Bioactive and Antioxidant Properties of Selenium enriched *Pleurotus fossulatus*

A Thesis Submitted
In partial fulfillment for the award for the
Degree of
Master of Science in Biotechnology

Submitted By:

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"Dedicated to my Family"

Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled "**Bioactive and antioxidant properties of selenium enriched *Pleurotus fossulatus***" in the partial fulfillment of the requirements for the award of the degree of Master in Science in Biotechnology, Department of Biotechnology and Environment Sciences, Thapar University , Patiala is an authentic record to my own work during a period of 6 months from January 2012 to June 2012 ,under the supervision of Dr. N. Tejo Prakash, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

Patiala Chandni Bansal

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……

This is to certify that the above statement given by the above candidate is correct and true to the best of my knowledge.

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Certificate

This is to certify that the project entitled "**Bioactive and antioxidant properties of selenium enriched *Pleurotus fossulatus***" being submitted by Ms. Chandni Bansal in partial fulfillment of the requirement for the award of degree for the Master of Science in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala ,is a bonafide work carried out under our guidance and supervision and that no part of this project has been submitted for the award of any other degree.

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Needless to say errors and ommisions are solely mine.

CHANDNI BANSAL

Summary

The present study was carried out to examine the antioxidant activity in oyster mushroom cultivated on Selenium rich substrate. *Pleurotus fossulatus* was cultivated on Se-rich wheat straw collected from the seleniferous belt of Punjab (India) and its potential to accumulate selenium from substrate was examined. Further, using different assay systems the modulations in the anti-oxidant profile of Se enriched mushroom was studied in comparison to the mushrooms cultivated on normal straw.The oyster mushrooms were observed to potentially mobilize selenium from Se-rich substrates to fruiting bodies, thus resulting in significantly high uptake. The antioxidant activity, as determined by various assays such as reducing power, 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging and metal chelating activity, was higher in experimental mushrooms when compared to control. Results obtained thus demonstrate that Se-fortified mushrooms through cultivation on straw containing organic forms of selenium, can be considered as an natural and effective dietary supplements of organic Se source for humans. The present study, thus, proposes the use of Se-rich agricultural residues as substrates for mushroom cultivation for human and livestock supplementation.

Table of Contents

1.0 Introduction

Selenium (Se), has evolved from its toxic properties to an essential trace element after a series of pioneering research endeavours over the past several decades. It was first recognized to be an essential trace element in 1957 and has been shown to be active in glutathione peroxidase (GSH-Px) (Schwaz and Foltz, 1957). Since its discovery in 1857 by a swedish chemist named Jons Jakob Berzelius selenium has been a subject of intense research. Se has an atomic weight of 78.96 and atomic number of 34. It shares similar chemical properties significantly with sulfur and to a lesser extent with tellurium. Selenium exists in four oxidation states: 0 (elemental Se), 2- (e.g, Na_2Se- Sodium Selenide ;(NH_2CH (COOH) $CH_2CH_2SeCH_3$– selenomethionine), 4+ (eg. Na_2SeO_3, sodium selenite; H_2SeO_3, selenious acid) and 6+ (e.g, Na_2SeO_4, sodium selenate; H_2SeO_4, selenic acid). Organoselenium compounds such as selenoaminoacids represent an important bio-available form of Se with selenomethionine being more bioavaliable than selenocysteine.

1.1 Selenium essentiality and biological role

Selenium is a well reported element for anti-oxidant properties in humans and animals. Biochemically, Se is a component of the enzyme glutathione peroxidase (GSH-Px), which along with superoxide dismutase (SOD), catalase (Cat) and vitamin E, protects against damage to cellular components by preventing the accumulation of peroxides in the tissue. Selenium is established as an essential trace mineral of fundamental importance to human health (Vanda Papp *et al*., 2007).

The World Health Organization report advises a Se intake of 40 µg/day as the average level needed to ensure meeting normative requirements of healthy adults. Similarly, the upper tolerable range for adults has been proposed to be 400 µg/day (FAO/WHO, 2002). The two faces of selenium, as nutrient and as a potent toxicant, make it a particularly important trace element in the health of both animals and humans. It is known primarily for its antioxidant activity and its therapeutic aspects, in addition to chemopreventive, anti-inflammatory, and antiviral properties (Rayman, 2000). Selenium

performs as an antioxidant through selenium-proteins. Selenoproteins have a key role in a variety of biological processes with some of them being associated with antioxidant defense mechanisms (Brenneisen *et al*., 2005). Selenium supplementation in acute and chronic exercise increases antioxidant activity, and thereby preventing lipid peroxidation (Zamora *et al*., 1995).

Selenium exerts its biological effect through variety of selenoproteins These selenoproteins include a number of glutathione peroxidases (GPx) including cellular GPx (GPx1) and phospholipid hydroperoxide GPx (PHGPx; GPx4), iodothyronine 50-deiodinases (IDI), sperm capsule selenoprotein and thioredoxin reductase, selenoprotein P (SePP) and selenoprotein W (Burk and Levander, 1999; Holben and Smith, 1999). Selenium functions in the body as an antioxidant, in thyroid hormone metabolism, redox reactions, reproduction and immune function (Rayman, 2000). Selenium deficiency in humans is rare, but is seen as Keshan disease, an endemic cardiomyopathy that occurs during preadolescent or adolescent years (Keshan Disease Research Group, 1979a, b) and as an endemic osteoarthritis, Kashin–Beck disease (Levander, 1987), both of which have been reported in low selenium areas of China. Low selenium status has also been associated with a number of chronic diseases such as cancer (Ip, 1998), cardiovascular disease (Ne`ve, 1996), asthma and many others (Rayman, 2000; Brown and Arthur, 2001).

1.2 Status of Seleniferous environment in India

For the past few years, the attention of several workers has been engaged in the study of the role of Se in Indian soil and plant and its impact on human and animal health. The soils can be classified as seleniferous or non-seleniferous depending on the Se level of non-accumulators (cultivated agricultural crops) grown on that soil. The soils containing as low as 0.1-0.5 mg Se/ kg (Ravikovitch and Margolin, 1957; Dhillon *et al*., 1992) are considered as seleniferous because the forages grown on such soils contain 0.4mg Se/ kg^{-}, i.e., the maximum permissible level for animal consumption. In India, the Se toxic sites identified included parts of Northwest region of Punjab (Dhillon and Dhillon, 1991), Haryana (Arora *et al*., 1975) and some locations in North-eastern states

of Assam and Meghalaya (Dey *et al*., 1999). Among all the locations identified, agriculturally rich belt along the border of Nawashahr-Hoshairpur districts of Punjab has gained prominence due to extensive research and agri-extension activity (Dhillon and Dhillon, 2003). The affected villages, covering over 1000 acres are Barwa, Bhano majra, Sikandarpur, Rakkar, Simbli, Jainpur, Mahenpur and Nazarpur (Dhillon and Dhillon, 1997). Preliminary report on chronic Se toxicity as a cause of hoof and horn diseases in livestock in seleniferous regions of Punjab was given by Gupta *et al*. (1982). Limited observations on human population in Se-rich regions of Punjab state had also shown signs of toxicity with selenosis in humans, mainly due to consumption of grains and vegetables harvested from Se-rich agricultural soils (Hira *et al*., 2003). Toxicity symptoms in humans include anorexia, watery diarrhoea, laboured breathing, elevated temperature and pulse rate, prostration and often death from respiratory failure. Toxicity to plants was seen in wheat, sugarcane that exhibited papery snow white chlorosis of leaves with pinkish coloration on the sheath and lower surface of leaves (Dhillon *et al*., 1992).

