition, the fermentation process greatly reduces the oligosaccharides that make beans hard to digest for some people. As the fermented products have quality improvements and may have many beneficial effects to human, they can also act as the health foods.

According to the description of Regulation of Health Food in Overseas Places: Overall Comparison (2001), it said that Hong Kong has no legal definition of 'health food'. Different terms such as dietary supplements, functional foods, nutraceuticals, designed foods and natural health products are used to refer to health foods. There is also no specific legislation governing health food products in Hong Kong. Health food products in Hong Kong are regulated under different ordinances depending on their ingredients.

In United States, there is also no legal definition of 'health foods or functional foods'. However there is a subset of foods called dietary supplements. Congress defined the term "dietary supplement" in the Dietary Supplement Health and Education Act (DSHEA) of 1994. A dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary

ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements can also be extracts or concentrates, and may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. The International Food Information Council (IFIC, 1998) defines functional foods as foods that provide health benefits beyond basic nutrition. This definition is similar to that of the International Life Sciences Institute of North America (ILSI, 1999), which has defined functional foods as foods that, by virtue of physiologically active food components, provide health benefits beyond basic nutrition. Although health foods or functional foods remain undefined under current US food regulations, they are usually understood to be any potentially healthful food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains (ADA, 1998).

1.3 Substrates

All solid substrates have a common feature: their basic macromolecular structure. In general, substrates for SSF are composite and heterogeneous products from agriculture or by-products of agro-industry. This basic macromolecular structure (e.g. cellulose, starch, pectin, lignocellulose, fibres etc.) confers the properties of a solid to the substrate. The structural macromolecule may simply provide an inert matrix (sugarcane bagasse, inert fibres, resins) within which the carbon and energy source (sugars, lipids, organic acids) are adsorbed. But generally, the macromolecular matrix represents the substrate and provides also the carbon and energy source (Raimbault, 1998).

1.3.1 Legumes

Legume grains occupy an important place in human nutrition. It is classified into two categories, "pulse" from the Latin which means pottage. It refers to the legume seeds containing only small amounts of fats. "Oilseed" is used to describe the legumes containing high proportion of fats (Nwokolo & Smartt, 1996). Several reports claim that inclusion of legumes in the daily diet has many beneficial physiological effects in controlling and preventing various metabolic diseases such as diabetes mellitus, coronary heart disease and colon cancer (Simpson *et al.*, 1981).

Legumes are inexpensive, it is considered as poor man's meat. They are generally good sources of carbohydrates and are rich in proteins (~18–25%). Soybean is a unique example that contains about 35–43% proteins. Legumes are the cheapest sources of supplementary proteins in developing countries (Tharanathan & Mahadevamma, 2003). The mature legume seeds are a good source of dietary fiber and minerals. Therefore seeds of legumes are a good alternative to costly meats and fishes.

1.3.2 Cereals

Cereal grains are considered to be one of the most important sources of dietary proteins, carbohydrates, vitamins, minerals and fiber for people all over the world. However, the nutritional quality of cereals and the sensorial properties of their products are sometimes inferior or poor in comparison with milk and milk products. The reasons behind this are the lower protein content, the deficiency of certain essential amino acids (lysine), the low starch availability, the presence of determined

antinutrients (phytic acid, tannins and polyphenols) and the coarse nature of the grains (Chavan & Kadam, 1989, Blandino *et al.*, 2002).

1.4 Edible mushroom

Mushroom, the reproductive fruiting body of higher fungi is considered as the queen of vegetables for a long time. Mushrooms have been recognized worldwide as a source of food due to high biological value of their proteins, apart from carbohydrates, fats, vitamins and minerals (Pandey, 1994).

Usually, edible mushrooms are used in fermentations. The reason of using edible fungi is due the fact that they are safe to consume. It can ensure that no mycotoxin formed during the process of solid-state fermentation. Since the fungi used in the process are edible, the fermentation products that also include the fungal mycelia could be generally regarded as safe (GRAS).

1.4.1 *Pleurotus spp.*

Pleurotus spp. is a common primary decomposer of wood and vegetal residues (Zadrazil & Kurtzman, 1981). Some species of *Pleurotus* are edible. They are naturally found in tropical and subtropical rainforest, and can be artificially cultivated. They are appreciated because of its delicious taste. This fungus has high quantities of proteins, carbohydrates, minerals (calcium, phosphorus, iron) and vitamins (thiamin, riboflavin and niacin) as well as low fat content (Bonatti *et al.*, 2004).

Pleurotus species have extensive enzyme systems capable of utilizing complex organic compounds, which occur as agricultural wastes and industrial by-products (Baysal *et al.*, 2003). Many wood decomposing fungi utilize lignocelluloses efficiently and this characteristic is due to their ability to metabolize lignin. *Pleurotus* species are found to be one of the most efficient lignocllulose decomposing types of white rot fungi. They require shorter growth time when compared to other mushrooms, they demand few environmental controls, their fruting bodies are not very often attacked by disease and pets and they can be cultivated in a simple and cheap way (Jwanny *et al.*, 1995). The nutrient compositions of two *Pleurotus* spp. fruiting bodies on different substrates are shown in Table 1.1.

1.4.1.1 *Pleurotus sajor-caju*

Pleurotus sajor-caju is one the most successfully cultivated specialty mushrooms and is now considered to be a delicacy. The different species of *Pleurotus* grow within a temperature range 15 to 30°C. *P. sajor-caju* can tolerate temperature up to 30°C although it fruits faster and produces larger mushroom at 25°C (Zadrazil & Kurtzman, 1981). Its cultivation on various lignocellulosic materials has been investigated by a number of researchers. *P. sajor-caju* has a very high saprophytic colonizing ability and can degrade wheat straw efficiently. It is known to produce 100% yields (100 kg fresh mushrooms per 100 kg dry straw) on unfermented pasteurizes rice straw (Shashirekha, 2002).

1.4.1.2 *Pleurotus nebrodensis*

Pleurotus nebrodensis is a rare edible mushroom that ubiquitously found in China, Xinjiang (Uygur Autonomous Region). It can also be named as White King oyster mushroom. The content of it includes 14.7% protein, 43.2% carbohydrate, 4.31% lipid and 15.4% dietary fiber (張噗安, 2000). The *P. nebrodensis* has healthy effect on immunity, such as inhibiting the HIV virus on attacking lymphocytes (柳曉嵐, 1998). The cultivated season starts in September and October and can be harvested at during December to February (王波等, 2003).

1.4.2 *Ganoderma lucidum*

Ganoderma lucidum is a type of fungus usually used in traditional Chinese medicine. Its fruiting body is called "Lingzhi" and contains various chemical substances, including more than 119 different triterpenes and several types of polysaccharides (Hsieh & Yang, 2004; Yang *et al.*, 2000) The polysaccharides of Lingzhi are the major source of its biological activity and therapeutic use. In China, Japan, Korea and Taiwan, *Ganoderma lucidum* has been a popular folk or oriental medicine used to treat various human diseases, such as hypertension, hypercholesterolemia, and gastric cancer (Mizuno *et al.* 1995). The nutrient compositions of *Ganoderma lucidum* fruiting body are shown in Table 1.1.

Recent studies on this fungus have demonstrated numerous biological activites, including antitumour and anti-inflammatory effects. Those studies also suggest that the carcinostatic substance in Lingzhi is a polysaccharide, 1, 3-β-D-glucan (Yang *et al.*, 2000). This polysaccharide seems to have promise as a new type of carcinostatic agent, which might be useful in immunotherapy (Mizuno *et al.*, 1995; Hsieh & Yang, 2004; Berovič *et al.*, 2003).

Because of its perceived healthy benefits, *Ganoderma lucidum* has gained wide popularity as a health food, in both Japan and China. The yield of wild *Ganoderma lucidum* is very low, and the price is high. Today, *Ganoderma lucidum* is being produced by artificial cultivation and fermentation.

1.5 Nutritional values of food and feed

1.5.1 Lipids

For animals, dietary fats and oils perform a variety of functions. Fats are the richest sources of energy on a weight basis and most adults eat up to 150 g of fat per day. Unfortunately an excessive energy intake results in fat being laid down as a "long-term" reserve with the concomitant increase in weight. Based on the FAO/WHO/UNU (1985) recommendation for energy intake and using a value of 0.9 kcal per g of digestible energy from fat, the dietary fat intake for 40 per cent of energy requirement are shown in Table 1.2.

As well as being a readily available source of high energy, fats and oils have essential food uses: they provide the dietary essential fatty acids which man can not synthesis; fats and oils are carriers for cell membranes. In cereals the oil droplets are concentrated in the subaleurone layers. In legumes, the fats located on the cotyledons. The fat content for a number of cereals and legumes is given in the Table 1.3.

1.5.2 Protein and amino acid

Proteins are fundamental components of all living cells. Protein is essential to:

1. Building body tissues during periods of growth and development and
2. Repairing and maintaining body tissues.

Proteins have numerous functions in the human body, including their vital role in muscle, hormones, enzymes, and various fluids and body secretions, and as antibodies in the immune system. Proteins also are essential to the transportation of lipids, fat-soluble vitamins, and minerals and contribute to homeostasis by maintaining a balance among body fluids (Stipanuk, 2000; Groff & Gropper, 2000).

Amino acids are the building blocks of protein. At least twenty of these amino acids commonly make up both plant and animal proteins. In the past, amino acids have typically been classified as essential or non-essential. However, it is becoming more common for the term "dispensable" to be used in place of non-essential and "indispensable" to be substituted for essential. By definition, indispensable amino acids are those that must be supplied by the diet because the body cannot synthesize

them in amounts sufficient to meet metabolic needs (Metges *et al.* 1999). Currently nine amino acids are classified as indispensable for all healthy human beings. The protein and indispensable amino acid required at different ages and the FAO/WHO requirement pattern (1985) of indispensable amino acids in different age groups are shown in Table 1.4.

1.5.3 Dietary fiber

Dietary fiber was recognized as an important food component in about the mid-1970s. Probably the most widely accepted definition for dietary fiber is that proposed by Trowell *et al.* (1976): "plant polysaccharides and lignin which are resistant to hydrolysis by digestive enzymes of man." Today, soluble and insoluble dietary fiber fractions may be isolated and analyzed (Manthey *et al.*, 1999). Such information will perhaps allow expansion of medical nutrition fiber therapy for the treatment and perhaps prevention of many conditions or diseases affecting humans.

Dietary fiber, in spite of the controversy about its definition, is derived from plant cells. The plant cell wall is of particular importance because it contains >95% of dietary fiber components including cellulose, hemicellilose, lignin, pectins, as well as some non-starch polysaccharides (Groff & Gropper, 2000).

Dietary fiber may be classified as water soluble fiber and water insoluble fiber. Water-soluble fiber in cereals is composed of nonstarchy polysaccharides, including some hemicellulose, pectin, gums, and mucilages. Water insoluble fiber is composed of nonstarchy polysaccharides, cellulose, lignin, and some hemicellulose (Manthey *et al.*, 1999).

There are several important physico-chemical properties of dietary fiber, such as increase viscosity by binding with water, increase bulk volume, binding of ions and bile acid (Wang *et al.*, 2001; Guillon & Champ, 2000). That can lower blood cholesterol concentration, and make people have longer satiety after intake of dietary fiber foods (Chu & Hanson, 2000)

1.5.4 Minerals

Of all foods, legumes can meet the recommended dietary guidelines for healthful eating most adequately; they are good source of vitamins and minerals (Cabrera *et al.*, 2003). Being a good source of minerals, legume seeds contain macro-elements such as calcium, magnesium, sodium, potassium and phosphorus as well as microelements such as copper, iron, manganese and zinc. Usually ash content is used for the determination of mineral content. Table 1.5 shows the ash content of different legumes before and after heat-treatments.

1.5.5 Carbohydrate

Carbohydrates are the major storage reserve of most seeds cultivated as food source. Starch is the carbohydrate most commonly found in seeds. It is stored in seeds in two forms, amylose and amylopectin. Most grains are composed of about 50-75% amylopectin and 20-25% amylose. Other carbohydrates that occur in non-storage forms are cellulose, pectins, and mucilages. Cellulose is a homopolymer of glucose. Hemicellulose is heteropolymer of a mixture of sugars. It is the major

form of stored carbohydrates in some seeds, particularly in certain endospermic legumes (Bewley & Black, 1994). Usually starch is absent from tissue where hemicelluloses are present in appreciable quantities. Free sugars are rarely the storage carbohydrate. Disaccharides (sucrose) and oligosaccharides (raffinose-series oligosaccharides) are commonly found as minor reserves in the embryo and reserve tissue. Lignin is the generic name for complex aromatic polymers. It gives rigidity to the plant cell wall. It is a three-dimensional aromatic polymer that surrounds the microfibrils, with some covalent attachment to the hemicellulose. It is composed of up to three monomeric units of cinnamyl alcohols (Bewley & Black, 1994; Gadd, 1994)

In general, natural fermentation of cereals leads to decrease in the level of non-digestible polysaccharides and oligosaccharides (Blandino *et al.*, 2003). Depolymerization of plant polysaccharide can also occurs through the production of extracellular enzymes of fungi. The fungi have two kinds of cellulolytic enzymes, endo-cellulases and ecto-cellulases, for cellulose digestion. Exoglucanases, can split glucose or cellobiose from the end of the polyglucose chain. Endoglucanases, can act randomly within the cellulose chain breaking chemical bonds and thus creating free ends on which the exo-glucanases act (Li *et. al.*, 2003).

1.5.6 Chitin

Chitin is the major structural component of the exoskeleton of invertebrates and the cell walls of fungi. The name 'chitin' is derived from Greek word 'chiton', meaning a coat of mail (Lower, 1984). It is the second most abundant biopolymer on the earth after cellulose and is a β(1→4)-linked glycan, but is composed of 2-acetamido-2-deoxy-β-D-glucose (N-acetylglucosamine), one of the most abundant polysaccharides named polyβ(1 → 4)-2-acetamido-2-deoxy-β-D-glucose (Fig.1.1) (Shahidi *et al.*, 1999; Kittur *et al.*, 2002; Ride & Drysdale, 1972). Chitosan is the name used for low acetyl substituted forms of chitin and is composed primarily of glucosamine, 2-amino-2-deoxy-β-D-glucose, known as (1 → 4)-2-amino-2-deoxy-β-D-glucose. Other then used as measurement of mold contamination and growth, chitin and chitosan also have antimicrobial activity and hypercholestemia effect (Koide *et al.*, 1998).

Separation of fungi from the tissues in which they grow is difficult and often impossible, the estimation of fungal biomass presents a number of problems (Sharma *et al.*, 1977). Attempts have been made to estimate the biomass of fungal mycelia in soil, based upon the determination of total hyphal lengths. However, this method is not suitable for fungal mycelia in tissue and agar plate. More recent models have attempted to include accessible parameters such as hyphal diameter and density, diffusion coefficients, yield coefficients, Monod kinetic constants, and so on (Ferret *et al.*, 1999). Chemical determination of chitin, a constituent of fungal cell walls, has an advantage in that it will reflect total mycelium based on chitin content (Whipps & Lewis, 1980; Bierstedt *et al.*, 1998; Chen & Johnson, 1983).

1.5.7 Phytic acid

Most raw seed products contain some antinutrional factors. These chemical substances, which although non-toxic, generate adverse physiological responses in animals consuming these seeds and, in many cases, interfere with the ultilization of nutrients in these products (Sindhu & Khetarpaul, 2001).

Phytic acid (PA) (myo-inositol hexakisphosphate) (Fig. 1.2) is classified as a kind of antinutrient. It is a natural plant compound with a unique structure that is responsible for its characteristic properties. Phytic acid has 12 replaceable protons, allowing it to complex with multivalent cations and positively charged proteins and thus can be found in many forms (Oatway *et al.*, 2001).

PA is found in most cereal grains, legumes, nuts, oilseeds, tubers, pollen, spores, and organic soils. It acts as the primary phosphorus reserve accounting for up to 85% of the total phosphorus in cereals and legumes. It is utilized during seed germination (Ayet *et al.*, 1997). It supports seedling growth by supplying biosynthetic needs of the growing tissues. Young seedlings use the end products of PA hydrolysis, particularly myo-inositol, for cell wall formation (Tsao *et al.*, 1997).

PA is generally considered as an antinutrient as it is a strong chelator of divalent minerals. Many studies indicate that phyates decrease minerial bioavailability by forming complexes with these minerals (Krebs, 2000). Many of the phytate mineral complexs are insoluble and, therefore, may become unavailable for absorption under normal physiological conditions. Because phyates are ionic in nature, they also react directly with charged groups of proteins or indirectly with the

negatively charged groups of proteins mediated by a positively charged mineral ion such as calcium. The resultant phytate-protein and phytate-minerial-protein complexes may also adversely influence protein digestion and bioavailability (Sathe & Reddy, 2002). However, some beneficial effects of phytic acid were recognized in recent years. Phytic acid has been shown to play a role as antioxidant (Graf & Eaton, 1990), inhibit of lipid peroxidation, prevent food products discoloration, increase nutritional quality, and prolong shelf life of foods (Oatway *et al.*, 2001). The PA may also have potential health benefits in management of blood glucose levels and cancer (Midorikawa *et al.*, 2001). Therefore the PA may act as chemopreventive agent. In fact, the studies of PA beneficial effects are still in developing stages. Therefore, in this research, the PA was still regarded as an antinutrient.

1.6 Protein digestibility

Digestibility is the first factor affecting the efficiency of diet proteins. When certain peptide links are not hydrolyzed in the digestive process, part of the protein is either excreted in the faeces, or transformed into a metabolic product by microorganisms present in the large intestine (Cruz, *et al.*, 2003).

Digestibility of raw seeds and products is usually very poor, improving with cooking, autoclaving, toasting or other forms of heat treatments. In general even well cooked seeds still have poorer protein digestibility than digestibility of animal protein. This is partly due to the presence of protease inhibitors (Osman, *et al.*, 2002). One of the major limitations of beans is the low nutritional value of their proteins, due to their poor digestibility and reduced content of sulphur amino acids (Nielsen,

1991; Genovese & Lajolo, 1996 & 1998).

1.7 Problem caused by fungal contamination

Mycotoxins have no biochemical significance in fungal growth and development (Moss, 1991), however, they have adversely affect human or animal health are found mainly in post-harvest crops such as cereal grains or forages (Janardhana *et al.*, 1999). Saprophytic fungi produce these toxins during storage or by endophytic fungi during plant growth. Mycotoxins are generally lipophilic and, therefore, they tend to accumulate in the fat fraction of plants and animals (Hussein & Brasel, 2001).