1.3 Selenium mobilization and biofortification

It has been seen that the vast majority of the world's population has sub-optimal Se intakes, and hence are at increased risk of exposure to several diseases such as cancer, heart diseases, viral diseases and other conditions that involve increased levels of oxidative stress (Combs, 2001). Adequate selenium supplementation is required to meet the normative requirements of healthy adults.

Although, it has been known since late 1800s that edible flora can accumulate extraordinary levels of metals and clean-up affected environments, the use of these systems as nutrient-supplements has been very limited. As many of the metals that can be hyperaccumlated are also essential nutrients, edible plants that accumulate elements such as selenium may be used as a natural source of mineral supplements for both animals and human beings, especially in areas that are mineral deficient. Plants have been providing the sole source of food, feed and fiber to society for many centuries. The concept of using plants in place of synthetic nutrient sources to supply the directly needed macro and

micro-elements is gaining significant attention and constitutes the first step towards biotechnology-based, nutritionally fortified food. The field of plant material nutrition has been around for a long time, but the idea of fortifying food at pre-harvest stage with essential minerals required for healthy diet is relatively new. By producing staple foods whose edible portions are denser in bioavailable minerals and vitamins, a process referred to here as "biofortification", researchers are attempting to provide farmers with crop varieties that can effectively reduce nutritionally related health problems through natural diet of human population. Among plant foods, wheat, pulses and certain vegetable crops are rich sources of selenium. Biofortification programme, propagated across the globe, is already focusing dominantly on three micronutrients viz., iron, zinc and vitamin A, as majority of world's population is suffering from their deficiencies. Selenium in food may vary from, 3 μg/ day in selenium deficient areas to about 7000 μg/ day (Fordyce, 2005; Rayman,2002) in areas where selenosis is endemic, and biofortification and supplementation of Se rich foods can be appropriate sources for facilitating bioavailability of selenium to human and livestock population.

1.4 Mushrooms as dietary sources of Selenium

Selenium deficiency can effectively tackled by supplementation through food sources necessitating the identification of appropriate sources of selenium. Different food sources of plant and animal origin are known to be potential dietary sources of selenium. The Selenium in the diet are generally available through wheat, bread, meat, fish, eggs and milk/dairy products. Among the crop plants, wheat, maize, pulses and certain vegetables crops such as *Allium sativum*, broccoli, garlic and mushrooms are known to be good sources of selenium. Edible mushrooms are known to be selenium accumulators (Ogra *et al*., 2004). Mushrooms have been a part of the human diet for thousands of years. Although mushrooms have long been appreciated because of their flavour, texture and medical properties, the recognition of their nutritional importance is recent. Mushroom consumption has markedly increased throughout the world; it includes different genera such as, *Auricularia*, *Flammulina*, *Ganoderma*, *Grifola*, *Hericium*, *Lentinus* (*Lentinula*), *Pleurotus*, *Trametes* (*Coriolus*), *Schizophyllum*, and *Tremella*

which have been demonstrated to possess significant medicinal properties (Wasser, 1995). The genus *Pleurotus* (Oyster mushroom) includes commercially important edible and medicinal species. Reported medicinal properties of this genus include reduction of cholesterol levels, antitumor activity, antiviral, antibacterial and immunomodulating activity. Mushroom species grow and yield on a spectrum of agricultural residues and by-products, such as paddy, wheat, barley, sunflower, maize and weed straws, jowar and bajra stalks, ground haulms, pod shells, cotton wastes, mango stiff, jute coir pith, sugarcane bagasse, water hyacinth, rubber wood dust and tree leaves (Khanna , 2003).

Each year, in Punjab, tons of agricultural residues are being generated which are either burnt as a regular practice or used for composting in some cases. The agri-wastes generated in seleniferous region of Punjab, contain significant levels of selenium accumulated in plant parts, as reported in leaves and straw of wheat, rice and cereals (Dhillon and Dhillon, 2003; Sharma *et al*., 2009), and therefore have potential use as substrates for cultivation of selenium enriched mushrooms, leading to their appropriate utilization

Extensive research has been carried out on Se uptake by edible mushrooms cultivated on substrates supplemented exogenously with inorganic selenium, but to the best of our knowledge no study has been carried out on mushrooms cultivated on substrates hyperaccumulated with selenium through natural processes. Therefore these naturally rich substrates can be used as raw material for cultivating se-enriched mushrooms. Moreover the use of these residues in production of enriched products (potential neutraceuticals) prevents their release into the environment, increases producer's income and leads to food product with high nutritional quality which can thereby be used to meet the normative requirements of people in Se deficient area. Keeping this in view, the present study aimed at exploring the cultivation of *Pleurotus fossulatus* on Se-rich wheat straw and determining its antioxidant profile as induced by enrichment of selenium.

2.0 Review of Literature

2.1 Selenium in Environment

2.1.1 *Occurrence, properties and uses*

Selenium (Se) is a comparatively rare, though being a widely distributed, element. Chemically, it is classified as a metalloid, with properties of both metals and non-metals. It is closely related to sulphur (S) in structure and function. Several oxidation states are possible for selenium with the main oxidation numbers being -II (selenide),0, +IV (selenite), and +VI (selenate) and the following redox processes are possible:

$$Se^{2-} \leftrightarrow Se^{0} \leftrightarrow SeO_3^{2-} \leftrightarrow SeO_4^{2-}$$

selenide element selenite selenate

It has six naturally occurring stable isotopes with varying degrees of abundance: ^{74}Se (0.9%), ^{76} Se (9.0%), ^{77}Se (7.6%), ^{78} Se (23.5), ^{80} Se (49.7%) and ^{82} Se (9.2%).

Se has distinctive physical properties, which account for its industrial use, in dry photocopiers, photoreceptors and semiconductors, as well as in the manufacture of glass and pigments. In addition, considerable quantities of selenium compounds are used as additives in fertilizers and as dietary supplements for farm animals and humans.

2.1.2 Distribution of selenium in environment

a. Environmental sources of selenium

Globally, total selenium concentrations typically lie in the range 0.01-2.0 mg/kg with an overall mean of 0.4 mg/kg (Fordyce, 2005). Much higher concentrations (1200 mg/kg) are found in soils derived from seleniferous parent materials, including shales, sandstones, limestones, slate and coal series (Johnson *et al*., 2010). The process of distribution of Se through the environment involves a variety of physical, chemical and biological activities including natural sources such as volcanic activity, weathering of rocks and soils, mineral formation, in addition to anthropogenic sources such as soil leaching, ground water transport, combustion of fossil fuels, agricultural inputs such as fertilizers, and release and disposal of sewage-sludge (Fordyce, 2005).

b. Selenium in soil

The Se concentrations of most soils in the world are low (normal range 0.01–2.0mg /kg; mean 0.4mg /kg), although concentrations ≤1200 mg /kg can occur in seleniferous soils (Fordyce, 2005). Seleniferous soils are widespread in the Great Plains of the USA, Canada, South America, China and Russia. Other notable inputs of Se to soils include atmospheric deposition of Se originating from volcanic activity, weathering of rocks, sea spray and volatilisation–recycling from biota. All these factors contribute to global Se cycling (Broadley *et al*., 2006). In North- eastern parts of Punjab, India, Se concentration in oats, corn and barley cultivated on seleniferous soils have been found to contain up to 35-41 mg/kg (Dhillon and Dhillon, 1990). Among non-accumulators, broccoli, wild mustard and other members of the genus Brassica (Bañuelos and Schrale, 1989), crucifers (Bisbjerg, 1972) and soybeans (Gupta and MacLoed, 1994) contain higher Se than other crops (Gupta and Gupta, 2000). In the Nawanshahr–Hoshiarpur region of Punjab, India, more than 1000 hectares of agricultural land is significantly affected by high levels of selenium (Dhillon and Dhillon, 2003). Studies were carried out to examine Se levels in soil and crops such as wheat grains, wheat husk, rice, maize and mustard using neutron activation analysis. The Se concentrations in soil and crop products were found to be ranging from 2.7 to 6.5 mg /kg and 13 to 670 mg/ kg, respectively, indicating significantly high selenium in these crop products. (Sharma *et al*., 2009)

Crop selenium uptake is influenced greatly by the availability and chemical species in soils.In organic phases occurs in three soil-phases- fixed,adsorbed, and soluble. Only soluble/adsorbed forms of selenium are thought to be bioavaliable for plant uptake. In addition, availability of selenate (+4) forms to plants varies markedly,with selenate (+6) taken up much more rapidly than selenite under most soil conditions.

c. Selenium in food

The Se content of food is highly dependent on the amount of Se in the soil, which varies from country to country and from region to region. The amount of selenium in the diet largely depends on where crops are grown and cultivated, the soil/fodder to which

animals are exposed, and the actual foods consumed. Levels usually found in different foods are in the microgram range: ~10-500 μg/kg. The main food groups providing selenium in the diet are cereals, meat, fish/eggs, and milk/dairy products. Certain plants, termed as hyperaccumulators, have the ability to accumulate Se from the soil to significantly high levels. Plants have been divided into three ecological types of 'non-accumulator', 'Se-indicator' and 'Se-accumulator' plants (Rosenfeld and Beath, 1964; Brown and Shrift, 1982; Dhillon and Dhillon, 2003). Non-accumulator plants are unable to grow on seleniferous soils and Se is toxic at tissue concentrations as low as 10–100 mg Se/g dry matter (Rosenfeld and Beath,1964; White *et al*., 2004), whereas Se-indicator plants can colonize both non-seleniferous and seleniferous soils and tolerate tissue Se concentrations approaching 1000 mg Se/g dry matter (Rosenfeld and Beath, 1964; Rodriguez *et al*., 2005).Among these are Brazil nuts, which can contain more than 50 mg/kg of the element which is 10^5 times levels found in most other foods (Dumont *et al*., 2006) Some of these rich sources of dietary Se, are listed in Table 1

d. Table 1. Selenium content of some selected foods and food groups

Food Groups	Se content (μg/g, fresh weight)
Cereals and cereal products	0.01-0.55
Meat, fish and eggs	0.01-0.36
Milk and Dairy products	<0.001-0.17
Vegetables and fruits	0.001-0.022
Selected high-Se foods	
Beef kidney	0.78-1.45
Crab	0.028-1.26
Lobster roe	0.08-4.43
Brazilian nuts	0.53-0.85
Broccoli	<0.001-0.46
Mushrooms	0.01-1.40

Source: Reilly (1996)

Selenium in foods and biological materials can exist in both organic and inorganic chemical forms (Lobinski *et al*., 2000; Dumont *et al*., 2006). In turn, the chemical form of the Se can affect its bioavailability from diet. In general, the organic forms, such as selenomethionine, are more bioavailable than the inorganic selenites or selenates.

d. Selenium intake: Global variation

Individual dietary selenium intakes across the world are estimated to range from 3 to 7000 μg/day (Fordyce, 2005; Rayman, 2002; Rayman, 2008; WHO, 2004).The highest levels of intake have been recorded in seleniferous regions of China [>4990 μg/day (Yang *et al*., 1983) and Venezuela (Rayman, 2004; Rayman, 2005). For European countries where estimates are available, mean intakes are typically <50 μg/day per person (Rayman, 2004; Rayman, 2005), which is close to or below the recommended nutrient intake level (Flynn *et al*., 2009; Rayman, 2008). In regions of relatively high selenium intakes in India, intakes were estimated to be 475 μg/day for woman and 632 μg/day for men, with >80% of the selenium intake provided through consumption of cereals grown in high selenium soil in the local region (Hira *et al*., 2003).

With respect to the selenium intake from habitual diet, the UK is one of the countries with the lowest estimated selenium intake. The mean selenium intake from food sources for men and women were 55 and 43μg/day respectively. In comparison, intakes in the United States are much higher: the mean intake was 133.5 ± 2.42 μg /day for men and 92.6 ± 1.57 μg/day for women (USARS, 2008).

The term essential trace element is often used to indicate an element with an established, estimated or suspected requirement of less than 1 mg /day usually indicated as μg /day (RDA, 1989). Two faces of selenium, as nutrient and as a potential toxin, make it particularly important trace element in the health of both animals and human. The narrow margin between beneficial and harmful levels has important implications for human activities that increase the amount of selenium in the environment. Selenium has three levels of biological activity (Hamilton, 2004): (i) trace concentrations are required for normal growth and development; (ii) moderate concentrations can be stored to maintain homoeostatic functions; and (iii) elevated concentrations can result in toxic effects. The World Health Organization states that a Se intake of 40μg/day is the average

intake level needed to ensure meeting normative requirements of healthy adults (Combs, 2001).