Examples of mycotoxins of greatest public health and agroeconomic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids (Hussein & Brasel, 2001). These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products (Shane, 1994).

Aflatoxins are the most studied (>5000 publications) group of mycotoxins and are produced by different species of the genus *Aspergillus*. Aflatoxins (Figure 1.3 – Figure 1.5) are dihydrofuran or tetrahydrafurano moieties fused to a coumarin ring (Erdogan, 2004).

Aflatoxins B_1 (Figure 1.3) is the most toxic in both acute and chronic aflatoxicoses. Naturally occurring mixtures of aflatoxin B_1 and other aflatoxins have been classified as group 1 human carcinogen (Bankole, 2004). These investigators illustrated the various relative potencies of different Aflatoxins and reported LD50 values of 0.36, 0.78, 1.70, and 3.44 mg/kg of ducklings consuming Aflatoxins B_1, Aflatoxins G_1, Aflatoxins B_2 or Aflatoxins G_2, respectively (Carnaghan *et al.*, 1963). The activated Aflatoxins B_1 metabolite (i.e. Aflatoxins B_1-8,9-epoxide) forms a covalent bond with the N7 of guanine (Lillehoj, 1991) and forms Aflatoxins B_1 -N7-guanine adducts in the target cells (Bailey, 1994). The results are G→T transversions, DNA repair, lesions, mutations, and subsequently tumor formation (Foster *et al.*, 1983). Table 1.6 shows the FDA's practical limits for aflatoxin in food and feeds.

1.8 Antioxidant

Lipid peroxidation plays an important role in the deterioration of foods during storage. Lipids and lipid-soluble substances that may be susceptible to oxidation are present in almost all foods. The problem of oxidation is one aspect of food preservation, especially when the oxidative products develop an unpleasant odour and taste. It is vital for food manufacturers to resolve the problem of lipid peroxidation in food. According to a very general definition, antioxidants are "substances capable of delaying, retarding or preventing oxidation processes" (Schuler, 1990). Antioxidant activity is a fundamental property important for life. Many of the biological functions, such as antimutagenity, anticarcinogenity and antiaging, among others, originate from this property (Cook & Samman, 1996).

Phenolic compounds were found in the mushrooms, which have antioxidant activity in the inhibition of low density lipoprotein (LDL) oxidation (Vinson *et al.*, 1995). For examples, the phenolic compounds such as variegatic acid and diboviquinone, which have been found in mushrooms (Kasuga *et al.*, 1995); they are excellent antioxidants.

The ingredients found in legumes and cereals have been shown to contain a number of components which are capable of inhibiting lipid oxidation including phytate, fiber and phenolics. Phytic acid inhibits lipid oxidation through its ability to chelate and inactivate prooxidant metals (Graf& Eaton, 1990). Polysaccharides including xanthan (Shimada *et al.*, 1997) and gum arabic have been reported to display antioxidant activity through unknown mechanisms. In addition, fibers from cereal flours have been reported to inhibit lipoxygenase-catalyzed oxidation of linoleic acid emulsons (Lehtinen *et al.*, 1997). Legumes and cereals also contain phenolic compounds, they are known to protect easily-oxidizable constituents of food from oxidation.

The intake of natural antioxidants has health promoting effects, it has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing (Cai *et al.*, 2004).

1.9 Research objectives

The objectives of thesis research are:

(1) To evaluate the change in nutritional values of selected cereals and legume seeds by means of bioconversion using three edible fungi.

(2) To assess the antioxidant activities of the bioconversion products obtained from solid-state fermentation of selected cereals and legume seeds substrates.

Table 1.1 Composition of *Pleurotus ostreatus, *Pleurotus sajor-caju** and *Ganoderma lucidum*** fruiting bodies, cultivated in rice straw, banana straw and unknown substrates.**

Component	Content (%)				
	Pleurotus ostreatus		*Pleurotus sajor-caju*		*Ganoderma lucidum*
	Banana straw	Rice straw	Banana straw	Rice straw	/
Total fat	5.97	6.32	5.26	4.99	3.85
Total carbohydrates	47.00	47.60	43.00	42.8	27.78
Ash	5.58	6.13	5.14	5.59	1.77
Total fibre	9.41	9.00	7.60	9.60	59.49
Total nitrogen	3.85	3.00	4.20	2.96	/
Total protein	16.90	13.10	18.40	13.00	7.18

*Bonatti *et al.* 2004

** Mau *et al.* 2001

Table 1.2 Estimated energy and fat requirements per day

Occupation	Work level	Energy requirement* (kcal)	Projected fat intake** (g)
Male office clerk (65 kg)	Light activity	2580	115
Subsistence farmer (58 kg)	Moderate activity	2780	124
Male heavy worker	Heavy activity	3490	155
Retired male (60 kg)	Very light activity	1960	87
Houswife (55 kg)	Light activity	1990	88
Female in developing country	Light activity	2235	99

*FAO/WHO/UNU 1995

**To provide 40 per cent of the required daily dietary energy (9 kcal $\equiv$ 1 g of dietary fat)

Table 1.3 The lipid content of cereals* and legumes**

Cereal	Lipid content (approximate %)
Oats	7.00
Oatmeal	6.20
Maize	4.50
Peral millet	5.40
Brown rice	2.30
Wheat rice	1.90
Barley	2.10
Sorghum	3.40
Moth bean	0.69
Black gram	0.58
Horse gram	0.45
Green gram	0.71
Haricot bean	0.90
Chick pea	4.16

*Robinson, 1987

**Bravo *et al.*,1999

Table 1.4 Protein and indispensable amino acid needs at different ages

Age group	Protein requirement (g / kg)	Indispensable amino acids (% of total)
Infant	1.8	43
Preschool child	1.2	32
School child	1	22
Adult	0.6	11

Data from FAO/WHO/UNU. Energy and protein requirements. Report of a joint FAO/WHO/UNU expert consultation. WHO Tech Report Ser 1985; 724.

Table 1.5 Ash content of different legumes before and after heat-treatments

	Raw seed	Heat treated
Canavalia ensiformis [a]	3.7 ± 0.1	3.6 ± 0.4
Mucuna pruriens [a]	5.3 ± 0.1	6.0 ± 0.8
Citrullus vulgaris [b]	3.3 ± 0.2	3.2 ± 0.2

a: Agbede & Aletor, 2004

b: Omafuvbe *et al.*, 2004

Table 1.6 FDA's practical limits for aflatoxin in food and feeds

Commodity	FDA tolerance Target
Corn and peanut products intended for finishing (i.e. feedlot) beef cattle	<300 ppb
Cottonseed meal intended as a feed ingredient for beef, cattle, swine or poultry (regardless of age or breeding status)	<300 ppb
Corn and peanut products and other animal feeds and feed ingredients (and cottonseed meal for dairy animals)intended for immature animals, or when the intended use is not known	<20 ppb
All feedstuffs other than corn such as: brazil nuts, foods, pistachio nuts, peanuts and peanut products	<20 pbb
Milk for the interstate milk shippers program	<0.5 ppb of AFM1

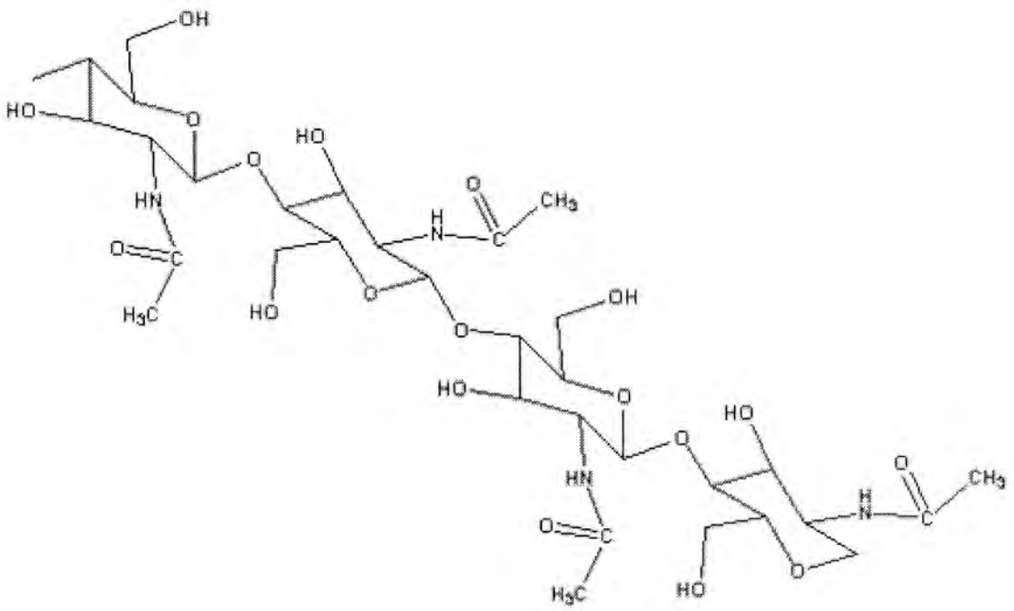


Figure. 1.1 Chemical structure of chitin

(Koide, 1998)

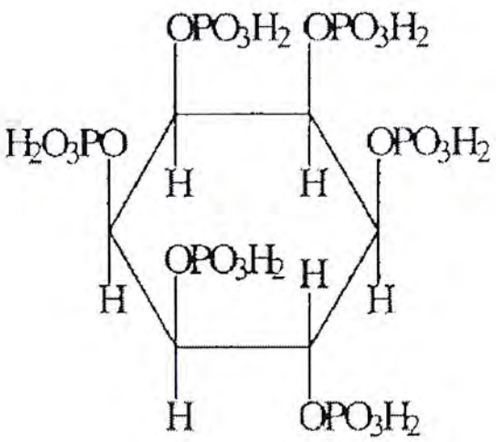
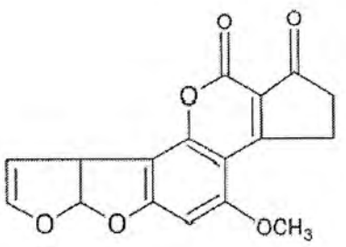
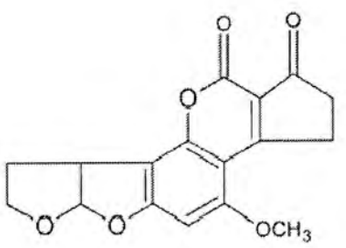


Figure 1.2 Basic structure of phytic acid

(Oatway *et al.*, 2001)

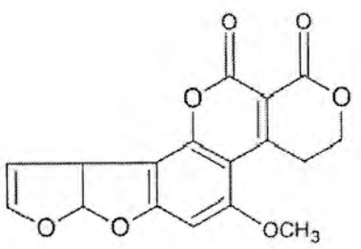


Aflatoxin B_1

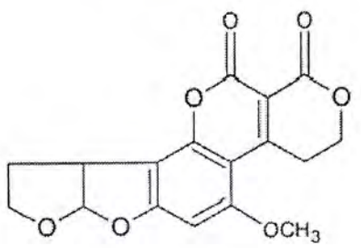


Aflatoxin B_2

Figure 1.3 Chemical structure of aflatoxin B (AFB_1 and AFB_2)
(Hussein & Brasel, 2001)

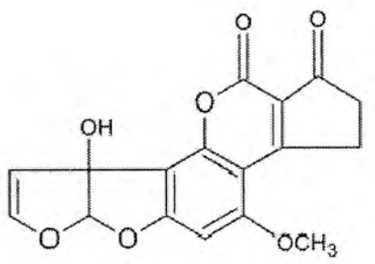


Aflatoxin G_1

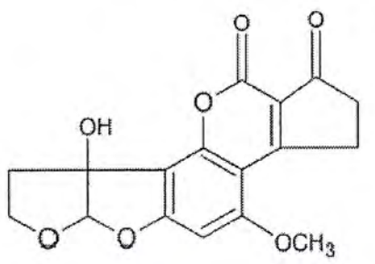


Aflatoxin G_2

Figure 1.4 Chemical structure of aflatoxin G (AFG_1 and AFG_2)
(Hussein & Brasel, 2001)



Aflatoxin M_1



Aflatoxin M_2

Figure 1.5 Chemical structure of aflatoxin M (AFM_1 and AFM_2)

(Hussein & Brasel, 2001)

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Plant Materials

The seeds from fifteen plant species were purchased from the local market. These include adzuki beans (*Vigna angularis*), barley (*Hordeum vulgare* L.), black soybean (*Glycine max* (L.) Merr.), buckwheat (*Fagopyrum esculentum*), chickpeas (*Cicer arietinum* L.), Hakmeitau bean (*Vigna sinensis*), lentils (*Lensculinaris esculenta*), millet (*Staria glauca* (L.) Beauv.), mung beans (*Vigna radiata* L.), navy beans (*Phaseolus vulgaris*), oats (*Avena sativa* L.), pinto beans (*Phaseolus vulgaris*), red kidney bean (*Phaseolus vulgaris*) soybean (*Glycine max* (L.) Merr.), split peas (*Lensculinaris esculenta*). The samples were manually screened for damage and defects.

2.1.2 Fungi Materials

Mycelia of *Pleurotus sajor-caju, Pleurotus nebrodensis and Ganoderma lucidum* were obtained form the Biology Department of the Chinese University of Hong Kong. The stock culture of the two *Pleurotus* species and the *Ganoderma lucidium* were grown for 10 days at 28°C on potato dextrose agar (PDA) and complete medium (Table 2.1), respectively. Sub-culturing was carried out weekly.

2.2 Sample Preparation

The fifteen types of seeds were milled into fine powders using a Sample Mill with screen size 0.5 mm (Cyclotec 1093, Tecator, Sweden). These powders were designated as raw seed powders.

For each plant species, fifteen grams of raw seed powder in Petri dishes was mixed with 25 ml distilled water and sterilized in 121°C for 20 minutes. These slurries were frozen by liquid nitrogen and freeze-dried in lyophilizer and then milled into fine powder. These samples were designated as Day-0 samples.

Solid-state fermentation was carried out in Petri dishes according to Yang *et al.* (2000) with modification. The Day-0 samples were inoculated with various fungal mycelia including *Pleurotus sajor-caju, Pleurotus nebrodensis and Ganoderma lucidum*. The Petri dishes were incubated at 28°C for 10 days. These samples were frozen by liquid nitrogen and freeze-dried in lyophilizer and then milled into fine powder as described above. They were designated as the fermented products.

All the raw seed powders, Day-0 samples and fermented product were subject to biochemical analysis and nutritional studies. They were stored in an air-tight container until used.

2.3 Fungal growth measurement

Pleurotus sajor-caju, Pleurotus nebrodensis and Ganoderma lucidum cultivated for 10 days separately. During the incubation period, discard any contaminated cultures were discarded. After 10 days, their diameters are measured and the growths of mycelia indifferent substrates are compared. After inoculation, the growth of fungal mycelia was monitored by measuring the diameter of the colony.

2.4 Proximate composition

2.4.1 Moisture determination

The moisture content was obtained by the moisture analyzer (Mettler LJ16, Greifensee, Switzerland). Two grams of sample powder, which was evenly distributed on an aluminium tray, were dried at 120°C. The moisture content of the sample was measured every 30 sec until three identical readings of moisture content were obtained consecutively. The last reading was accepted to be the moisture content (%) of the sample.

2.4.2 Ash determination

The ash content was determined according to the Official Methods of Analysis (4.1.10) (AOAC, 1995). Two grams of sample powder was weighed into a preweighed porcelain crucible and placed in a temperature-controlled furnace preheated to 600°C. This temperature was maintained for 4 hours. After ashing, the crucible was cooled in a desiccator, and the amount of ash in the sample was determined gravimetrically.

2.4.3 Crude lipid determination

Five grams of the sample powder was placed inside a fat-free thimble, and covered by some preweighed defatted cotton wool. The loaded thimble was then placed in a Soxhlet apparatus (Teactor, Hoganas, Sweden) preheated to 140°C. A preweighed aluminium cup, which contained up to two-third of hexane, was placed in the apparatus as well. During the lipid extraction, the thimble was soaked in the hexane at 140°C for 1hour. Next the thimble was kept at a position above the hexane and rinsed by condensed hexane for another hour in order to extract as much lipid as possible. After direct desolventization in the apparatus, the dried aluminium cup containing the residual oil was further dried in an oven (80°C) overnight. After cooling in a desiccator, the amount of residual oil (crude lipid) in the cup was determined gravimetrically.

2.4.4 Dietary fiber content determination

2.4.4.1 Insoluble dietary fiber (IDF)

The IDF was prepared according to the Official Methods of Analysis (32.1.17) (AOAC, 1995) with some modification. One gram of sample powder was added in 40 ml 0.05 M Mes-Tris buffer (pH8.2) followed by the addition of 50 µl heat stable α-amylase (Sigma, A3306) and the mixture was incubated at 95-100°C for 30min. After cooling to 60°C, 100 µl protease (Sigma, P3910) was added to the mixture and incubated at 60°C for 30 min with continuous agitation. After that, 5ml 0.561 N hydrochloric acid was dispensed into the mixture, and the pH of the mixture was adjusted to 4.0 – 4.7 at 60°C by using 1 N sodium hydroxide or 1 N hydrochloric acid. The mixture was then added with 300µl amyloglucosidase (Sigma, A9913), and incubated at 60°C for 30 min with continuous agitation. The enzyme digestate was centrifuged at 3000g for 20 min. The supernatant was used for preparation of soluble dietary fiber elsewhere. The residue (containing IDF) was washed with 10ml water (70°C) twice, 40 ml 95% ethanol, and 40ml acetone, and further dried in oven at 80°C. The IDF content in the sample could be obtained after correcting the weight of the dried residue for its protein and ash.

IDF = (weight of residue) – (weight protein + weight of ash)

2.4.4.2 Soluble dietary fiber (SDF)

The SDF was determined according to the Official Methods of Analysis (32.2.17) (AOAC, 1995) with some modification. The supernatant obtained from 2.4.4.1 was combined with the portion of aqueous washings which was obtained during rinsing the residue. The combined solution (containing SDF) was mixed with 4 volumes of 95% ethanol, and was kept at RT (24°C) for 1h to precipitate the SDF. After centrifuged at 3000g for 20 min, the pellet was washed with 40 ml 95% ethanol and 40 ml acetone, and then dried in oven at 80°C. The SDF content in the sample was obtained after the weight of the dried pellet was corrected for its protein and ash.