2.2 Biological roles of selenium

Historically, the perspective of selenium has changed dramatically over the last century. Initially, selenium was widely considered to be a toxic agent in mammals. In the 1930s, this element was found to be responsible for several illnesses. Thus, it was quite surprising when Schwarz and Foltz reported in 1957, that selenium prevented liver necrosis in rats under vitamin E deficiency. This finding and a report that selenium was important in anaerobic growth of *Escherichia coli* (Pinsent,1954) when grown in glucose provided evidence that selenium was an important nutrient for mammals and for certain bacteria.

2.2.1 Antioxidant, anti-inflammatory, and immune-stimulatory roles of selenium

Selenium exerts its biologic functions largely through its presence in selenoproteins. Only a few of the 25 identified mammalian selenoproteins have so far been functionally characterized. Most of these selenoproteins exhibit enzymatic redox function *via* selenocysteine (Sec), which confers their catalytic or antioxidant activities. Cellular processes so far demonstrated to require selenoproteins include biosynthesis of dNTPs for DNA, removal of damaging or signaling peroxides, reduction of oxidized proteins and membranes, regulation of redox signaling, thyroid hormone metabolism, selenium transport and storage and potentially protein folding. Some low-molecular weight selenium compounds, such as methyl selenic acid, methyl-selenocysteine, and SeMet, have been found efficient as antitumorigenic agents in animal studies and *in vitro* models (Ganther, 1999). In functionally characterized selenoenzymes, Sec is part of the catalytic group within their active site and is directly involved in redox reactions (Zhong & Holmgren, 2000).

Selenocysteine, the 21st amino acid, present in selenoproteins having have important enzyme functions in humans. Glutathione peroxidase (of which at least five forms exist) has an antioxidant role in reducing damaging hydrogen peroxide and

lipid/phospholipid hydroperoxides produced in eicosanoid synthesis by the lipoxygenase and cyclo- oxygenase pathways (Spallholz *et al.,* 1990). This function reduces damage to lipids, lipoproteins and DNA, and hence reduces risk of cardiovascular disease and cancer (Neve, 1996). Moreover, selenite inhibits tumour necrosis factor-alpha-induced expression of adhesion molecules that promote inflammation (Zhang *et al*. 2002). There is a growing body of evidence to suggest that Se (especially in the sodium selenite form) can alleviate conditions associated with high levels of oxidative stress or inflammation (Prabhu *et al*. 2002; Davis *et al*. 2002; Vunta *et al*. 2008; Chen *et al*. 2009). These include asthma (Jahnova *et al.,* 2002), diabetes (Kowluru *et al,* 2001), arthritis (Peretz *et al.,* 2001), muscular dystrophy (Kurihara *et al.,* 2000), cystic fibrosis (Kauf *et al.,* 1994), acute pancreatitis (Castano *et al.,* 2000), osteoarthritis (Kurz *et al.,* 2002), systemic inflammatory response syndrome (Angstwurm *et al.* 1999) and kwashiorkor (Ashour *et al.* 1999). In addition, Schrauzer (1998) discussed the application of selenite therapy to viral haemorrhagic fever, acute septicaemia and lymphoedema. Another group of selenoenzymes, the thioredoxin reductases are involved in reduction of nucleotides in DNA synthesis, regeneration of antioxidant systems, and maintenance of intracellular redox state (Allan *et al.,* 1999).

The thyroid gland has the highest Se concentration of any human organ (Kohrle, 1999), and Se is involved in thyroid metabolism through the iodothyronine deiodinases, which catalyse the production of active thyroid hormone, T3, from thyroxine, T4 (Beckett *et al.,* 1987). The iodine deficiency diseases goitre and myxedematous cretinism are more prevalent in central Africa in those regions which are deficient in both iodine and Se (Vanderpas *et al.,* 1993), and in such areas supplementation with both nutrients is indicated. Se has a role in many aspects of the immune response to infections. Se deficiency reduces immunocompetence, involving impairment of neutrophil, macrophage and polymorphonuclear leukocyte activity (Boyne & Arthur, 1986; Spallholz *et al.* 1990). Se supplementation of even supposedly Se-replete individuals is immunostimulatory, and involves enhancement of natural-killer-cell and lymphocyte activity as well as enhancement of proliferation of activated T-cells (Kiremidjian- Schumacher *et al.,* 1996).

Selenium compounds have shown protective effects against various cancers. Both organic and inorganic forms of selenium have been used. The prototypes most commonly used are sodium selenite and selenomethionine. The majority of the epidemiologic and prospective studies conducted in different worldwide regions have shown an inverse relation between selenium status and risk of cancer and cancer mortality (Surai, 2006). Several case–control studies also confirmed that selenium levels in blood, serum, hair, and toenails are lower in cancer patients than in control subjects (Surai, 2006). A significant inverse correlation between baseline selenium and death of esophageal and gastric cancer (Wei *et al*., 2004), as well as lung cancer risk (Reid *et al*., 2002), has been found. These findings suggest that selenium supplementation may have protective effects against some types of cancer in individuals with lower dietary selenium intake.

2.3 Health effects of Selenium

The effects of selenium on the organism are concentration dependent, ranging from essential to antioxidant in the nanomolar-micromolar range to potentially prooxidant at concentrations above what is required for maximal selenoprotein synthesis (Vinceti *et al*., 2001). At even higher concentration, selenium compound may accumulate and redox cycle with intracellular thiols, leading to oxidative stress and damage to cellular components, thus having toxic effects.

2.3.1 Selenium toxicity

In animals, the amount of dietary Se necessary to cause chronic toxicity is influenced by the form of Se, type of diet, and species (5 mg/kg in cattle, 2 mg/kg in sheep). Farm animals raised in certain seleniferous regions of China and the U.S. have developed toxicity diseases from consumption of Se-rich plants native to these regions.(ICPS,1987). Environmental contamination caused by drainage of seleniferous irrigation waters into certain wildlife habitats has increased concern over toxicity issues associated with this element.(Levander, 1991). Garlic breath odour is indicative of excess

Se exposure in humans, although more consistent signs of human selenosis include morphological changes in fingernails and hair loss as observed in parts of China where the average human dietary intake has been recorded as 900 μg/day (Dietary Reference Values for food, energy and nutrients – UK, 1991). Based on such observations, the U.K. Department of Health has recommended the maximum safe Se intake from all sources to be 450 μg (5.7 μmol) / day for adult males, which corresponds to 6 μg/kg/d.(Longnecker *et al*., 1991). In seleniferous regions (Venezuela and South Dakota), daily intakes of 300 to 724 μg/d have been reported, but with no detectable adverse effects (Reilly, 1993; Flodin, 1990). Peripheral neuropathy has been reported in the U.S. following consumption of "health food" supplements containing almost 200 times the stated dose of Se. Although several biochemical mechanisms have been proposed for Se poisoning, no specific index has been defined as yet.