SDF = (weight of residue) – (weight protein + weight of ash)

2.4.4.3 Total dietary fiber (TDF)

The total dietary fiber was the sum of the IDF and SDF.

2.4.5 Crude protein determination

Two to three mg of sample powder was accurately weighed into an aluminium capsule, which then compressed into a dice by using 2 forceps. The compressed aluminium dice was transferred into the auto-sampler of CHNS/O Elemental Analyzer (Perkin Elmer 2400, Connecticut, USA) to determine the nitrogen content of the sample at 970°C. The crude protein was calculated by multiplying the nitrogen content by a factor of 6.25.

2.4.6 Carbohydrate determination

The content of carbohydrate (%) in the sample was calculated by subtracting the percentage of crude lipid, crude protein, total dietary fiber, and ash from one hundred percent.

2.4.7 Glucose determination

Glucose was determined using the Total Glucose Kit (Sigma, 510DA), 0.2 g sample powder was extracted in 1.8 ml distilled water. 1 ml of Barium hydroxide (Sigma, 14-3) and Zinc sulfate solution (Sigma, 14-4) each were added to the extract respectively. The mixture was mixed and centrifuged at 4000g for 10 min. Five ml enzyme-color regent* was added to 0.5 ml supernatant, and the mixture was incubated at 37°C for 30 min. Following the same procedure, a blank and a standard were prepared by adding 0.2 ml distilled water and glucose standard solution (Sigma, 635-100) respectively. The absorbance of the sample and standard solution were measured at 450nm against the blank. The glucose concentration was calculated as follows:

Glucose content (mg/dL) = $(A_{sample}/A_{standard})$ x 100

Where A: absorbance

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* Enzyme-color reagent: It was prepared by dissolving 1 capsule of PGO enzyme (Sigma, 510-6) in 100ml distilled water and adding 1.6 ml of the color reagent solution o-Dianisidine Dihydrochloride (Sigma, 510-50) to it.

2.4.8 Chitin

The content of chitin in the sample was determined by the method of Lena (1993). Five hundred mg of sample powder was hydrolysed in 20 ml of 1 N sodium hydroxide. The mixture was boiled at 120°C for 15 min. After cooling, the pellet was collected by centrifugation at 4000g for 30 min. The pellet was then washed with 20 ml of distilled water by centrifugation at 4000g for 30 min, until the supernatant become colorless. The supernatant was then measured at 260 nm and 280 nm to ensure that the reading was zero. The pellet was then demineralized using 5 ml 2N hydrochloric acid. The reaction mixture was heated at 95°C overnight and the supernatant was collected by centrifugation. Excess amount of 12 N sodium hydroxide was added to the supernatant in a preweighed centrifuge bottle and the precipitate formed was collected by centrifugation at 4000 g for 30 min. The pellet with centrifuge bottle was dried in 105°C oven for 24 h. The percentage of chitin content of the sample was calculated according to the following equation:

Chitin content (%) = (Wf - Wi) / Ws x 100%

Where Wf = dry weight of chitin (g)

Wi = dry weight of centrifuge bottle (g) and

Ws = dry weight of sample (g)

2.4.9 Phytic acid determination

Phytic acid was determined according to the method of Haug and Lantzsch (1983), two grams of sample powder was stirred with 50 ml 0.2 N hydrochloric acid for 3 h, and was centrifuged at 3000g for 20 min. Five hundred µl supernatant and 1 ml ferric solution* were added into a screw-cap test tube, and were heated in a boiling water bath for 30 min. After cooling to RT (24°C) under running tap water, 2 ml 2', 2' – bipyridine solution** was added into the cooled solution, and the mixture was then measured at 519 nm against distilled water. The content of phytates in the sample was calculated from a standard calibration curve, which was made with a series of phytate standard solution***.

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*Ferric solution: It was prepared by adding 0.2 g ammonium iron (III) sulphate dodecahydrate in 100ml 2 N hydrochloric acid, and making up to 1L with distilled water.

**2', 2' – dipyridine solution: It was prepared by dissolving 10 g 2', 2 – bipyridine (Sigma, D7505) and 10 ml thioglycollic acid (Sigma, 55179) in distilled water, and making up to 1 L with distilled water.

***Phytate standard solution: A stock solution of phytate was prepared by dissolving 0.15 g phytic acid dodecasodium salt (Sigma, P8810) in 100ml distilled water.

2.5 *In vitro* protein digestibility

The *in vitro* protein digestibility (IVPD) was determined by the multi-enzyme method of Hsu *et al.* (1977). Sample powder was mixed with distilled water to give a suspension (each ml containing 6.25mg protein). The aqueous suspension was adjusted to pH 8.0 with 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, and incubated at 37°C. Fifty ml of suspension and 5ml of an enzyme mixture* were mixed, and incubated at 37°C exactly for 10 min with continuous stirring. The pH change of the mixture after 10 min was recorded, and used to calculate the percent of IVPD (Y) using the equation as shown below:

$Y = 210.464 - 18.10X$ (where X is the pH change after 10 min)

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* Enzyme mixture: It was prepared by dissolving enzymes in distilled water, adjusted to pH 8.0 with 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, and maintained in an ice bath. Each ml enzyme solution contained 1.6 mg porcine pancreatic trypsin (Sigma, T4665); 3.1 mg bovine pancreatic chymotrypsin (Sigma, C4129), and 1.3 mg porcine peptidase (Sigma, P7500).

2.6 Aflatoxin determination

The aflatoxin content of the sample can be measured by using RIDASCREEN® Aflatoxin Total Kit (R-Biopharm, R4701). The principle of the test was based on the antigen-antibody reaction. The wells in the microtiter strips were coated with capture antibodies directed against anti-aflatoxin antibodies. Aflatoxin standards or the sample solutions, aflatoxin-enzyme conjugates and anti-aflatoxin antibodies were added to the wells. Free and enzyme conjugated aflatoxin competed for the aflatoxin antibody binding sites (competitive enzyme immunoassay). At the same time, the capture antibodies also bound the aflatoxin-antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow.

To measure the aflatoxin content in the samples, 0.2 g of sample powder was extracted in 1 ml 70% methanol at RT for 10 min with continuous agitation. The supernatant was obtained by centrifugation at 4000g for 20 min. One hundred µl of the filtrate was diluted with 600 µl of dilution buffer. Fifty µl of aflatoxin standard solutions or prepared sample were added to separate wells on the microtiter plate (coated with capture antibodies). Fifty µl of diluted enzyme conjugate and diluted antibody solution were added to each well respectively. The microtiter plate was incubated for 30 min at room temperature in dark. The liquid was poured out of the wells and the plate was tapped upside down vigorously against absorbent paper to ensure complete removal of liquid from the wells. The wells washed with 250 µl

distilled water and this washing procedure was repeated two times. Fifty µl of substrate and chromgen were added to each well respectively. The microtiter plate was incubated for 30 min at room temperature in dark. One hundred µl of the stop solution was added to each well with gently mixing and measured the absorbance at 450 nm against air blank. A series of aflatoxin standard solution* was used to plot a calibration curve. The content of aflatoxin in the sample was calculated from a standard calibration curve by using the % absorbance of the samples. The percentage of aflatoxin content of the sample was calculated according to the following equation:

$(A_{standard}$ or $A_{sample} / A_{zero\ standard}$*) x 100 = % absorbance

Where A: absorbance

---

* Six aflatoxin standard concentrates: 0 ppb (zero standard), 0.5 ppb, 1.5 ppb, 4.5 ppb, 13.5 ppb, 40.5 ppb aflatoxin B_1 in methanol.

* $A_{zero\ standard}$: stand for the absorbance of aflatoxin standard concentrate with 0 ppb.

2.7 Antioxidant activity assay

2.7.1 Ferric reducing antioxidant powder (FRAP) assay

The total antioxidant ability measured by FRAP assay was according to Benzie& Strain (1996). The reduction of ferric tripyridyltriazine (Fe^{III}- TPTZ) complex to its ferrous form, which has an intense blue color can be monitored by measuring the change in absorption at 593 nm.

Freshly prepared FRAP reagent* was warmed to 37°C. Weigh 0.5 g of sample powder and extracted with 1 ml absolute methanol for 24 h. The supernatant of the extract was obtained by centrifugation at 4500 g for 15 min. Forty μl of sample was added to 1.2 ml FRAP reagent. The mixture was then measured at 593 nm against FRAP reagent blank. Absorbance reading was taken every minute. In this study the reaction was monitored for up to 8 min, but the 4-min reading were selected for calculation. Absorbance change was translated into FRAP value (in μM) by relating the ΔA_{593nm} of test sample to that of a standard solution** of known FRAP value.

ΔA_{593nm} (0 to 4 min) sample /ΔA_{593nm} (0 to 4 min) standard × FRAP value of standard

---

*FRAP reagent was prepared by mixing 25 ml acetate buffer (300 mmol/L), 25 ml TPTZ solution (10 mmol/L) (Sigma, T1253), and 2.5 ml $FeCl_3.6H_2O$ solution (20 mmol/L) (RDH, 12321).

**Aqueous solution of known Fe (II) concentration ($FeSO_47H_2O$) and ascorbic acid (Sigma, A2708) in the range of 100-1000 μmol/liter were used for calibration.

2.7.2 Trolox equivalent antioxidant capacity (TEAC) assay

The free radical scavenging abilities of the sample was assessed by the method of Miller *et al.* (1996). This technique was used to measure the relative ability of antioxidant substances by comparing with standard amounts of the synthetic antioxidant Trolox (6-hydroxy- 2,5,7,8-tetramethyl-chroman -2-carboxylic acid), a water-soluble vitamin E analogue.

$ABTS^{\cdot+}$ radical cations were prepared by passing a 5 mM aqueous stock solution of ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline- 6-sulphonic acid diammonium salt) (Sigma, A1888) through manganese dioxide (MnO_2) with Whatman filter papers (No. 1). Excess MnO_2 was removed from the filtrate by passing it through a 0.2μM Whatman PVDF syringe filter. This solution was then diluted in a 5 mM phosphate buffered saline (PBS) at pH 7.4 to an absorbance of 0.7 (± 0.02) at 734 nm and pre-incubated at 30°C prior to use. Fresh $ABTS^{\cdot+}$ radical cation solution was prepared each day. A 2.5 mM Trolox stock solution (Fluka, 56510) was prepared in PBS. Ultrasonication was required to dissolve the crystals of Trolox. Fresh solutions were prepared daily by diluting 2.5 mM Trolox with PBS.

As described in 2.7.1, 0.5 g of sample powder was extracted with 1 ml absolute methanol for 24 h. The supernatant of the extract was obtained by centrifugation at 4500 g for 15 min. After addition of 50μl Trolox or sample to aliquots of 1ml of $ABTS^{\cdot+}$ solution, each solution was vortexed for exactly 30s. The absorbance was taken at 734 nm at exactly 1min after the initiation of mixing. Decrease in absorbance at 734 nm was recorded at an interval of 30s for up to 6min at 30°C. PBS blanks and methanol blanks were run in each assay. The dose-response

curve for Trolox was obtained by plotting the absorbance at 734 nm as a percentage of the absorbance of the remaining $ABTS^{\cdot+}$ solution against concentration. The activities of sample were assayed at five concentrations which had been determined to be within the range of the dose-response curve. Using the Trolox dose-response curve, the mean Trolox equivalent antioxidant capacity (TEAC) value was derived for each tested compound.

2.8 Statistical analysis

Data were presented as mean ± SD, using one-way analysis of variance (ANOVA) with Microsoft® Excel made analyses. Difference with $P < 0.05$ were considered statistical significant. Multiple comparisons between means were done with t-Turkey test using SPSS software.

Table 2.1 Composition of Complete Medium for *Ganoderma lucidum* (1L).

Composition	**Weight/g**
Magnesium sulfate-7-hydrate	1.02
Potassium dihydrogen orthophosphate	0.46
di-potassium hydrogen orthophosphate	1
Peptone	2
Glucose	20
Agar	20
Yeast Extract	2
Thiamin/HCL	0.005

Chapter Three: Results

3.1 Mycelia growth

3.1.1 Growth diameter

The growth of mycelia on different substrates a period of 10 days is shown in Table. 3.1. The three fungal mycelia (*Pleurotus sajor-caju*, *Pleurotus nebrodensis* and *Ganoderma lucidum*) grew on all fifteen kinds of seed substrates. However, different seed substrates supported fungal growth differently. Different fungal species had different growth rates on the same substrate.

Based on the growth diameter all these fungal species grew best on substrates prepared from mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and read kidney bean. On the other hand, lentil and navy bean were the most inferior substrates for mycelia growth. The colony diameters on these two substrates were about 3 cm and 5 cm respectively. Overall, mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and read kidney bean were the best substrates for the growth of fungal mycelia. Adzuki bean, barley, black soybean, chickpea, millet, soybean and split bean were average. Lentil and navy bean were comparatively worse substrates.

After statistical analysis, in black soybean, mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and read kidney bean, it could not draw any conclusion on which fungal species grew better than the other two species. For the cultivation of *G. lucidum*, the mycelial growth rate of Adzuki bean, barley, millet, navy bean, soybean and split bean were significantly with lower than the both *Pleurotus* spp. On the other hand, the *P. sajor-caju* was significant growth best with the substrates barley, millet, navy bean, split bean and chickpea. Moreover, none of the *P. nedrodensis* fermented product had significantly highest or lowest growth. For examples, there was no difference of both *Pleurotus* spp growth on Adzuki bean (6.0 cm and 6.5 cm). For barley and chickpea, it was also no significant different between *P. nedrodensis* and *G. lucidium* (barley: 7.1 cm and 7.0 cm; chickpea: 6.2 cm and 6.1 cm). Therefore, it can be said that the growth ability of *P. sajor-caju* amongst the three fungal species was the best, *P. nebrodensis* was second, and *G. lucidium* was the worst.

3.1.2 Chitin

Chitin was another parameter that was used for measurement of mycelia growth. The chitin content of all fermented products are shown in Table 3.2. The results were very similar to that of the growth diameter. In general, the chitin amount of *P. sajor-caju* fermented products was the highest, *P. nebrodensis* was the second and *G. lucidum* was the least. This general trend was found in almost all 15 kinds of cereal and legume substrates, except for buckwheat fermented products where there was no significant difference between *P. nebrodensis* and *G. lucidum*.

Regardless of the fungal species, the fermented products of mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and red kidney had the highest chitin content. In *P. sajor-caju*-grown substrates, the order of top six chitin contents was Hakmeitau bean (9.86 %), red kidney bean (8.22 %), buckwheat (7.65 %), oat (6.86 %), mung bean (6.36 %) and pinto bean (5.70 %). For *P. nebrodensis*, the fermented products of red kidney bean (7.64 %) and Hakmeitau bean (7.51 %) had higher chitin content than that of buckwheat (5.78 %), mung bean (5.63 %), oat (5.56 %) and Pinto bean (4.64 %). For *G. lucidum*-grown substrates, there was no significant difference amongst these six fermented products, but all of them were higher in chitin content than the rest of fermented products. All fermented products of lentil and navy bean had lowest amount of chitin. In the *G. lucidum*-grown substrate of lentil-fermented products, the chitin content was the lowest (0.84 %).

From the results of 3.1.1 and 3.1.2, mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and read kidney bean were found to be the best substrates for the growth of *Pleurotus sajor-caju*, *Pleurotus nebrodensis* and *Ganoderma lucidum*. Therefore they were selected for further bioconversion studies.

3.2 Weigh loss in sample preparation

The six selected samples were frozen by liquid nitrogen and freeze-dried in lyophilizer and then milled into fine powder. For the following bioconversion studies, all the samples (excluding raw seed powders) were weighed as freeze-dried weight. In general, the weight of the freeze-dried samples was around 12 g, which was 2 g less than the unfermented substrates.

3.3 Proximate composition

3.3.1 Moisture content

For the moisture content (Table. 3.3), there was no significant difference between all the fermented products of the three fungi and the Day-0 samples. However, in raw seed powder, buckwheat (13.87 %) had highest moisture content, followed by pinto bean (13.03 %), red kidney bean (11.73 %), oat (11.57 %), mung bean (10.80 %) and Hakmeitau bean (10.40 %). In most cases, the raw seed powder also had higher moisture content than the corresponding fermented product and Day-0 samples.

3.3.2 Ash

The ash content is an indication of the mineral content. As shown in Table 3.4, the ash content of cereals were lower than the legumes. The buckwheat had the lowest ash content. Its ash content was 1.34 % in raw seed powder, 1.32 % in Day-0, 1.85 % in *P. sajor-caju*, 1.70 % in *P. nebrodensis* and 1.81 % in *G. lucidum* fermented products, and oat had the second lowest ash content, in raw seed powder (1.91 %), Day-0 (1.90 %), *P. nebrodensis* (1.92 %)and *G. lucidum* (2.10 %) fermented products. On the other hand, the ash content of pinto bean and red kidney bean were highest among *P. sajor-caju* (pinto: 4.02 %; kidney: 3.92 %), *P. nebrodensis* (pinto: 4.17 %; kidney: 4.12 %) and *G. lucidum* (pinto: 4.04 %; kidney: 3.80 %) fermented products.

In buckwheat, Hakmeitau bean, pinto bean and red kidney bean, all the fungi fermented products had significantly higher ash content then the raw seed powder and Day-0. In addition, there was no significant difference in the ash content between the *P. sajor-caju*, *P. nebrodensis* and *G. lucidum* fermented products when they grew on different substrates. Moreover, in all six selected cereals and legumes, there was no significant difference between the raw seed powder and the Day-0 sample.

3.3.3 Crude lipid

As shown in Table 3.5, the lipid contents of oat sample including raw seed powder (5.50 %), Day-0 (5.23 %), *P. nebrodensis* (5.92 %) and *G. lucidum* fermented products (6.51 %) were the highest among the six selected substrates. The lipid content of most samples of mung bean and red kidney bean were the lowest (<1 %).

In the cases of oat, buckwheat, Hakmaitau bean and pinto bean, the raw seed powder and Day-0 sample had very similar lipid contents. In the cases of mung bean and red kidney bean, the lipid content of raw seed powder was reduced after autoclaving. This heat-included reduction in lipid content was not observed in other seed substrates. After fermentation, it was found that the lipid content of *P. sajor-caju*-fermented products of mung bean, oat, Hakmeitau bean and red kidney bean were significantly lower than that of *P. nebrodensi*-fermented products and *G. lucidum*-fermented products.

3.3.4 Dietary fiber

3.3.4.1 Insoluble dietary fiber (IDF)

The IDF contents of all samples varied form 5.68 % to 19.39 % (Table 3.6). In general, cereals had lower IDF content than legumes. None of the oat or buckwheat fermented products had more than 10 % of IDF. In legume seed substrates, the IDF was reduced as a result of solid-state fermentation. In contrast, the buckwheat fermentation with *P. sajor-caju* and oat fermentation with *G. lucidum*, they had increased IDF after fermentation.

3.3.4.2 Soluble dietary fiber (SDF)

Table 3.6 and 3.7 show that in all the six substrates studies, the SDF contents were much lower than IDF content. *Pleurotus nebrodensis* fermented pinto bean product had 7.68 % of SDF, which was the highest among all the samples. However, it was lower than the IDF of mung bean raw seed powder 2.5 folds.

The effects of bioconversion on the change in the SDF are as follows:

(1) *P. nebrodensis* fermentation increased SDF in oat and pinto bean.

(2) *G. lucidum* fermentation increased SDF in Hakmeitau bean.

(3) *P. sajor-caju* and *P. nebrodensis* fermentations increased SDF in red kidney bean.

3.3.4.3 Total dietary fiber

Total dietary fiber composes of IDF and SDF. The total dietary fiber content was calculated as the sum of IDF and SDF. As shown in Table 3.8, among the six selected substrates, mung bean had the highest content of total dietary fiber content (25.99 %), whereas buckwheat had the lowest content (12.58 %). In general, the total dietary fiber contents of substrates were reduced during the course of solid-state fermentation.

3.3.5 Crude protein

Table 3.9 shows the protein contents of various substrates before and after solid-state fermentation with the three fungal mycelia. In general, cereal substrates had lower protein content than beans. Autoclaving had no effect on the protein content of a substrate. After fermentation for 10 days, the protein contents of some products increased. This was obvious in substrates fermented with *P. sajor-caju*. Figure 3.1 shows the percentage increase in protein content of various substrates inoculated with different fungal mycelia. All *P. sajor-caju*-fermented products showed an increase in protein content, ranging from 28% in Hakmeitau bean to 139% in oat. On the other hand, most of the *P. nebrodensis* fermented products showed no difference from the unfermented substrate. Only buckwheat (6.41 %) and red kidney bean (20.93 %) fermentation had protein enrichment. In *G. lucidum*-fermented products of buckwheat (8.83 %), Hakmeitau bean (15.81 %), pinto bean (79.13 %) and read kidney bean (24.44 %), protein enrichment was observed.

3.3.6 Carbohydrate content

In most cases, no significant change in carbohydrate of the substrates of the Day-0 and fermented substrates after fermentation for 10 days was observed. (Table 3.10)

3.3.7 Glucose content

As shown in Table 3.11, the glucose content of all substrate were low, i.e. 0.67 mg/g in mung bean, 1.05 mg/g in buckwheat, 0.22 mg/g in oat, 2.48 mg/g in Hakmeitau bean, 1.13 mg/g in pinto bean and 0.82 mg/g in red kidney bean.

Autoclaving resulted in an increase of glucose content in the substrates. The glucose content of autoclaved Day-0 samples was 3.49 mg/g in mung bean, 5.74 mg/g in buckwheat, 1.00 mg/g in oat, 4.70 mg/g in Hakmeitau bean, 1.95 mg/g in pinto bean and 2.01 mg/g in red kidney bean. Moreover, the heating effect was more obvious in oat and buckwheat substrates. The glucose content of all fermented products were higher than that of the corresponding non-fermented substrates.

3.3.8 Phytic acid (PA)

A phytic acid calibration curve Fig. 3.2 was used to determinate the PA content of the samples. Table 3.12 shows that the phytic acid contents of different

substrates and their fermented products. Among the six seed substrates, oat contained the most PA (0.43%), whereas Hakmeitau bean contained the least (0.14%).

There was no difference in the PA content between raw seed powders and Day-0 samples, indicating that this compound was heat stable as the Day-0 samples had subject to autoclaving. In general, solid-state fermentation by all three mushroom mycelia reduced the PA content in all substrates examined. As shown in Fig. 3.3, among the 3 fungal species, the *P. nebrodensis* had the lowest ability to reduce phytic acid. The reduction was only observed in the mung bean (29.89 %) and Hakmeitau bean (11.76 %).

On the other hand, with *P. sajor-caju* and *G. lucidum* were able to degrade phytic acid present in the seed substrates effectively. These two organisms could remove over 70 % of PA in mung bean and Hakmeitau bean during the 10-day fermentation period (Fig. 3.3)

3.4 *In vitro* protein digestibility (IVPD)

As shown in Table 3.13, red kidney bean had the highest IVPD irrespective nof original substrates (raw seed powder: 79.42 %; Day-0: 79.24 %) or fermented products (*P. sajor-caju*: 81.02 %; *P. nebrodensis*: 80.96 %; *G. lucidum*: 85.06 %). Among all cereals and legume seeds examined, buckwheat had the lowest IVPD. Autoclaving did not have any effect on the IVPD of all substrates except Hakmeitau bean. The IVPD of Hakmeitau bean changed from 76.46 % in raw seed powder to 78.46 % in autoclaved sample when subject to autoclaving.

In general, solid-state fermentation could enhance the IVPD of all substrates studied. Results show that *G. lucidum* was most effective in enhancing the IVPD of the substrates. The change in IVPD of *G. lucidum*-fermnted products ranged from 0.8% in mung bean to 15.6% in Pinto bean.

3.5 Aflatoxin

Table 3.14 shown that four of the six selected seed substrates were contaminated with aflatoxin. The detectable amounts of aflatoxin were 5.92 ppt in oat, 31.49 ppt in buckwheat, 48.99 ppt in mung bean and 25.83 ppt in red kidney bean. For the unfermented substrates and fermented products, no mycotoxin was detected in Hakmeitau bean and pinto bean. The contaminated aflatoxin was heat-stable, as its amount did not change after autoclaving. In most cases, the aflatoxin contents of the fermented products remain unchanged after fermentation with all three mushroom mycelia. It seems that no any aflatoxin generated during the fermentation process.

3.6 Antioxidant ability

3.6.1 Ferric reducing antioxidant power (FRAP) assay

Figure 3.6 to Figure 3.9 showed the FRAP values of the three fungal mycelia, each substrate and its fermented products in different concentrations. The relative FRAP activities of different substrates & fermented products to the antioxidant

standard $FeSO_4 7H_2O$ are shown in Table 3.15 and Table 3.16.

In general, the antioxidant activity of Hakmeitau bean and its fermented products was higher than other samples. For raw seed powder of Hakmeitau bean, its antioxidant powder was 1.507 times higher than that of the standard $FeSO_4 7H_2O$. It was the highest antioxidant activity among all the samples. In contrast, the unfermented substrates and fermented products of oat and mung bean had the lowest antioxidant activity than the others. Especially for the fermented products with *P. sajor-caju* and *P. nebrodensis*, the antioxidant activity was only 10-30 % as the $FeSO_4 7H_2O$.

In all six kinds of cereals and legumes substrates, there was a trend of decrease in antioxidant activities. The raw seed powder had highest antioxidant powder, and then decreased in Day-0, and the Day-10 samples had the lowest antioxidant activities. As the bioconversion of the seed substrates by the fungi mycelia was lasted for 10 days. Therefore the comparison between Day-10 and fermented products were carried out. Excluding the fermented products of *P. sajor-caju* with oat, Hakmeitau bean and *P. nebrodensis* with oat, all others products had higher antioxidant activities than the Day-10 unfermented products. In addition, the fermented products of *G. lucidum* fermented with mung bean, buckwheat, oat and red kidney bean had higher antioxidant activities than the other two *Pleurotus* fermented products. However, this trend was not found in the fungal mycelia extracts. The mycelia of *G. lucidum* did not have the highest antioxidant activities. It was only higher than *P. nebrodensis* but similar to *P. sajor-caju.*

When compared with the fermented products and the fungal mycelia, all the fermented products with mung bean, and the oat substrates fermented with *P. nebrodensis* and *G. lucidum* which had lower antioxidant activities than that of the mycelia extracts. For the rest of the fermented products, all of them had higher antioxidant ability than the mycelia extracts.

3.6.2 Trolox equivalent antioxidant capacity (TEAC) assay

The results of Trolox equivalent antioxidant capacity (TEAC) assay (Figure 3.11) were quite similar to that of the Ferric reducing antioxidant powder (FRAP) assay. Among all the samples, Hakmeitau bean had the highest TEAC value in Day-0 (0.0084) and Day-10 (0.0043). Oat had the lowest one in Day-0 (0.0006) and Day-10 (0.0008). In Hakmeitau bean and red kidney bean, the TEAC values of Day-0 autoclaved substrates were higher than the Day-10 unfermented substrate. On contrast, different from FRAP assay, only the products of *G. lucidum* fermented with red kidney bean had significantly higher TEAC value than Day-10.

Among the three fungal mycelia extracts, the mycelia of *G. lucidum* had the highest TEAC value (0.0102), while the *P. nebrodensis* had the lowest TEAC value (0.0006). For the *G. lucidum*, its fermented products with mung bean (0.0036), buckwheat (0.0037), Hakmeitau bean (0.0033) and red kidney bean (0.0027) had higher TEAC values than that of the other two fungal species.

When compared with the fermented products and the fungal mycelia, all the fermented products had lower TEAC than the mycelia extract of *G. lucidum*. By contrast, the mycelia extract of *Pleurotus nebrodensis* had lower TEAC values than the *P. nebrodensis* fermented products.

Table 3.1 The growth of fungal species on various substrates.

Substrate	Diameter of fungal colony (cm)		
	Pleurotus sajor-caju	***Pleurotus nebrodensis***	***Ganoderma lucidum***
Adzuki bean	6.0 ± 0.8^{b}	6.5 ± 1.3^{d}	4.7 ± 1.0^{c}
Barley	7.9 ± 0.8^{e}	7.1 ± 1.0^{e}	7.0 ± 0.3^{f}
Black soybean	6.0 ± 0.3^{b}	6.0 ± 1.0^{c}	5.7 ± 0.5^{d}
Buckwheat	8.3 ± 0.0^{f}	8.3 ± 0.0^{f}	8.2 ± 0.3^{g}
Chick pea	6.9 ± 0.5^{d}	6.2 ± 0.6^{cd}	6.1 ± 0.3^{e}
Lentil	3.1 ± 0.8^{a}	3.0 ± 0.3^{a}	2.8 ± 0.6^{a}
Millet	7.6 ± 0.5^{e}	7.1 ± 0.8^{e}	6.7 ± 0.8^{f}
Mung bean	8.3 ± 0.0^{f}	8.3 ± 0.0^{f}	8.3 ± 0.3^{g}
Navy bean	6.4 ± 0.9^{c}	4.5 ± 0.5^{b}	4.1 ± 0.3^{b}
Oat	8.3 ± 0.0^{f}	8.3 ± 0.0^{f}	8.3 ± 0.0^{g}
Pinto bean	8.3 ± 0.0^{f}	8.3 ± 0.3^{f}	8.2 ± 0.5^{g}
Red kidney bean	8.3 ± 0.0^{f}	8.2 ± 0.3^{f}	8.1 ± 0.8^{g}
Soybean	6.5 ± 0.8^{c}	6.0 ± 0.3^{c}	5.6 ± 0.8^{d}
Split pea	7.7 ± 1.3^{e}	7.1 ± 1.0^{e}	4.3 ± 0.5^{b}
Hakmeitau bean	8.3 ± 0.0^{f}	8.2 ± 0.3^{f}	8.1 ± 0.5^{g}

Values are means ± standard deviations of triplicate samples.
Values in the same column with different superscript are significantly different ($p<0.05$).

Table 3.2 Chitin content of different fermented products (%).

Substrate	Chitin content (%)		
	Pleurotus sajor-caju	***Pleurotus nebrodensis***	***Ganoderma lucidum***
Adzuki bean	3.16 ± 0.04^{b}	2.38 ± 0.02	1.80 ± 0.06^{c}
Barley	4.32 ± 0.01^{e}	3.19 ± 0.04^{b}	2.57 ± 0.07^{c}
Black soybean	3.61 ± 0.06^{bcd}	2.86 ± 0.03^{b}	1.94 ± 0.01^{abc}
Buckwheat	7.65 ± 0.11^{i}	5.78 ± 0.17^{d}	4.97 ± 0.29^{d}
Chick pea	3.64 ± 0.01^{cd}	2.77 ± 0.02^{b}	2.07 ± 0.02^{abc}
Lentil	1.82 ± 0.01^{a}	1.19 ± 0.03^{a}	0.84 ± 0.02^{a}
Millet	3.87 ± 0.02^{de}	2.98 ± 0.02^{b}	2.21 ± 0.01^{bc}
Mung bean	6.36 ± 0.11^{g}	5.63 ± 0.11^{d}	4.86 ± 0.15^{d}
Navy bean	1.96 ± 0.01^{a}	1.39 ± 0.01^{a}	1.01 ± 0.01^{ab}
Oat	6.86 ± 0.06^{h}	5.56 ± 0.06^{d}	4.33 ± 0.14^{d}
Pinto bean	5.70 ± 0.23^{f}	4.64 ± 0.23^{cd}	4.34 ± 0.12^{d}
Red kidney bean	8.22 ± 0.07^{j}	7.64 ± 0.07^{e}	5.22 ± 0.07^{d}
Soybean	3.79 ± 0.05^{d}	2.98 ± 0.05^{b}	2.37 ± 0.04^{c}
Split pea	3.29 ± 0.04^{bc}	2.47 ± 0.04^{ab}	1.90 ± 0.03^{abc}
Hakmeitau bean	9.86 ± 0.08^{k}	7.51 ± 0.08^{e}	5.33 ± 0.12^{d}

Values on dry weight basis and are means ± standard deviations of triplicate samples. Values in the same column with different superscript are significant different ($p<0.05$).

Table 3.3 Moisture content of different substrates and fermented products.

Moisture (%)

Substrate	Unfermented		Fermented		
	Raw seed powder*	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	11.57 ± 0.03^{b}	6.43 ± 0.13^{a}	6.67 ± 0.17^{a}	6.77 ± 0.14^{a}	6.70 ± 0.11^{a}
Buckwheat	13.87 ± 0.03^{c}	5.87 ± 0.15^{a}	6.50 ± 0.11^{b}	6.65 ± 0.16^{b}	6.67 ± 0.06^{b}
Mung bean	10.80 ± 0.05^{c}	5.27 ± 0.16^{a}	7.10 ± 0.05^{b}	6.7 ± 0.13^{b}	7.02 ± 0.06^{b}
Hakmeitau bean	10.40 ± 0.05^{b}	6.23 ± 0.08^{a}	6.25 ± 0.35^{a}	6.52 ± 0.14^{a}	6.48 ± 0.23^{a}
Pinto bean	13.03 ± 0.03^{c}	6.13 ± 0.06^{a}	6.75 ± 0.125^{b}	6.87 ± 0.16^{b}	6.75 ± 0.09^{b}
Red kideny bean	11.73 ± 0.03^{b}	6.13 ± 0.03^{a}	6.63 ± 0.15^{a}	6.63 ± 0.21^{a}	6.8 ± 0.05^{a}

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.4 Ash content of different substrates and fermented products.

Ash (%)

Substrate	Unfermented		Fermented		
	Raw seed powder*	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	1.91 ± 0.04 [a]	1.90 ± 0.1 [a]	2.14 ± 0.09 [a]	1.92 ± 0.03 [a]	2.10 ± 0.01 [a]
Buckwheat	1.34 ± 0.14 [a]	1.32 ± 0.01 [a]	1.85 ± 0.10 [b]	1.70 ± 0.07 [ab]	1.81 ± 0.04 [b]
Mung bean	2.87 ± 0.19 [a]	2.91± 6 0.1 [a]	2.83 ± 0.03 [a]	2.78 ± 0.01 [a]	2.95 ± 0.03 [a]
Hakmeitau bean	3.09 ± 0.04 [ab]	3.01 ± 0.04 [a]	3.35 ± 0.01 [c]	3.20 ± 0.02 [b]	3.35 ± 0.04 [c]
Pinto bean	3.21 ± 0.03 [a]	3.25 ± 0.11 [a]	4.02 ± 0.08 [b]	4.17 ± 0.10 [b]	4.04 ± 0.01 [b]
Red kideny bean	3.11 ± 0.02 [a]	3.13 ± 0.13 [a]	3.92 ± 0.01 [b]	4.12 ± 0.05 [b]	3.80 ± 0.07 [b]

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.5 Lipid content of different substrates and fermented products.

Lipid (%)

	Unfermented		Fermented		
Substrate	**Raw seed powder***	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	5.50 ± 0.07^{ab}	5.23 ± 0.10^{ab}	4.75 ± 0.05^{a}	5.92 ± 0.02^{bc}	6.51 ± 0.3^{c}
Buckwheat	1.86 ± 0.08^{a}	1.81 ± 0.01^{a}	2.4 ± 0.17^{b}	2.41 ± 0.03^{b}	2.41 ± 0.09^{b}
Mung bean	1.02 ± 0.03^{d}	0.71 ± 0.01^{bc}	0.45 ± 0.03^{a}	0.64 ± 0.04^{b}	0.86 ± 0.04^{c}
Hakmeitau bean	1.08 ± 0.02^{ab}	1.19 ± 0.03^{b}	0.90 ± 0.1^{a}	1.52 ± 0.03^{c}	1.44 ± 0.06^{c}
Pinto bean	1.396 ± 0.02^{ab}	1.38 ± 0.03^{b}	1.10 ± 0.05^{a}	1.25 ± 0.03^{b}	1.39 ± 0.01^{b}
Red kideny bean	0.94 ± 0.03^{c}	0.57 ± 0.03^{a}	0.74 ± 0.03^{b}	1.14 ± 0.02^{d}	1.23 ± 0.01^{d}

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.6 Insoluble dietary fiber content of different substrates and fermented products.