2.3.2 Selenium deficiency

Selenium has the narrowest range between dietary deficiency (< 40 μg/day) and toxic levels (>400 μg/day). Selenium deficiency is more widespread than selenosis. Selenium is essential for life, adequate amounts of this element are required for optimal human health. Many of its physiologic roles are directly attributed to its presence within selenoproteins. It has been recognized as antioxidant and its presence is related with the reduction of certain types of cancer and other diseases. However, in several regions of the world the content of selenium in diet has been estimated insufficient to reach the correct activity of protective selenoenzymes. It justifies the growth interest in the production of selenium-enriched food and nutritional supplements and in parallel the necessity to develop new analytical methodologies for selenium characterization in these matrices.

Moderate selenium deficiency has been linked to many conditions, such as increased cancer and infection risk, male infertility, decrease in immune and thyroid function, and several neurologic conditions, including Alzheimer's and Parkinson's disease (Rayman, 2000). Two endemic diseases related to insufficient intake of Se have been elucidated in China in a region where the soil is depleted of Se leading to low Se concentration in crops and vegetables.

Keshan disease: is a cardiovascular disease, which mainly affects children and young women. The Main clinical features of Keshan disease are acute and chronic episodes of a heart disorder characterized by cardiogenic shock and/or congestive heart failure. Dilation of the heart is commonly observed (Ge and Yang, 1993). Supplementation of individuals with selenium tablets (as sodium selenate) has been effective in preventing the development of Keshan disease (Yang, 2006). Kashin –Beck disease is an endemic, chronic, degenerative ostreoarthropathy that is present in selenium-deficient areas in the world, and is mainly found in a diagonal belt from northeast to southwest China, and also in Mongolia, Siberia, and North Korea. This disease might occur due to combination of several factors but dominantly including low Se-intake and high oxidative stress.

2.4 *Biofortification of Se*

As a potential step towards finding a solution for elevated Se in the environment, as well as nutritional Se deficiency, better knowledge of Se metabolism in higher plants is crucial. Plant uptake and metabolism of Se can be exploited for the purposes of genetic biofortification (i.e. the development of high-Se crop cultivars combined with Se fertilization) and phytoremediation (plant-mediated removal of excess Se from soil or water). It is for these reasons that there is a resurgence of interest in Se in higher plants and, during the past few years, significant progress has been made in this area (Zhu,Y.G. *et al*., 2009). To increase mineral concentrations in edible tissues, without loss of yield, there must be increased uptake by roots (of minerals present in the soil solution) or leaves (for foliar applied minerals), effective redistribution within the plant to the edible portion, and accumulation in edible tissues in a nontoxic form (Welch and Graham, 2005).Fortification within the edible plant enhances nutritional quality for all types of food materials. A new paradigm for agriculture in the 21st century was proposed (Welch and Graham, 2000) that views agriculture as an instrument for public health and focuses attention on the role of agriculture in delivering nutrients to humans and animals in balanced amounts that can sustain maximal physical and mental activity of the humans.

The biofortification concept seeks to bring the full potential of agricultural and nutrition science to bear on the persistent problem of micronutrient malnutrition.

2.5 *Fortified Crops*

Salt fortified with Se has been used in parts of China where the soil is naturally depleted (Lyons *et al*., 2004a). In Japan and some other Asian countries several different kinds of Se-enriched foods are marketed on the grounds of their health-enhancing properties. In China a Se-rich drink which, it is claimed, helps to prevent ageing and heart disease, uses Se-rich green tea as the source of the element (Reilly, 1996). Products based on garlic, which can contain relatively high levels of Se, as well as Se-rich nuts and other foods, are also available. An intriguing possibility is the development of products using Se-enhanced `designer' eggs from hens fed fortified layer rations (Cantor, 1997).

Cruciferous vegetables, including broccoli, typically contain low amounts of Se (0.1-0.3 μg /g DW), but when grown in the presence of adequate Se, many have the unique ability to accumulate concentrations of Se many orders of magnitude above normal. For example broccoli grown on the west side of the central valley of California may contain up to 0.007 mg /kg DW (Banuelos, 2002) and broccoli grown under experimental conditions may accumulate as much as 0.950 mg /kg DW (Finley *et al*., 2000a). Similarly aqueous extracts of mushroom (*Agaricus bisporus*) grown on substrates supplemented with 100mg/l selenite (enriched through spraying) showed a selenium concentration of 0.051.8 ± 0.005 mg /kg (Cremades, 2012).

2.6 *Mushrooms: Potential selenium fortifiers*

Over the past decade, in an attempt to find natural sources of antioxidants, many species of fruits, vegetables, herbs, cereals, seeds and mushrooms have been investigated for antioxidant activities (Wong and Chye, 2009). Amongst these sources, harmless substances are of great interest. In this context, due to their high volume of production using mechanised systems, seasonal independence and their safety and acceptance by

consumers, edible mushrooms, can be very important for obtaining extracts with antioxidant activity, especially selenium-enriched extracts.

In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues such as cassava bagasse, sugar cane bagasse, sugar beet pulp, coffee pulp/husk, apple pomace, etc towards variety of bioprocess applications. Several processes have been developed that utilize these as raw materials for the production of bulk chemicals and value-added fine products such as ethanol, single cell protein (SCP), mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, etc. (Pandey, 1992, 1994). Application of agro-industrial residues in bioprocesses on the one hand provides alternative substrates, and on the other hand helps in solving pollution problems, which their disposal may otherwise cause. Thus these agricultural residues can effectively be used as substrates for mushroom cultivation.

Mushrooms constitute a very important and highly appreciated source of food worldwide. They are appreciated, not only for their texture and flavour, but also for their nutritional value. Different edible species of mushroom such as *Agaricus, Pleurotus, Volvariella, Ganoderma* and *Lentinus* are cultivated worldwide. *Pleurotus* mushrooms have high nutritional value and can be a good source of protein, carbohydrates, vitamins, calcium and iron (Schmidt *et al*., 2003). Furthermore, these mushrooms have important medicinal properties, such as anti-tumour and immunostimulatory activity, as observed in rats (Sarangi *et al*., 2006). The products derived from *Pleurotus* mycelia can promote biological responses during cancer treatment in humans and have been used as antitumourogenic drugs (Sarangi *et al*., 2006). Edible mushrooms are a good source of protein and fibre and make a useful contribution to vitamin and mineral intake (Kalac, 2009). It is known that some mushroom species have the capacity to accumulate selenium (Kalac and Svoboda, 2000). The amount of selenium found in mushrooms is dependent on the species, the stage of maturity, the amount of selenium in soil, and the substrates used for growth of cultivated species (Kalac, 2009). Mushrooms have known antioxidant properties provided by different compounds such as phenolics, ergothioneine (ERGO) and selenium (Se) (Mau *et al*., 2002; Werner and Beelman , 2002; Beelman and Royse, 2006). Selenium content in mushrooms is species specific (Stijve, 1977). *Boletus edulis* for example, may have concentrations of up to 17 mg/kg DW, while wild *Agaricus* spp.

may contain 2.7 mg/kg DW and *P. cornucopiae* and *Grifola frondosa* may have less than 0.5 mg/kg DW (Beelman and Royse, 2006). Researchers have found that supplementation of the substrate with sodium selenite (Na_2SeO_3) results in an increase in the Se content in mushroom mycelium and basidiomata (Werner and Beelman, 2002, Beelman and Royse, 2006). Werner and Beelman (2002) demonstrated that Se accumulated in *A. bisporus* basidiomata when the substrate was supplemented with aqueous solutions of Na_2SeO_3 at different concentrations and that the Se uptake by *A. bisporus* basidiomata was linearly related to concentration in the compost.