Insoluble dietary fiber (%)

	Unfermented	Fermented		
Substrate	**Day-0**	***Pleurotus sajor-caju***	***Pleurotus nebrodensis***	***Ganoderma lucidum***
Oat	7.09 ± 0.05^{b}	5.68 ± 0.14^{a}	7.57 ± 0.08^{b}	9.32 ± 0.24^{c}
Buckwheat	6.86 ± 0.14^{ab}	8.472 ± 0.01^{c}	7.45 ± 0.01^{b}	6.70 ± 0.11^{a}
Mung bean	19.39 ± 0.27^{c}	17.15 ± 0.11^{b}	16.07 ± 0.05^{b}	14.26 ± 0.19^{a}
Hakmeitau bean	12.89 ± 0.08^{c}	9.29 ± 0.01^{a}	8.95 ± 0.03^{a}	12.12 ± 0.08^{b}
Pinto bean	17.79 ± 0.20^{b}	14.11 ± 0.08^{ab}	12.18 ± 0.13^{a}	13.59 ± 1.00^{a}
Red kideny bean	13.92 ± 0.06^{c}	12.94 ± 0.05^{ab}	11.30 ± 0.21^{a}	12.23 ± 0.16^{ab}

Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.7 Soluble dietary fiber content of different substrates and fermented products.

Soluble dietary fiber (%)

	Unfermented	Fermented		
Substrate	**Day-0**	***Pleurotus sajor-caju***	***Pleurotus nebrodensis***	***Ganoderma lucidum***
Oat	5.75 ± 0.04^{a}	5.43 ± 0.02^{a}	6.30 ± 0.06^{b}	6.42 ± 0.10^{b}
Buckwheat	5.72 ± 0.01^{c}	4.41 ± 0.01^{a}	5.25 ± 0.331^{b}	5.82 ± 0.02^{c}
Mung bean	6.61 ± 0.07^{a}	5.77 ± 0.15^{a}	6.15 ± 0.11^{a}	5.81 ± 0.14^{a}
Hakmeitau bean	6.84 ± 0.13^{a}	7.65 ± 0.12^{a}	7.40 ± 0.09^{a}	7.34 ± 0.02^{c}
Pinto bean	6.05 ± 0.03^{a}	5.72 ± 0.09^{a}	7.68 ± 0.13^{b}	5.40 ± 0.07^{a}
Red kideny bean	5.53 ± 0.02^{b}	7.84 ± 0.03^{c}	6.80 ± 0.14^{c}	4.82 ± 0.02^{a}

Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.8 Total dietary fiber content of different substrates and fermented products.

Total dietary fiber (%)

	Unfermented	Fermented		
Substrate	**Day-0**	***Pleurotus sajor-caju***	***Pleurotus nebrodensis***	***Ganoderma lucidum***
Oat	12.84 ±0.01 [b]	11.11 ± 0.16 [a]	13.87 ±0.14 [b]	15.74 ±0.35 [c]
Buckwheat	12.58 ± 0.09 [a]	12.89 ± 0.01 [a]	12.69 ±0.03 [a]	12.52 ±0.13 [a]
Mung bean	25.99 ±0.34 [c]	22.93 ± 0.05 [b]	22.218 ± 0.07 [b]	20.07 ±0.04 [a]
Hakmeitau bean	19.73 ±0.21 [b]	16.94 ± 0.11 [a]	16.36 ± 0.06 [a]	19.45 ±0.06 [b]
Pinto bean	23.84 ± 0.23 [b]	19.84 ± 0.17 [a]	19.86 ±0.01 [a]	18.98 ±0.93 [a]
Red kideny bean	19.45 ± 0.04 [bc]	20.77 ±0.02 [c]	18.10 ± 0.35 [ab]	17.05 ±0.18 [a]

Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.9 Protein content of different substrates and fermented products (%).

	Protein (%)				
	Unfermented		Fermented		
Substrate	**Raw seed powder***	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	12.41 ± 0.02 [a]	13.72 ± 1.61 [a]	32.86 ± 0.23 [b]	13.98 ± 0.17 [a]	15.90 ± 0.07 [a]
Buckwheat	13.47 ± 0.55 [a]	14.63 ± 0.15 [a]	19.20 ± 0.88 [b]	15.56 ± 0.03 [ab]	15.92 ± 0.05 [ab]
Mung bean	22.66 ± 0.33 [a]	22.66 ± 0.24 [a]	32.00 ± 0.19 [d]	27.25 ± 0.03 [b]	28.52 ± 0.13 [c]
Hakmeitau bean	26.03 ± 0.41 [a]	26.03 ± 0.02 [a]	33.24 ± 0.57 [c]	27.38 ± 0.05 [a]	30.15 ± 0.21 [b]
Pinto bean	22.84 ± 1.17 [a]	22.56 ± 0.35 [a]	29.29 ± 0.77 [b]	25.23 ± 0.17 [a]	40.42 ± 0.31 [c]
Red kideny bean	20.34 ± 0.07 [a]	20.16 ± 0.24 [a]	27.15 ± 0.93 [b]	24.38 ± 0.08 [b]	25.08 ± 0.05 [b]

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.10 Carbohydrate content of different substrates and fermented products.

Carbohydrate (%)

	Unfermented	Fermented		
Substrate	**Day-0**	***Pleurotus sajor-caju***	***Pleurotus nebrodensis***	***Ganoderma lucidum***
oat	59.73	42.89	57.69	53.40
buckwheat	63.98	56.96	61.10	60.86
mung bean	42.16	34.56	40.57	40.72
Hakmeitau bean	43.78	39.56	44.87	38.79
pinto bean	42.75	38.39	42.63	28.14
red kideny bean	50.29	41.81	45.70	45.96

Calculated by difference (=100 - crude lipid - crude protein - IDF - SDF - ash - moisture)

Table 3.11 Glucose content of different substrates and fermented products.

Glucose (mg/g)

	Unfermented		Fermented		
Substrate	**Raw seed powder***	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	0.22 ± 0.05 [a]	1.00 ± 0.01 [b]	6.52 ± 0.01 [cd]	6.583 ± 0.06 [d]	6.4 ± 0.03 [c]
Buckwheat	1.05 ± 0.02 [a]	5.74 ± 0.04 [b]	6.90 ± 0.03 [d]	6.90 ± 0.03 [d]	6.45 ± 0.04 [c]
Mung bean	0.67 ± 0.01 [a]	3.49 ± 0.05 [b]	6.10 ± 0.04 [c]	6.34 ± 0.06 [d]	6.25 ± 0.04 [cd]
Hakmeitau bean	2.48 ± 0.04 [a]	4.70 ± 0.03 [b]	6.17 ± 0.01 [d]	5.58 ± 0.05 [c]	6.17 ± 0.02 [d]
Pinto bean	1.13 ± 0.03 [a]	1.95 ± 0.02 [b]	6.06 ± 0.03 [c]	6.16 ± 0.02 [c]	6.01 ± 0.03 [c]
Red kideny bean	0.82 ± 0.01 [a]	2.01 ± 0.04 [b]	6.93 ± 0.02 [cd]	6.76 ± 0.05 [c]	6.95 ± 0.02 [d]

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.12 Phytic acid content of different substrates and fermented products.

Phytic acid (%)

Substrate	Unfermented		Fermented		
	Raw seed powder*	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	0.43 ± 0.01 [b]	0.40 ± 0.05 [b]	0.39 ± 0.01 [b]	0.41 ± 0.00 [b]	0.34 ± 0.02 [a]
Buckwheat	0.21 ± 0.01 [b]	0.21 ± 0.01 [b]	0.17 ± 0.00 [a]	0.21 ± 0.01 [b]	0.18 ± 0.0 [a]
Mung bean	0.21 ± 0.00 [c]	0.19 ± 0.02 [c]	0.05 ± 0.01 [a]	0.12 ± 0.01 [b]	0.04 ± 0.005 [a]
Hakmeitau bean	0.14 ± 0.00 [b]	0.14 ± 0.00 [b]	0.04 ± 0.01 [a]	0.12 ± 0.00 [b]	0.04 ± 0.01 [a]
Pinto bean	0.29 ± 0.01 [c]	0.26 ± 0.00 [c]	0.08 ± 0.02 [a]	0.27 ± 0.00 [c]	0.21 ± 0.01 [b]
Red kideny bean	0.22 ± 0.01 [b]	0.23 ± 0.00 [b]	0.16 ± 0.01 [a]	0.24 ± 0.01 [b]	0.24 ± 0.00 [b]

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

**Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.13 *In vitro* Protein digestibility (IVPD) of different substrates and fermented products.

IVPD (%)

Substrate	Unfermented		Fermented		
	Raw seed powder*	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	79.06 ± 0.74 [a]	79.12 ± 0.21 [a]	80.20 ± 0.05 [ab]	79.54 ± 0.15 [ab]	81.59 ± 0.40 [b]
Buckwheat	72.66 ± 0.05 [a]	73.27 ± 0.09 [a]	73.60 ± 0.16 [a]	75.95 ± 0.13 [b]	79.69 ± 0.41 [c]
Mung bean	76.58 ± 0.37 [a]	77.43 ± 0.41 [a]	78.00 ± 0.19 [b]	79.57 ± 0.07 [ab]	78.06 ± 0.39 [ab]
Hakmeitau bean	74.65 ± 0.68 [a]	78.46 ± 0.10 [b]	77.61 ± 0.21 [b]	77.22 ± 0.19 [b]	82.01 ± 0.11 [c]
Pinto bean	72.84 ± 0.84 [a]	72.36 ± 1.03 [a]	77.04 ± 0.74 [b]	79.75 ± 0.13 [b]	83.64 ± 0.48 [c]
Red kideny bean	79.42 ± 0.16 [ab]	79.24 ± 0.39 [a]	81.02 ± 0.46 [b]	80.96 ± 0.09 [ab]	85.06 ± 0.40 [c]

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.
*Values are on dry weight basis and are means ± standard deviations of triplicate samples.
Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.14 Aflatoxin concentration of different substrates and fermented products.

Aflatoxin concentration (ppt)

	Unfermented		Fermented		
Substrate	**Raw seed powder***	**Day-0****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	5.92 ± 0.01 [a]	5.50 ± 0.59 [a]	9.21 ± 0.21 [a]	10.25 ± 2.59 [a]	7.91 ± 1.00 [a]
Buckwheat	31.49 ± 0.12 [b]	28.99 ± 1.06 [bc]	29.65 ± 0.89 [b]	35.95 ± 0.21 [c]	11.08 ± 0.36 [a]
Mung bean	48.99 ± 0.12 [b]	47.74 ± 0.41 [b]	49.20 ± 0.27 [b]	36.45 ± 2.68 [a]	38.84 ± 1.29 [ab]
Hakmeitau bean	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
Pinto bean	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.71 ± 0.50 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
Red kideny bean	25.83 ± 0.12 [a]	23.58 ± 0.77 [a]	15.96 ± 0.50 [a]	21.04 ± 2.56 [a]	25.16 ± 1.89 [a]

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

Values in the same row with different superscript are significantly different ($p<0.05$).

Table 3.15 The relative FRAP activities of fungal extract to the antioxidant standard $FeSO_4 7H_2O$.

	FRAP ratio
Pleurotus sajor-caju	0.557
Pleurotus nebrodensis	0.124
Ganoderma lucidum	0.558

Values are on dry weight basis and means ± standard deviations of triplicate samples.

Table 3.16 The relative FRAP activities of different substrates & fermented products to the antioxidant standard $FeSO_4 7H_2O$.

FRAP ratio

	Unfermented			Fermented		
Substrate	**Raw seed powder***	**Day-0****	**Day-10****	***Pleurotus sajor-caju****	***Pleurotus nebrodensis****	***Ganoderma lucidum****
Oat	0.302	0.195	0.118	0.095	0.119	0.324
Buckwheat	0.925	0.962	0.659	0.810	0.893	1.101
Mung bean	0.363	0.248	0.112	0.266	0.122	0.381
Hakmeitau bean	1.507	1.242	0.873	0.690	1.140	0.791
Pinto bean	0.419	0.341	0.349	0.890	0.565	0.566
Red kideny bean	0.689	0.402	0.370	0.679	0.685	0.847

*Values are on fresh weight basis and are means ± standard deviations of triplicate samples.

*Values are on dry weight basis and are means ± standard deviations of triplicate samples.

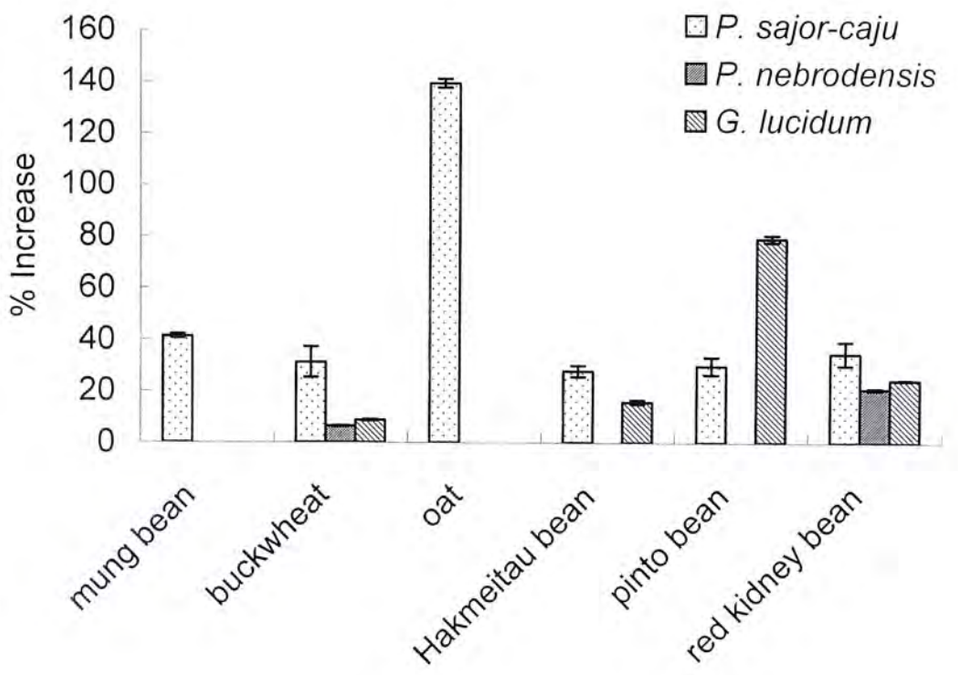


Figure 3.1 Increase in protein content of different substrates after fermentation.

Results are on dry weight basis and are means ± SD of triplicate measurements.

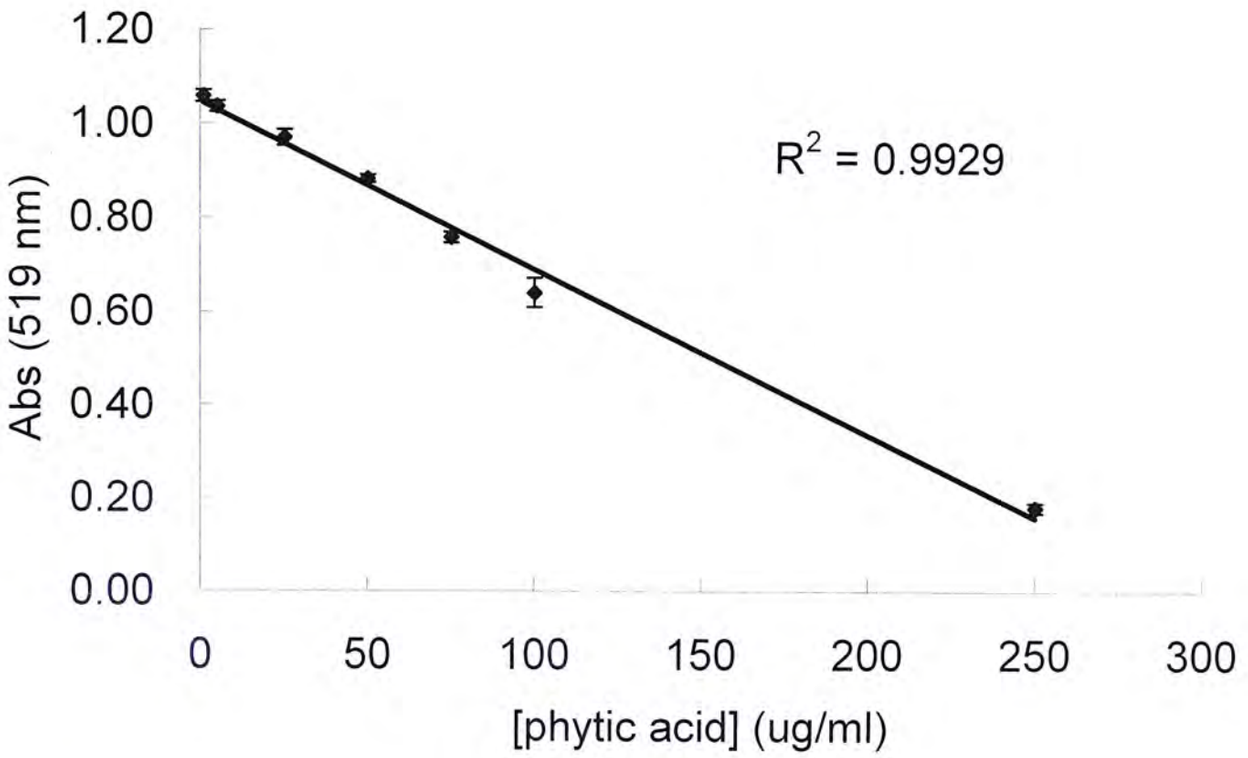


Fig. 3.2 Calibration curve for phytic acid determination.

Results are means ± SD of triplicate measurements.