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The review of literature, although indicated extensive studies on Se uptake by edible mushrooms cultivated on substrates supplemented with Se exogenously, there is limited evidence on Se uptake and biological properties of the Se fortified mushrooms. The earlier observations reported by various researchers also show that Se uptake and speciation studies were carried out only in mushroom cultivated on substrates enriched with Se through its supplementation in the growth or aqueous media. To the best of our knowledge, no study is available on accumulation and speciation of Se in mushrooms grown on agri-residues hyper-accumulated with Se through natural processes. Keeping in view (a) the studies on Se uptake in mushrooms and (b) the anticipated benefit facilitating a re-use of agri-residues in view, the present study examined

(a) Cultivation of oyster mushroom (*Pleurotus fossulatus*) on Se-rich agricultural residues,

(b) Determine the uptake of selenium by *P. fossulatus* cultivated on se-rich residue, and

(c) Examine the anti-oxidant and related properties of Se-enriched fruiting bodies.

3.0 Material and Methods

3.1 Sample collection

The selenium rich agricultural residues of wheat crop collected from the village of Jainpur (31°13' N, 76°21' E), Nawanshahr- Hoshiarpur region, Punjab, India), were used as substrates for cultivation of Se-enriched mushrooms. Similarly non-seleniferous (control) agricultural residues collected from Patiala (Punjab, India) were used for cultivation of control mushrooms.

3.2 Mushroom Cultivation

3.2.1 Culture collection

The strain of *Pleurotus fossulatus* was procured from National Research Centre of Mushroom (NRCM), Solan (Himachal Pradesh, India). This fungus was cultured on Potato dextrose agar (PDA) medium and was stored at 4ºC till use.

3.2.2 Spawn preparation

Selenium rich wheat grains were washed and boiled (25 minutes) till it get softened. Boiled wheat was thoroughly mixed with 2% calcium sulphate and 0.5% calcium carbonate to absorb the excess moisture. The resultant mixture was filled in plastic bags (approximately 500 gm/bag) and was inoculated with the desired culture in the form of fungal discs (5mm). The inoculated bags were incubated at 25°C for 7 days until there was visible growth of dense mycelium (completely run spawn). Similar procedure was followed for preparation of spawn for control mushroom by using non-enriched wheat grains.

3.2.3 Spawning of substrates

Prior to spawning, selenium rich wheat straw was disinfected by dipping in water containing 1.5% formalin. The treated straw was then inoculated with spawn (approx. 3.0 %) and was filled in plastic bags (3-4 kg/bag). The spawned bags were then incubated in growth chamber at 25 ± 3°C for approximately 15 days in suspended position till entire straw got tightly bound by mycelia (spawn run). The plastic layers of completely

colonized bags were torn off and were left suspended until fruiting. Similar conditions were followed to grow control mushrooms, with non-Se wheat straw as the substrate. Fruiting bodies (Figure 1) of *Pleurotus fossulatus* were collected after 4-5 days, by cutting the base of the stipe with sterile surgical blade, weighed and dried at 40ºC for near complete dehydration.

Fig 1. Fruiting of *P.fossulatus cultivated on Se-rich wheat straw*

3.3 Determination of total selenium

Working standard of solutions of selenium in the range of 2.0 -10.0 ng/ml was prepared in 0.1 N HCl . Dried fruiting bodies of *P.fossulatus* (control and experimental) from the first flush were crushed to powder with the help of mortar and pestle. Selenium content of powdered samples were analysed using florescence spectrometer after a triacid (nitric acid+ perchloric acid+ hydrochloric acid) digestion (Levesque and Vendette, 1970) followed by reaction with 2,3-diaminonaphthalene (DAN). The Se content was also determined in substrate. In brief, 100 mg portion of the sample (fruiting body/straw) was weighed accurately in an acid washed kjeldahl flask containing 5 ml concentrated nitric acid. After 30 min at room temperature, 2 ml of perchloric acid (72%) was added and kept on kjeldahl digestion unit for 20 min until emission of $HClO_4$ fumes and

condensation of acid in the neck of the flask. The process of digestion was considered to be complete when the condensation ring reached the top of the neck. The mixture was then allowed to cool to room temperature. To facilitate the reduction of selenate (SeO_4^{2-}) to selenite (SeO_3^{2-}), 2 ml of 1.0 N HCI was added and the flask was placed in the water bath (100^o C) for 15 min. The digest was quantitatively transferred to graduated tubes (50 ml), washed and made to volume (25 ml) with 0.1N HCl. 1 ml of the digest was added to 200 µl of 1:1 formic acid and 200 µl of stabilizing solution (A 0.04 M solution of Na_2EDTA which contained 10% hydroxylamine hydrochloride ($NH_2OH.HCl$). The digest was titrated to pH 1.8 with 4.0 N NH_4OH and placed in a water bath at 50^0C for 10 min. 2 ml DAN (0.1% in 0.1 M HCl) was added to the reaction mixture, shaken thoroughly (20 sec) and kept in water bath at 50º C for 30 min. After cooling to room temperature, 5 ml of cyclohexane were added, and the contents were vigorously mixed, and allowed to separate. The organic phase (-3 ml) was separated and assayed fluorimetrically (Perkin-Elmer -LS45) at an excitation wavelength of 360 nm and emission wavelength of 520 nm.

3.4 Basic Composition of Mushrooms

Carbon, hydrogen and nitrogen content (%) was determined in powdered sample of mushroom by using CHN analyzer (Flash EA 1112 series). Total protein content in the mushroom was determined by the method of Lowry (1951).

3.4.1 Mushroom Extraction

Prior to use, the fruiting bodies were milled until a fine powder was obtained. One gram of the samples (in case of estimations of total phenols and total antioxidants) and 100mg in case of other assays were subjected to stirring with 10 ml of 20% and 90% methanol for 2 h respectively, at room temperature using ultrasonicator bath, and filtered through Whatman 1 paper. The resultant extracts were stored at 4°C till use.