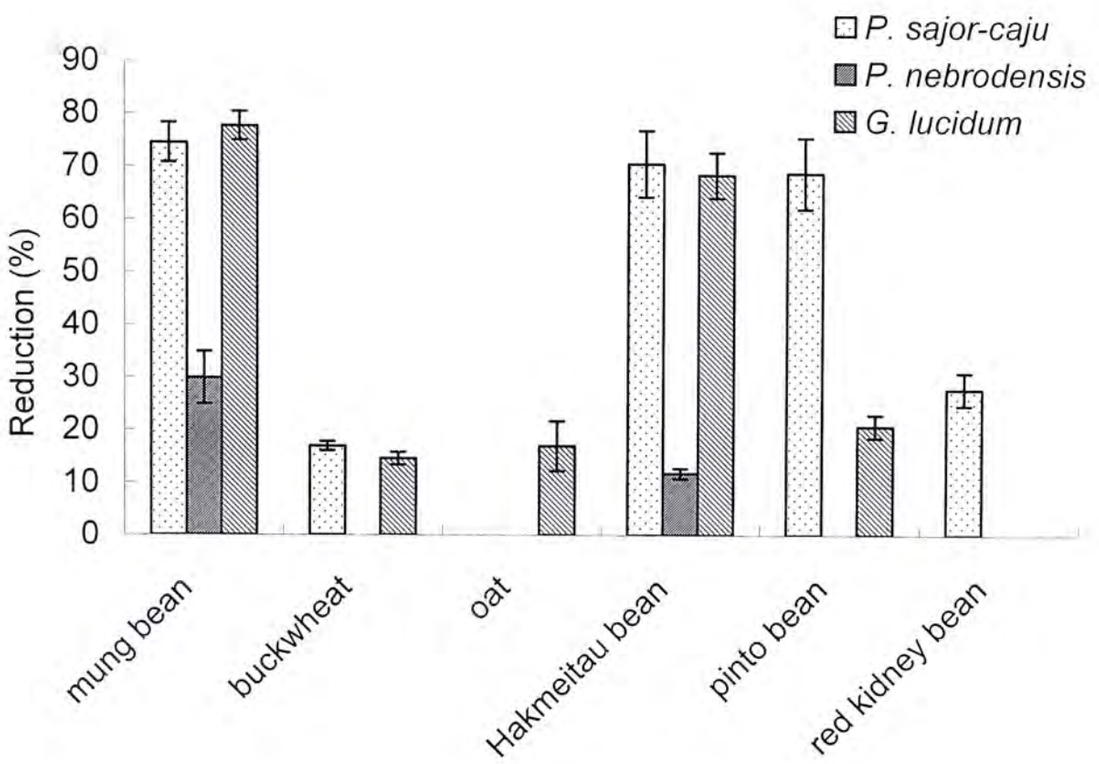


Figure 3.3 Phytic acid reduction of fermented products after fermentation

Results are on dry weight basis and are means ± SD of triplicate measurements.

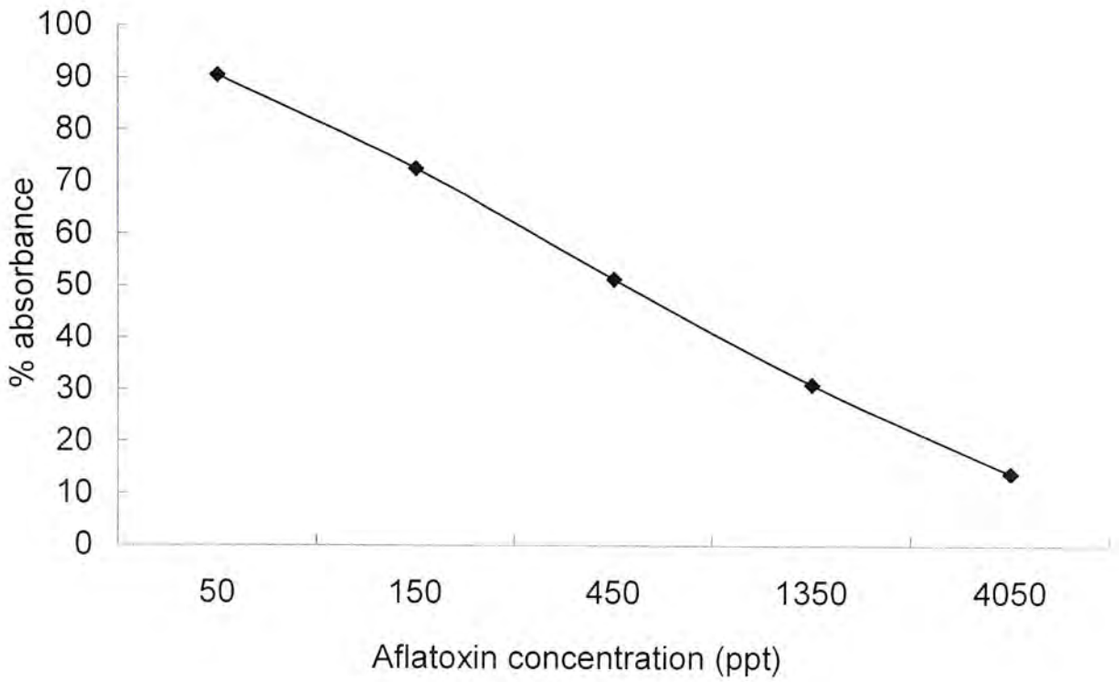


Fig. 3.4 Calibration curve for aflatoxin determination.

Values were means ± SD of triplicate measurements.

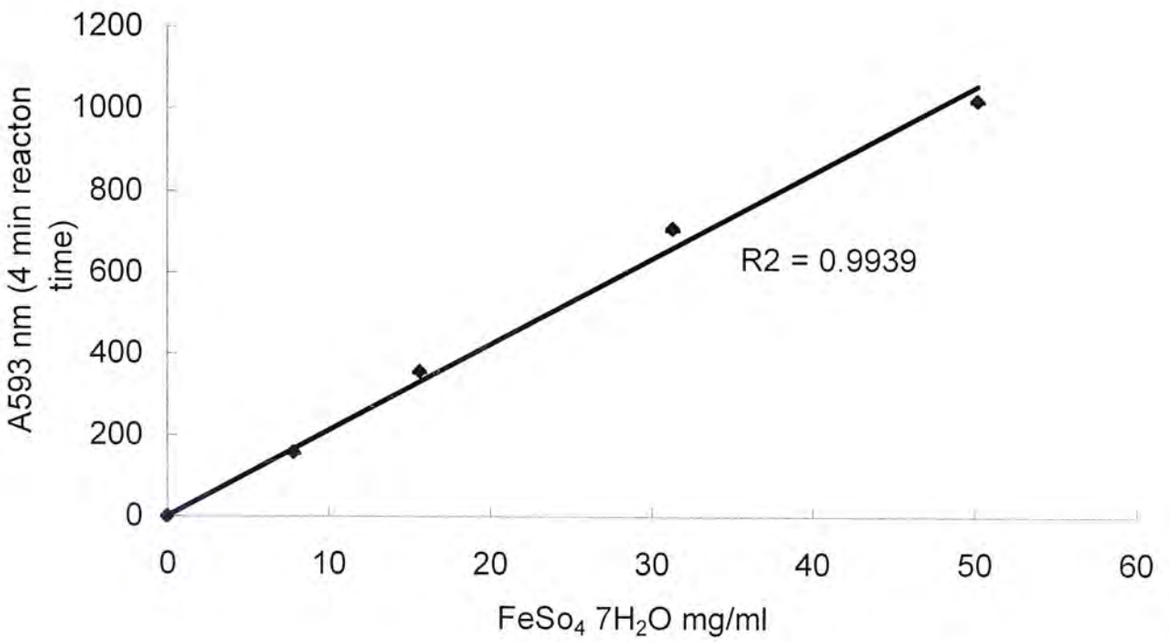


Figure 3.5 Calibration curve for FRAP assay of antioxidant activity.

Values were means ± SD of triplicate measurements.

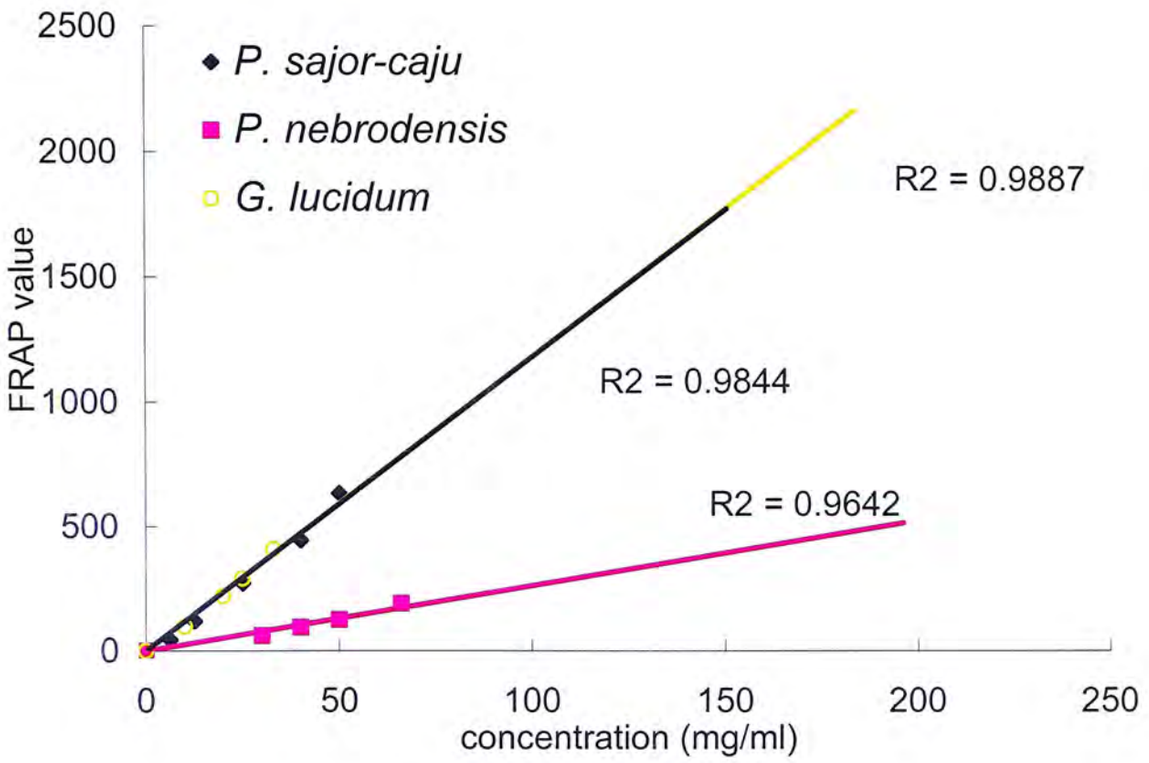


Figure 3.6 Dose–response curves of antioxidant activities of fungal extracts as determined by the FRAP assay.

Values were mean of triplicate measurements.

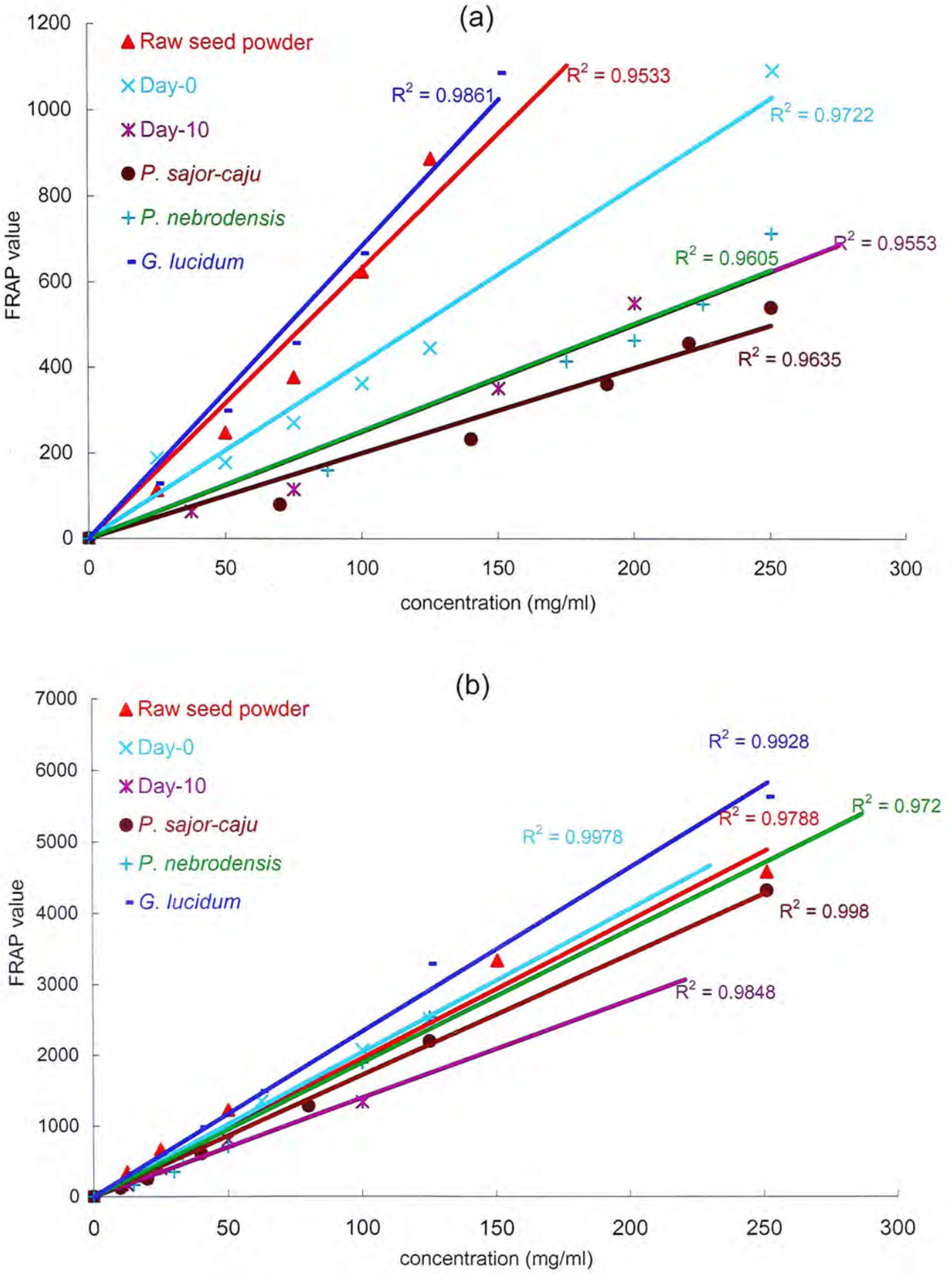
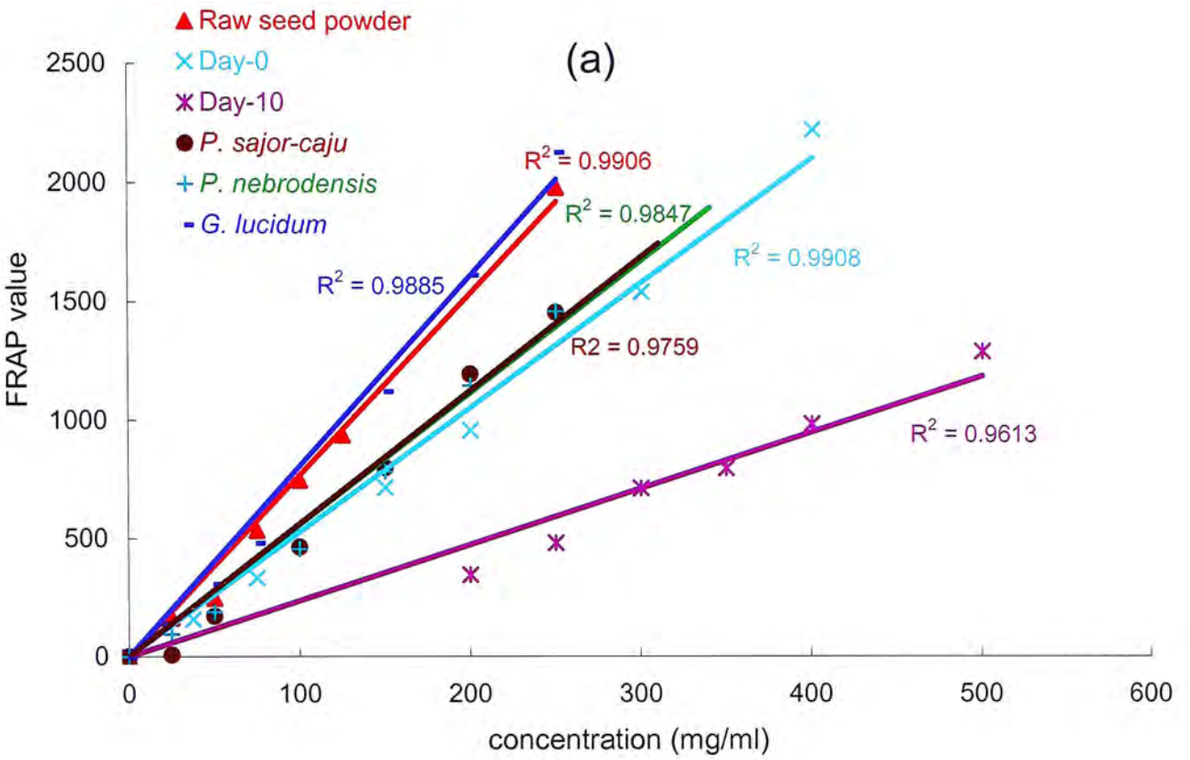


Figure 3.7 Dose–responsecurves of antioxidant activity as determined by the FRAP assay. (a) Buckwheat & (b) Oat

Values were mean of triplicate measurements.



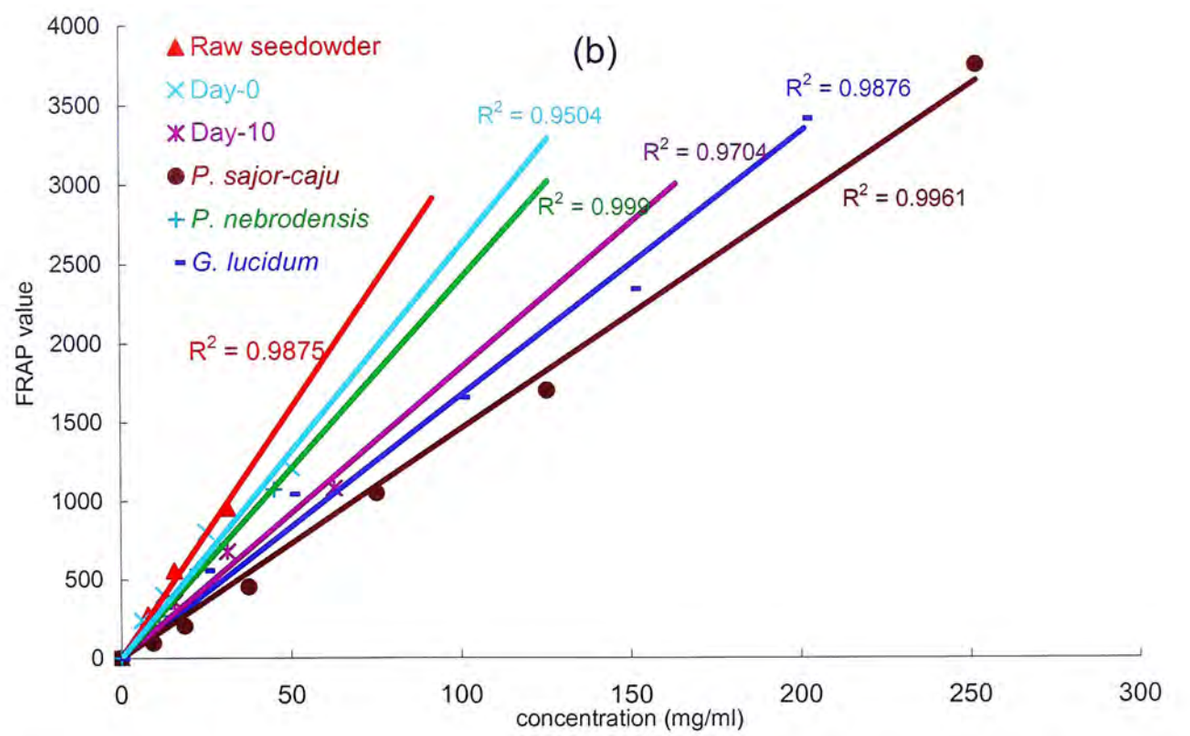


Figure 3.8 Dose–responsecurves of antioxidant activity as determined by the FRAP assay. (a) Mung bean & (b) Hakmeitau bean

Values were mean of triplicate measurements.

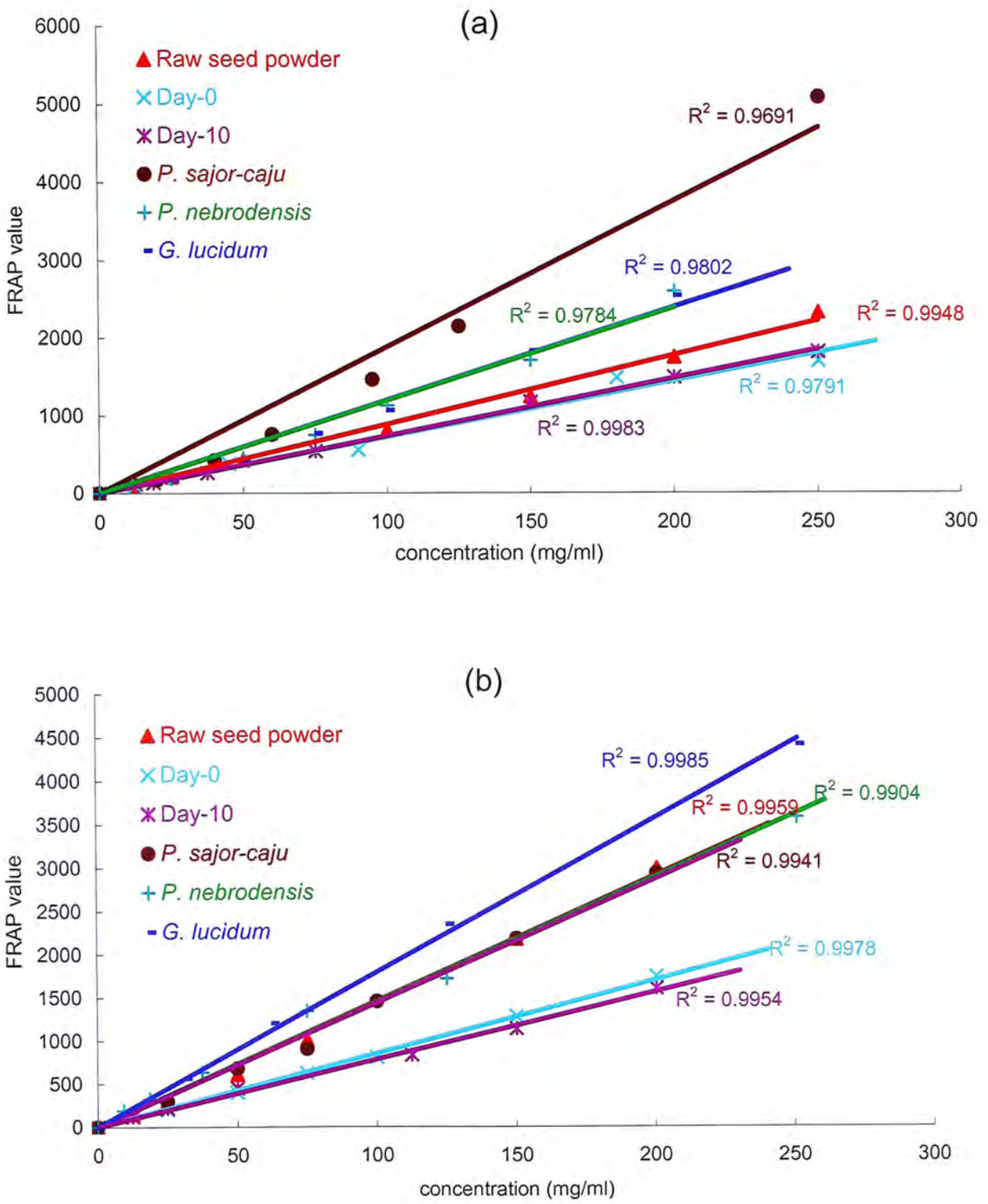


Figure 3.9 Dose–response curves of antioxidant activity as determined by the FRAP assay. (a) Pinto bean & (b) Red kidney bean

Values were mean of triplicate measurements.

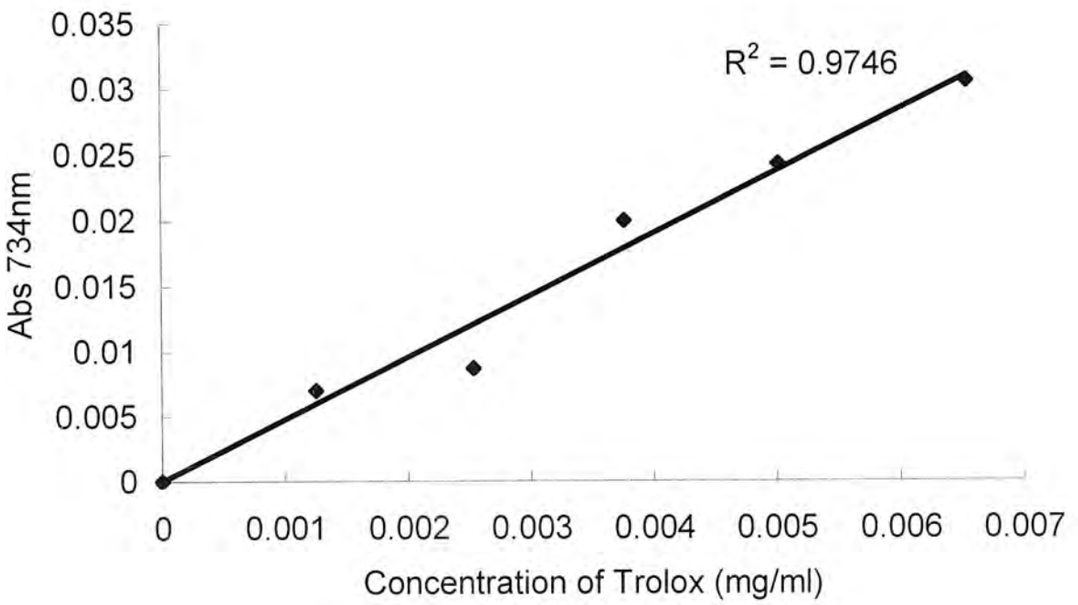


Figure 3.10 Dose-response curve of the antioxidant activity of Trolox as determined by the $ABTS^+$ scavenging method.

Values were means ± SD of triplicate measurements.

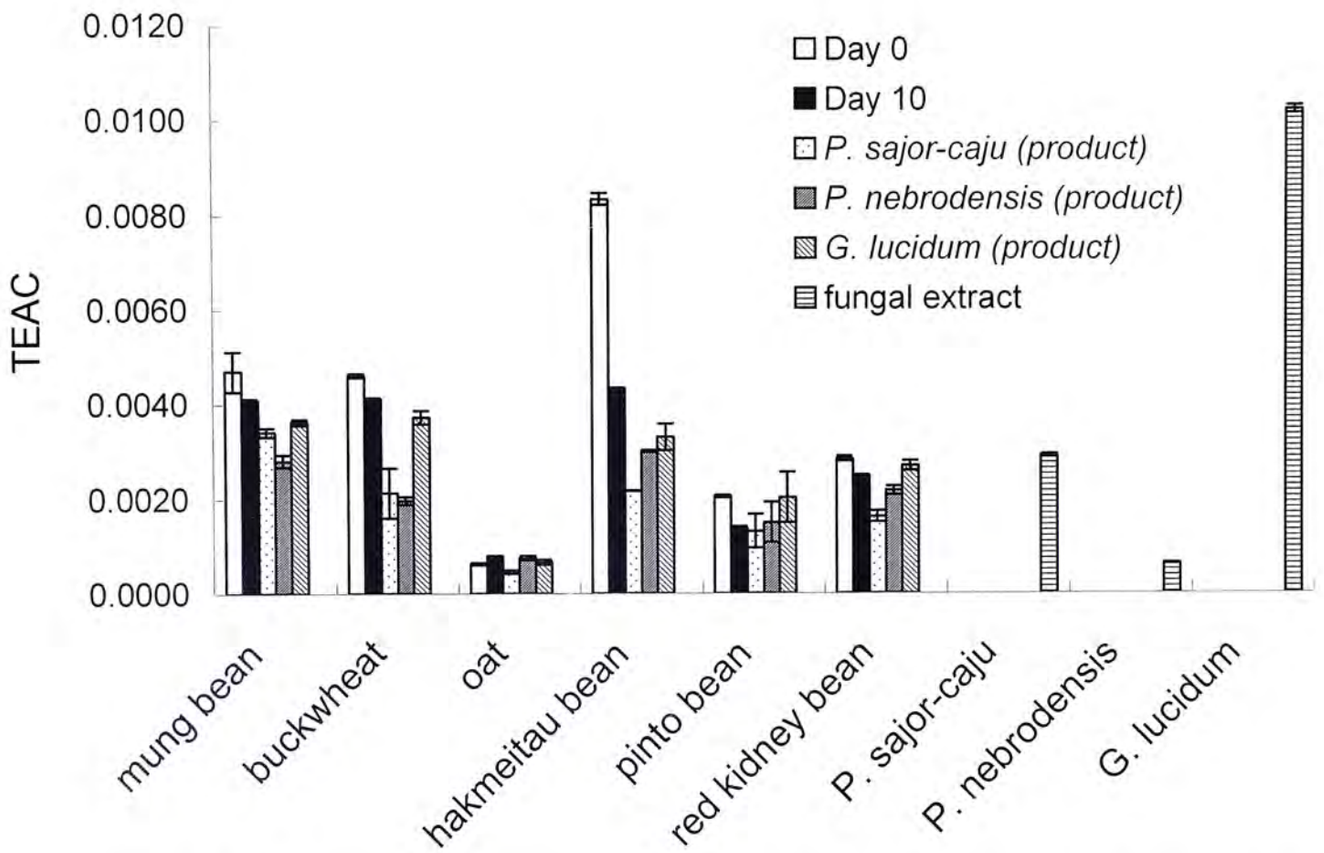


Figure 3.11 The antioxidant capacities of selected mycelia, substrates and fermented products to scavenge the $ABTS^{+}$ radical cation.

TEACs are on dry weight basis and are means ± SD, each performed in triplicate at 5 separate concentrations.

Chapter Four: Discussions

4.1 Mycelia growth

Both growth diameter (Table 3.1) and chitin content (Table 3.2) were used to measure the growth of mycelia in all kinds of agricultural substrates. The results of these two parameters were quite similar. Results show that, mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and red kidney bean supported good substrates and had higher mycelia growth rates than other substrates.

Fungi are heterotrophic organisms and they need to get all nutritive elements from the substrate. Such substrate must contain a carbon source such as glucose, nitrogen and ions (Chang & Miles, 1989). Mung bean, buckwheat, oat, Hakmeitau bean, pinto bean and red kidney were the better media for the mycelia growth. It might be due to their nutrient compositions are more optimal for mycelia growth.

In general, the growth of *P. sajor-caju* and *P. nebrodensis* on the substrates were better than *G. lucidum*. It is because the *Pleurotus* species has lower growth requirement than *G. lucidum*. The carbon sources suitable for the growth of *Pleurotus* species mycelia are: starch, glucose, fructose, mannose, sucrose, pectin, cellulose and lignin. The nitrogenous sources utilized by *Pleurotus* are: peptone, yeast powder, ammonium sulfate, asparagines, serine, alanine and glycine (Chang & Miles, 1989). However, the growth requirement of *G. lucidum* is higher. It is a kind of basidiomycete that requires vitamin B1 in the culture medium. The content of thiamine in the substrates might be not high enough to provide optimal growth for the *G. lucidum* mycelia.

4.2 Weigh loss in sample preparation

The weight of the freeze-dried samples was about 2 g less than the starting materials. It is due to the following reasons:

1. The moisture content of the fermented samples after freeze-drying was lower than that of the raw seed powder substrates.
2. Some of the sample powders were lost during the process of milling.
3. The nutrient contents of the substrates were used for the growth of mycelia.

4.3 Proximate composition

4.3.1 Moisture

As shown in the Table 3.3, the moisture content of the raw seed power was higher than that of the fermented products and Day-0 autoclaved seed substrates. It is because the latter two samples were prepared from lyophilized substrates. The freeze-drying process reduced the moisture content to about 6 to 7 %. The moisture content of the raw seed powder prepared from dry seeds was around 10.5 – 14.0 %. High moisture content of seeds would encourage the growth of pathogens (Pietronave *et al.*, 2004). In fact, the moisture content of the fermented products in this research is quite low, and it can decrease the chance of bacterial contamination.

4.3.2 Ash

The ash content of both cereals and legume samples were shown in Table 3.4. Results showed that cereals had lower mineral content than legumes. Since the solid-state fermentation is a close system, and no any additional nutrient was added during the cultivation period, therefore the mineral content of the final fermented products should be the same as the initial substrates.

On the other hand, the fermented products of buckwheat, Hakmeitau bean, pinto bean and red kidney bean had significantly higher ash content then the corresponding raw seed powder and Day-0 sample. This increased mineral content might come form the fungal inoculations. In addition, the process of fermentation provided an optimum pH for the activities of phytase, which can degrade the phytates. Phytates are the salt of phytic acids, which form complex with polivalent cations, eg. Fe^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+}& proteins. Therefore, decrease the amount of phytates can increase the amount of free Fe^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2} in several folds (Vats& Banerjee, 2004). In addition, there was no significant difference between raw seed powder and Day-0 substrates. It was agreed with Table 1.5, the ash contents of 3 various seeds did not change significantly after heating (Agbede & Aletor, 2004; Omafuvbe *et al.*, 2004)

4.3.3 Crude lipid

As shown in Table 3.5, the lipid contents of both cereals (buckwheat & oat) were higher than the legumes in all samples (raw seed powder, autoclaved seeds and fermented products). The reason of cereals having higher lipid contents than legumes is due to cereal consists of large storage lipids, composed of triacylglycerols and hydrolysis products (Lehtinen *et al.*, 2003), which serve as a reservoir for energy and building blocks for germinating seeds and amphipathic phospholipids in membranous structures, which help to maintain microstructure in grain (Lehtinen *et al.*, 2003).

The lipid content of mung bean and red kidney bean in Day-0 sample were significantly lower than that of the raw seed powder. It is because subjecting the moist foods to high temperatures resulted in lipid hydrolysis (Penfield & Campbell, 1990). The esterification reaction in which the acyl group is transferred from the glycerol to water is generally referred to as lipid hydrolysis (Ferrer *et al.*, 2000). Lipid hydrolysis involves breaking the ester linkage between glycerol and a fatty acid and requires one molecule of water for break down every ester linkage. In addition, the resulting glycerols and short-chain fatty acids are more digestible (Briggs & Calloway, 1984). As the Day-0 unfermented substrates were autoclaved, hence lipid hydrolysis occurred and the lipid content in the samples was decreased.

In general, the *P. sajou-caju* fermented products had lower lipid content than the autoclaved seed substrates. It is due to the secretion of extracellular lipase by *P. sajou-caju* to the substrates. As most of the fatty acids found in plant seeds were esterified to a specific alcohol molecule, glycerol. Therefore, the lipid content of *P. sajou-caju* fermented products was lower than that of unfermented substrates.

In contrast, only the *P. sajou-caju* fermented products had such lipid reduction and it was not found in the other two fungal species. It is because different fungi produce different kinds and amounts of lipase. Therefore the degree of lipid hydrolysis is different. The reason *P. nebrodensis* and *G. lucidum* fermented products having higher lipid content than Day-0 samples might be due to the conversion of the carbon source in the substrate to the fungal lipids.

The lipid contents of the three fungi fruiting bodies are shown Table 1.1. It is ranging from 3.86 – 6.32 %. Except from the oat-fermented products, all the rest of the fermented products had lower lipid content than the fungi fruiting bodies. None of them had higher 2.41 % of lipids. It seems that the fermented products had low lipid contents than that of the unfermented substrates and the fungal fruiting bodies.

4.3.4 Dietary fiber

The Food and Nutrition Board of the Institute of Medicine recommends the daily dietary fiber intakes are 25 grams for adult women and 38 grams for adult men up to age 50. For men and women over 50, the recommendations decrease to 30 and

21 grams respectively.

As shown in Table 3.8, the total dietary fiber (TDF) content of legumes substrates and its products were higher than that of cereal samples. In addition, the amount of insoluble dietary fiber (IDF) in the samples was higher than soluble dietary fiber.

For the insoluble dietary fiber (Table 3.6), all of the fermented products of legumes and some of the cereal-fermented products had lower fiber content than Day-0 samples. The insoluble dietary fibers are composed of cellulose, hemicellulose and lignin. The enzymes that produced by the mycelia can degrade all these insoluble dietary fibers. Therefore the amount of insoluble dietary fiber was reduced after fermentation. The cellulose was broken down by the cellulase secreted by the mycelia extracellularly (Bhat *et al.*, 1997). The lignin was also degraded by mycelial ligninolytic enzymes. The ligninolytic enzymes, lignin peroxidase (LiP) and manganese peroxidase (or manganese-dependent peroxidase) (MnP) were described in 1983 and 1984 in the fungus *Phanerochaete chrysosporium* (Camarero *et al.*, 1999). Recently, two peroxidases produced in solid-state fermentation and liquid cultures of *Pleurotus eryngii* have been cloned too (Camarero *et al.*, 1999). These two ligninolytic enzymes are able to degrade the lignin. The xylanase can also degrade the hemicellulose. Next to cellulose, xylan is the most abundant structural polysaccharide in nature. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes: the endoxylanases which randomly cleave β-1,4-linked xylose (the xylan backbone); the β-xylosidases which hydrolyze xylooligomers; and the different side-branch-splitting enzymes, e.g., α-glucuronidase and α-arabinosidase, acetylxylan esterase, and acetyl esterase, which liberate other sugars (glucuronic acid arabinose) that are attached as branches to the backbone

(Biely, 1985).