3.5 Analysis of Antioxidant properties

3.5.1 Total phenol content

Total phenolic content of both the extracts (20 & 90% methanol) was analysed using the Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965) using quercetin as standard, with some modifications. Briefly, in 0.1 ml of sample was added in 0.1 ml of Folin and Ciocalteu's phenol reagent. The mixture was incubated at room temperature for 3 min followed by addition of 0.1 ml of saturated Na_2CO_3 solution. The final volume was made up to 1 ml with distilled water. The reaction mixture was incubated for 90 min in dark condition. The absorbance was measured at 765 nm using UV-visible spectrometer (Hitachi –U 2900). Gallic acid at concentrations of 0.05-0.10 mg/ml was used to make standard curve. The mean values were expressed as mg of Gallic acid equivalents (GAE) per gram of extract.

3.5.2 Lipid Peroxidation assay by TBARS method

Lipid peroxides were extracted by grinding the sample with 3 ml of 5% (w/v) metaphosphoric acid and 100 ml of 2% (w/v) butyl hydroxytoluene (in ethanol). Homogenates were filtered and centrifuged at 15,000g for 20 min. The chromogen was formed by mixing 500µl of supernatant, 50 µl of 0.2% (w/v) butyl hydroxytoluene (BHT), 250 µl of 1% (w/v) Thiobarbituric acid (TBA) (in 50 mm NaOH), and 250 µl of 25% (v/v) HCl, and by incubating the reaction mixtures at 95°C for 30 min (Minotti and Aust, 1987). A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 50 mm NaOH. The reaction was stopped by cooling the samples in an ice bath. For determination of thiobarbituric acid reactive substances (TBARS), the chromogen formed was extracted by adding 2 ml of 1-butanol, the tubes were vigorously shaken, the organic (upper) phase was separated by low speed centrifugation and assayed fluorimetrically (Perkin-Elmer-LS45) at an excitation wavelength of 532 nm and emission wavelength of 550 nm. Calibration curves were made using Malondialdehyde (MDA;Sigma) in the range of 0.5-5.0 µM.

3.5.3 Total antioxidant activity

Total antioxidant activity of methanolic extract of *P. fossulatus* was measured according to the method (phospho-molybdenum assay) outlined by Imran *et al*. (2011). Different concentrations of BHT (0.1 – 1.0 mg/ml) were prepared. 0.3 ml of each of the methanolic extracts was taken in test tube to which 10 ml of 0.6 M sulphuric acid, 10 ml of 28 mM sodium phosphate and 10 ml of 4 mM ammonium was added. 0.3 ml of methanol served as blank. All the tubes were incubated at 95° C for 90 min and cooled to room temperature. The optical density was measured at 695 nm using UV-visible spectrometer (Hitachi –U 2900).

3.5.4 Reducing power

The Fe-reducing power of the extract was determined by the method of Oyaizu (1986) with a minor modification. 25 µl of methanolic extracts (20 & 80 %) were mixed with 475 µl phosphate buffer (0.2 M, pH 6.6) and 250 µl potassium hexacyanoferrate (0.1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 250 µl of trichloroacetic acid (10%) was added to terminate the reaction. The reaction mixture was centrifuged at 3500 g for 10 min. The upper portion of the solution (500 µl) was separated and diluted with 500 µl distilled water and 100 µl $FeCl_3$ solutions (0.01%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated better reducing power. BHT (0.01- 0.10 mg/ml) was used as a positive control.

3.5.5 2,2 Diphenyl 1-picryl hydrazyl (DPPH) Scavenging Assay

The scavenging activity of the methanolic extracts (20 and 80 %) from mushroom on DPPH radicals was measured according to the method of Chu *et al*. (2000). An aliquot of 900 µl of 0.1 mM (DPPH) (Sigma) taken in methanol was added to a test tube with 100 µl of mushroom methanol extract. Methanol was used as a control. The reaction mixture was mixed at room temperature and the absorbance was determined immediately

by measuring at 520 nm using UV-visible spectrometer (Hitachi –U 2900). The scavenging activity (%SA) of DPPH radicals was calculated using equation [%SA= (1-Abs in the presence of sample/Abs in the absence of sample) × 100]. Quercetin (0.05-0.50 mg /ml) was used as standard.

3.5.6 Metal chelating activity

The chelating activity of the both extracts (20 and 90 % methanol) for ferrous ions was measured following the ferrozine method (Dinis *et al*., 1994). 50 µl of the extract was mixed with 50 µl of ferrous chloride ($FeCl_2$, 2 mM). After 5 min, the reaction was initiated by the addition of 5 mM ferrozine (0.1 ml), and the total volume was adjusted to 3 ml with deionized water or ethanol. Then, the mixture was shaken vigorously and incubated at room temperature for 10 min. The absorbance of the mixture was determined at 562 nm. The metal chelating activity of the mushroom extracts was calculated as: % chelating activity = $[(A_{negative} - A_{sample}) / A_{negative}] \times 100$, where A is absorbance. EDTA was used as positive control while absence of extract of the mushroom was the negative control.

3.6 Statistical analysis

All estimations were carried in triplicates except for the CHN analysis and total protein content which was carried in duplicates. The comparison between Se and non-Se samples were drawn using student ‘t’ test using Graphpad Prism Ver.5.0.

4.0 Results and Discussion

The present study was focused to understand the modulations induced by selenium rich extracts of *Pleurotus fossulatus* cultivated on Se-rich wheat straw on various anti-oxidant properties. To monitor the Se-induced transformations, the selenium enriched mushroom (*P.fossulatus)* were cultivated on selenium rich post harvest agricultural residues and the resulting fruiting bodies were furthur analyzed for various bioactive and antioxidant properties. The same analysis was followed for non enriched mushrooms (as control) cultivated on normal agricultural residues.

4.1 Elemental composition (CHN) of mushroom

The organic composition i.e. Carbon (C), hydrogen (H) and nitrogen (N) content, expressed as percent dry weight of mushrooms was studied in both Se-rich and control mushrooms. The CHN content was nearly the same in fruiting bodies of both selenium enriched (39.1, 3.57, 6.88 %) and control mushrooms (38.9, 4.6, 6.55 %). These results were comparable to CHN content studied in *Pleurotus ostreatus* grown on normal wheat straw (Yildiz *et al*.,1998). Total protein content of mushrooms were also studied using Folin Lowry method (Table 1). Total protein content in methanolic extracts of selenium enriched mushrooms (307 ± 4.5 mg/g dw) was found to be significantly higher ($p < 0.001$) than control mushrooms (282 ± 2.4 mg/g dw). The total protein content obtained was higher than the protein (213.9 ± 1.09 mg/g dw) content reported in selenium enriched *Lentinus edodes* mycelial methanolic extracts. (Turlo *et al*., 2010). Similarly, total protein content (205 - 246 mg/g dw) of different species of *Pleurotus* grown on non-enriched substrates, as reported by Alam *et al*. (2008), was found to be lower than our findings.