For the soluble dietary fiber (SDF) (Table 3.7), more than half of the fermented products had similar or lower fiber content than the Day-0 autoclaved samples. It is because of the presence of polygalacturonase. Since pectin is an important constituent of soluble dietary fiber in plant cell wall, the major pectin-hydrolyzing enzyme, polygalacturonase which cleaves the α-1,4 linkages of pectin or polygalacturonic acid, was expected to be commonly produced by fungi involved in the decomposition of vegetable matter (Sakamoto *et al.*, 2003). In *P. sajor-caj*-fermented oat, *P. sajor-caj*-fermented red kidney bean, *P. nebrodensis*-fermented oat, *P. nebrodensis*-fermented red kidney bean and *P. nebrodensis* -fermented pinto bean products, they had higher soluble dietary fiber contents than Day-0 substrates. It might due to the conversion of carbohydrates in substrates to SDF by the mycelia.

From the research, although most of the fermented products had lower TDF than the unfermented substrates, the mycelia broke down the indigestible polysaccharides to simpler sugars. These sugars are more digestible by human intestine. In addition, all the *Pleurotus* fermented products had higher TDF content than the *Pleurotus ostreatus* and *Pleurotus sajor-caju* fruiting bodies (Table 1.1). Therefore, it can say that the fermented products had TDF improvement when compared with fungi fruiting bodies.

4.3.5 Crude protein

As shown in Table 3.9, the protein content of raw seed powder and Day-0 autoclaved seeds had no significant difference. Proteins denature when exposed to heat. The protein peptide chain unfolds as weaker bonds structure (Penfield & Campbell, 1990). Since autoclaving does did not break down the peptide bonding of proteins. The amount of protein nitrogen present in the autoclaved seed substrates remained the same.

In general, the protein contents of more than half of the fermented products were higher than Day-0 autoclaved substrates. Especially in *P. sajor-caju* fermented products, their protein contents were higher than that of the other two species. It showed that the *P. sajor-caju* had better protein conversion ability than others. Some researchers claimed that *P. sajor-caju* might be capable of nitrogen fixation (Ginterota & Gallon, 1979), to convert the atmospheric nitrogen into compounds, such as ammonia. Biological nitrogen fixation can be represented by the following equation:

$$\mathbf{N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi}$$

This reaction is performed by using an enzyme complex termed nitrogenase. This enzyme consists of two proteins; an iron protein and a molybdenum-iron protein. Iron protein donates electrons to N_2 and the NH_3 is the final product. The NH_3 can convert to proteins.

Recently, Jayasinghearachchi & Seneviratne (2004) has done a research on *Pleurotus* spp. nitrogen fixation to find out its nitrogenase activities. They found that nitrogen fixation of the fungi associated with diazotrophs. Nitrogenase activity absent when the fungus or the bradyrhizobial strain was alone. However, the strong evidence of nitrogen fixation ability in *P. sajor-caju* is still deficient. Further studies on nitrogenase activities are needed to confirm such suggestion.

The protein contents of the *Pleurotus sajor-caju*, *Pleurotus ostreatus* & *Ganoderma lucidum* fruiting bodies are shown Table 1.1. It is ranging from 7.18 % (in *G. lucidum*) to 18.40 % (in *P. sajor-caju*). When compared with the fermented products, all the *P. sajor-caju* and *G. lucidum* fermented products had higher protein contents than that of its fungi fruiting bodies.

In fact, over estimation of protein content in the fermented products might occur. As the protein content was calculated from multiplying the total nitrogen content by 6.25. The reason for multply by 6.25 is due to cereals and legumes contained 8-15% nitrogen as non-protein nitrogen. However, for the fungi, the amount non-protein nitrogen was higher than cereals and legumes. As the fermented products composed of both seed substrates and fungi. Therefore, by using the factor 6.25, the calculated the protein content may be higher than that of true protein content.

4.3.6 Glucose concentration

As shown in table 3.11, the glucose content of Day-0 autoclaved seeds was higher than the raw seed powder. It was because upon exposure to moist heat, starch granules experienced the physical transformations of gelatinization and pasting. As heat is applied, the starch granule swells with water (gelatinization) and, if held at high temperatures for several minutes, eventually implodes (pasting) (Collado & Corke, 1999).

Upon heating, granule integrity is lost and small amount of glucose, amylose, one of two starch fractions was released (Wandanider, 1997). Therefore, the glucose content of autoclaved seeds was higher than raw seed powder. The heat treatment of carbohydrates is predominantly concerned with enhancing digestion. Simple sugars were small enough polymers that they are readily absorbed into blood vessels in the small intestine. The digestibility of a carbohydrate is related to its polymer size and structure (Botham *et al.*, 1996).

All the glucose content of fermented products was higher than that of the Day-0 samples and raw seed powders. It is because there are many extracellular enzymes secreted by the fungi to break down the polysaccharides of the seed substrates. For example, α-amylase hydrolyzes α-1,4-glycosidic linkages in starch to produce maltose and oligosaccharides of various lengths. All species of thermophilic fungi studied so far secrete amylase (Maheshwarl *et al.* 2000). Glucoamylase is an exoacting enzyme which hydrolyzes α-1,4- glycosidic linkages and, less frequently, α-1,6- glycosidic linkages from the nonreducing end of starch, producing

β-D-glucose as the sole product. The growth in a starch medium initiates both glucoamylase and α-amylase production simultaneously (Rao *et al.,* 1981). In addition, the enzyme system of fungi can increase the polysaccharide digestibility of the fermented products. The cellulase system in fungi is considered to comprise three hydrolytic enzymes, endo-(1,4)- β-D-glucanase, exo-(1,4)-b-D-glucanase and b-glucosidase, which work together to release glucose form cellulose (Bhat & Bhat, 1997). The actions of these enzymes will increase the glucose content of the fermented products. For the process of bioconversion, the non-digestible lignocelluloses are degraded by the fungal secretions. Hence, the human intestine can absorb the carbohydrates of the fermented products more easily.

4.3.7 Phytic acid (PA)

The phytic acid content (Table 3.12) of Day-0 unfermented substrates was remained unchanged after heat treatment. It was because the phytic acid was stable to heat. This suggestion was agreed by Vijayakumari *et al.* (1998), as the phytic acid content of *Vigna aconitifolia* and *Vigna sinensis* had the same phytate content after heat-treated.

After fermentation with fungi, most of the fermented products had lower phytic acid content than the unfermented substrates and the raw seed powder. It was due to the presence of phytase. Phytase (*myo*-inositol hexakisphosphate phosphohydrolases) catalyzes the hydrolysis of PA to the mono-, di-, tri-, tetra-, and pentaphosphates of *myo*-inositol and inorganic phosphate (Mullaney *et al.*, 2000).

Both seed substrates and fungi had phytase, the seed substrates had 6- phytase which was inactive in dry seeds due to lack of moisture for their activation. Adding water to the substrates can activate the phytase. The fungi had another kind of phytase, 3-phytase, which can also hydrolyze the phytic acid into myo-inositol and inositol phosphates and use them for their growth (Vats & Banerjee, 2004, Oatway *et al.*, 2001). However, 70% to 80% of phytate's enzymatic activity was lost when it is exposed to temperatures between 55 °C and 90 °C (Wyss *et al.,* 1998). As the seed substrates in this experiment were autoclaved, this heat treatment denatured the 6-phytase, so the action of 3-phytase secreted by the fungi was the main cause of phytate reduction.

The fungi used in solid-state fermentation can secrete phytase, which is able to degrade the PA in the substrates. This phenomenon was described by Vats & Banerjee. (2004). The production of phytase from *Aspergillus* spp. has been achieved by solid-state fermentation. It was reported that there was a complete reduction of phytic acid content in canola meal using SSF by *Aspergillus* spp..

Figure 3.2 shows that, *P. sajor-caju* and *G. lucidum* fermented products had higher phytic acid reduction than *P. nebrodensis*. It might be due to the mycelia of *P. nebrodensis* secreting less phytase than the other two fungal species. In fact, the high phytase activity of *P. sajor-caju* and *G. lucidum* within this research is a hypothesis only. Further analysis of *P. sajor-caju*, *P. nebrodensis* and *G. lucidum* mycelia phytase activities is needed to prove this assumption.

4.4 *In vitro* protein digestibility (IVPD)

The *in vitro* protein digestibility (Table 3.13) of autoclaved Hakmeitau bean substrate was higher than its raw seed powder. It was because after heat treatment, the protein denatured, and weaker the bonding structure (Penfield & Campbell, 1990). Unfolded or denatured protein is more readily hydrolyzed (Brace et al., 1987), leading to increased solubility. It is thus digested by proteolytic enzymes more easily (Wandanider *et al.*, 1997). In addition, the relatively low value of in vitro protein digestibility of the raw sample may be partly attributed to the presence of trypsin inhibitor. Giami *et al.* (2001) suggested that boiling the seeds for the 20 minutes resulted in decreases in trypsin inhibitor (37.7%). As the Day-0 samples were autoclaved, high temperature can also reduce the factor of trypsin inhibitor. However, this heat-induced increase in digestibility only observed in Hakmeitau bean but not in other seed substrates. It is because some trypin inhibitors are stable to heat (eg. mung bean) (Liener & Kakade, 1969). Therefore, it was suggested that the effect of heat on protein digestibility for this experiment was not such important.

In most cases, fermented products had higher *in vitro* protein digestibility than the substrates. It was because the mycelia secreted protease extracellularly (Andrade *et al.*, 2002). Some of the protein peptide linkages were broken down and the proteins became more digestible.

4.5 Aflatoxin

For the EU equal limits for aflatoxins, 2 ppb for aflatoxin B_1 and 4 ppb for total aflatoxins. Table 1.7 also shown the Food and Drug Adminstration (FDA)'s practical limits for aflatoxin in food and feeds.

The competitive enzyme immunoassay used in aflatoxin detection is highly sensitive. Table 3.14 shown that a minute amount of aflatoxin was present in some of the substrates, which including oat, buckwheat, mung bean and red kidney bean. However, the highest amount of aflatoxin detected in mung bean was only 48.99 ppt, a value far below the safety limit according to both EU standard (4 ppb) and FDA standard (<20 ppb). In addition, the contaminated aflatoxin was heat-stable, as its amount did not change after autoclaving. No significant change in the aflatoxin content was detected in the substrates after solid-state fermentation with the three edible mushroom mycelia. Therefore, the amount of aflatoxin in all fermented products was also lower than the EU and FDA safety limits and they are supposed to be safe to consume. Further toxicity test may be needed to confirm this.

4.6 Antioxidant activity

For the Ferric reducing antioxidant powder (FRAP) assay (Table 3.15 & Table 3.16), it showed that the totally antioxidant ability of raw seed powder was higher than Day-0. It was because the autoxidation occurred. Energy in the form of heat caused a hydrogen atom to escape the polymer chain (eg. polysaccharides and polyphenolic compounds). The result was a polymer radical [R˙] and a free hydrogen formation. The polymer radical [R˙] reacted with a molecule of oxygen [O_2] to form a peroxyl radical [ROO˙]. The peroxyl radical then removed another hydrogen atom from the polymer chain, forming unstable hydroperoxides [ROOH], alcohols [ROH] and new hydrocarbon free radicals [R˙]. Autoxidation continues unless counter measures are taken to halt the process (Gutteridge & Halliwell, 1994). For the Day-10, the antioxidant ability was further lower than Day-0, it was because of further autoxidation occurred. This result was agreed by the Trolox equivalent antioxidant capacity (TEAC) assay. It also showed that Day-0 autoclaved seed substrates had higher antioxidant ability than Day-10. In additions, Hakmeitau bean had the highest antioxidant in both assays.

For both assays, the antioxidant ability of fermented products was lower than Day-0. It was because the fermented products had great phytic acid reduction. Phytic acid can inhibits lipid oxidation through its ability to chelate and inactivate prooxidant metals (Graf & Eaton, 1990). After fermentation, the amount of PA decreased and its antioxidant activities also decreased.

On the FRAP assay, most of the fermented products had higher total antioxidant than Day-10. It might due to during the cultivation of mycelia on the substrates, new products (eg. phenolic compounds) were formed. The generation of these new compounds will improve the antioxidant activity of the products. However, in the TEAC assay, only the *G. lucidum*-red kidney bean fermented products had significantly higher TEAC value than Day-10. It was due to the TEAC assay was used to measure the free scavenging properties (Miller *et al.*, 1996), on the other hand the FRAP assay was used to measures the ferric reducing ability. It was used to find out the total antioxidant activities of the samples (Benzie & Strain, 1996). Therefore, the results of antioxidant activities by using these two assay methods had such differences.

For the TEAC assay (Figure 3.3), the mycelia extract of *G. lucidum* had higher antioxidant activity than the other two fungal species and its fermented products also had higher antioxidant ability than the other two fungal species fermented products in both assays. Although, the mycelia of *P. nebrodensis* and it fermented products had lowest antioxidant power, almost all of the fermented had higher antioxidant activity than the mycelia. Therefore, it can say that the products of *P. nebrodensis* had antioxidant activity improvement.

4.7 Bioconversion ability

Among the three of fungi investigated, the mycelia of *P. sajor-caju* had largest diameters and highest chitin contents when they grew on most of the substrates. Therefore the growth of *P. sajor-caju* mycelia was better than that of *P. nebrodensis* and *G. lucidum*. For the lipid content, *P. sajor-caju* was the only fungal species that can lower the lipid content of the fermented products. Moreover, the lipid content of *P. nebrodensis* and *G. lucidum* fermented products was higher than the unfermented substrates. In addition, among three kinds of fungi fermented products, it had the highest phytic acid reduction ability. Furthermore, its fermented products had the highest protein content and improvement in *in vitro* protein digestibility. Therefore *P. sajor-caju* had better bioconversion ability than *P. nebrodensis* and *G. lucidum*.

4.8 Best substrate

For the comparison between the fermented products, it was found that Hakmeitau bean was the best substrates for bioconversion. It was due to the following reasons:

1. It supported the highest growth of the fungal mycelia. Since the highest chitin content was determined on the Hakmeitau bean fermented products.

2. The lipid content of the Hakmeitau bean fermented products was quite low (0.9 % to 1.5 %), which was much lower than the oat fermented products (4.75 – 6.57 %).
3. The Hakmeitau bean fermented products had the highest protein content (27.38 – 30.15 %).
4. Very low amount of PA content (0.04 – 0.12 %) was found in Hakmeitau bean fermented products. It also had the second highest amount of PA reduction after bioconversion (around 70 % reduction in *P. sajor-caju* and *G. lucidum* fermentation).
5. None of the Hakmeitau bean fermented product was contaminated by aflatoxin.
6. The Hakmeitau bean fermented products had the highest relative FRAP activity (1.140) in *P. nebrodensis* fermentation.

4.9 Functional foods

When compared the fermented products in this research to the well-known Indonesian fungi fermented product "Tempeh", it was very similar that both of the products had phytate reduction. In addition, the fermentation process hydrolyzed the polysaccharides to simpler sugar that can improve the products digestibility. However, it was found that nutritional values of the fermented products in this research were better than Tempeh (Table 4.1). They had higher protein, carbohydrate, fiber and ash, but lower lipid than Tempeh. Moreover, the fermented products in this research had one more advantage than Tempeh, they had improvement in antioxidant activities. The intake of antioxidants has health promoting effects, it has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other

diseases associated with ageing (Cai *et al.*, 2004). Therefore, the fermented products had potential to act as functional food.

4.10 Limitation of the methodology and future development

In this research project, the experimental protocols were very straight forward. It evaluated the nutritional values and antioxidant activity of the fermented products in comparison with the starting materials. It was found that the nutritional values of the fermented products were improved. It may due to the enzymatic activities of the fungal mycelia. However, this phenomena observed had no experimental evidences to support. Therefore, further experiments are needed.

For the future development, the activities of phytase, protease, nitrogenase, α-amylase, glucoamylase, etc. should be done to find out the reason of change in nutritional values. In addition further toxicity testes (eg. MICROTOX®) should be done to ensure that the fermented products are safe to consume.

The International Life Sciences Institute of North America (ILSI, 1999) defined functional foods as foods that, by virtue of physiologically active food components, provide health benefits beyond basic nutrition. As the fermented products in this have improvements in antioxidant activities. They have potential to become functional foods, therefore the active components to cause high antioxidant activity should also be found.

Proximate composition of "Tempeh" and fermented products.

	Composition (%)	
Proximate Nutrients	Tempeh[+]	Range of 3 fungi fermented products
Protein	19.7	14.0 - 40.4
Lipid	7.7	0.7 - 6.5
Carobhydrate	17	34. 6 - 57.7
Fiber	4.8	11.1 - 22.3
Ash	1.4	1.8 - 4.1

[+] Data from USDA Nutrient Database for Standard Reference

Chapter Five: Conclusion

In general, the mycelia of all these mushrooms grew on all fifteen tested substrates. Five of the substrates support better growth and they were selected for further studies. They were hakmeitau bean (*Vigna sinensis*), mung bean (*Vigna radiata* L.), pinto bean (*Phaseolus vulgaris*), red kidney bean (*Phaseolus vulgaris*), buckwheat (*Fagopyrum esculentum*) and oat (*Avena sativa* L.). There was at most about 1 to10 % of chitin found in the fermented products. Further analyses are needed to find out the reason of difference in growth rate. After fermentation, it was found that all the fermented products were free of aflatoxin contamination. Since both of the substrates and mycelia are edible, the products are supposed to be safe to consume. Further toxicity test may be needed to confirm this.

Among the three fungi studied, *P. sajor-caju* is a better candidate to be used in bioconversion of cereal grains and legume seeds.

As a result of bioconversion, the protein content and protein digestibility of the fermented products were improved. It had higher glucose content and the antinutrient phytic acid level was also reduced. In addition, from the results of FPAP assay, it was found that the total antioxidant activity of the substrates decreased after the 10-day storage. However, most fermented products had higher antioxidant activity than Day-10 unfermented substrates. Therefore, it can conclude that bioconversion can improve the nutritional value and enhance the antioxidant activity of the substrates. The high antioxidant activity may attribute to the "functionality" quality of the fermented products.

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