The chemical composition of edible mushrooms determines their nutritive value. Selenium is a potent antioxidant and it is nutritionally facilitated through its various selenoproteins and is involved in regulation of cellular pro-oxidant insults that damage cell organelles and DNA. Among the different selenoproteins, glutathione peroxidase (GPx), primarily protects the cell from various pro-oxidants. Other dominant selenoproteins reported in mushrooms include iodothyronine 5-deiodinases and

thioredoxin reductases (Gergely *et al*., 2006). Since mushrooms contain relatively high protein levels, and can accumulate large amounts of selenium, it is reasonable to expect that selenium could be incorporated in proteins.

Table 1. Total protein levels (n=3); and elemental content (n=2) in Se-enriched and control mushrooms.

Mushroom Sample	Elemental Analysis (%)			Total Proteins (mg/g dw)
	Carbon (C)	Hydrogen (H)	Nitrogen (N)	20% Methanol
Se	39.1	3.57	6.88	307 ± 4.5
Non-Se	38.9	4.60	6.55	282 ± 2.4 **

**p<0.001

4.2 Selenium content in fruiting of *P.fossulatus* grown on Se-rich agricultural residues

The selenium content in straw collected from Se rich and control (non-Se) sites, and in fruiting bodies of Se-rich rich mushroom cultivated on the said substrates are presented in Table 2. The fruiting bodies harvested from Se-rich straw containing a total Se concentration of 24.0 ± 0.2 μg/g were noted to accumulate significantly higher ($P < 0.0001$) selenium upto 37.2 ± 0.6 μg/g as compared to control/ non-Se mushroom (3.57 ± 0.53 μg/g) cultivated on non-Se straw (1.9 ± 0.8 μg/g). The extent of accumulation was notably higher to selenium concentration reported in case of *Pleurotus eryngii* (4.6 and 9.3 μg/g) cultivated on substrates supplemented with 5.0 and 10.0 mg/kg of sodium selenite respectively (Estrada, 2009), but lower than those reported in *P.ostreatus* (57.6 μg/g) cultivated on substrate supplemented with 3.2 μg/g of sodium selenite (da Silva et al., 2012).

Similarly, many investigations have been carried for growing Se-enriched mushrooms on substrate supplemented with selenium wherein dominantly inorganic forms of selenium compounds were used for supplementation of Se (Costa-Silva *et al*., 2011).

Table 2. Total selenium content in mushrooms (n=3) cultivated on selenium rich and control wheat straw as substrates

Sample	Se (μg/g dw)	
	Wheat straw	Fruiting bodies
Se	24.0 ± 0.2	37.2 ± 0.6
Non-Se	1.90 ± 0.8	3.57 ± 0.53
	***	***

(*** p<0.0001)

However, to the best of our knowledge, there is no report on the mobilization of selenium by mushrooms such as *Pleurotus fossulatus* cultivated on substrates naturally enriched with Se. Among the crop plants, wheat, maize, pulses and certain vegetable crops grown in the region have been found to hyperaccumulate significantly high levels of Se (Dhillon and Dhillon, 1991; Sharma *et al*., 2007, Cubadda *et al*., 2009) that are envisaged to have potential physiological and pharmacological benefits. Therefore, use of such substrates can facilitate the availability of bioaccessible forms of selenium during growth of mushrooms as compared to those that are exogenously supplemented.

4.3 Selenium induced transformations on bioactive and antioxidant properties of mushrooms

The influence of the Se hyperaccumulation in *P. fossulatus* was examined on the antioxidant properties of mushrooms using various assay systems.

4.3.1 Total phenol content

The term polyphenol refers to a complex group of compounds that includes in their structure an aromatic ring bearing one or more hydroxyl groups. They include simple phenols such as phenolic acids and derivatives, as well as complex structures such as flavones, flavonoids, anthocyanins, among others. Among the antioxidant compounds, polyphenols have gained importance due to their large array of biological actions that include free radical scavenging, metal chelation and enzyme modulation activities, and inhibition of LDL oxidation, among others (Rodrigo and Bosco, 2006). In addition, selenium is well known for its association with antioxidant mechanisms in humans and animals. The total phenolic content, expressed as mg of GA/ g dw of mushrooms, is

shown in Table 3. The amount of phenolic compounds in the methanol (20%) extracts from the se-enriched mushroom (11.15 ± 0.37 mg GA/g dw) was significantly higher ($p<0.001$) than control (9.32 ± 0.18 mg GA/ g dw). Similarly the total phenol content in 90% methanolic extract from Se-enriched mushroom (7.10 ± 0.17 mg GA/g dw) was also found to be significantly higher ($p<0.05$) than the control (6.45 ± 0.10 mg GA/g dw). However mushrooms extracted in 20% methanol showed higher phenol content than those extracted in 90 % methanol. The results obtained are higher than the total phenol content (4.0 ± 0.32 mg/g) reported in case of Se-enriched fruiting of *Agaricus bisporus* (Cremades, 2012) but comparable to phenol content (14.3mg/g in water extracts; 7.4 mg/g in methanolic extracts) reported in case of *P.sajorcaju* (Puttaraju *et al*., 2006) grown on non enriched substrates.

Table 3. Total phenols in Se-enriched and control mushrooms (n=3)

Sample	Total phenols (mg GA/g dw)	
	90% methanol extract	20% methanol extract
Se	7.10 ± 0.17	11.1 ± 0.37
Non-Se	6.45 ± 0.10	9.32 ± 0.18
	*	**

*p <0.05, ** p <0.001

It had been reported that the antioxidant activity of plant materials is well correlated with the content of phenolic compounds. Polyphenols, such as BHT (butylated hydroxytoluene) and gallate, are known to be effective antioxidants (Velioglu *et al*., 1998). Mushrooms contain various polyphenolic compounds recognized as an excellent antioxidant due to their ability to scavenge free radicals by single-electron transfer (Hirano *et al*., 2001). Some common edible mushrooms, which are widely consumed in Asian cuisine, have currently been found to possess antioxidant activity, and are very well correlated with their total phenolic content (Cheung and Cheung, 2005; Cheung *et al*., 2003). Increase in the phenol content in Se-enriched mushrooms may be due to the influence of selenium in promoting the synthesis and activity of antioxidant metabolites, a feature that has been observed in the present study. Various researchers have earlier

defined selenium role in inducing antioxidant capacity by facilitating increase in tocopherol, and phenolic compounds in plants (Xu *et al*., 2003). Due to the particular structure and arrangement of functional groups in phenols, they have immense potential to stabilize free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Shahidi and Wanasusdara, 1992).

4.3.2 DPPH scavenging potential

Antioxidant tests could be based on the evaluation of lipid peroxidation or on the measurement of free radical scavenging potency (hydrogen-donating ability). The radical scavengers donate hydrogen to free radicals, leading to non toxic species and therefore to inhibition of the propagation phase of lipid oxidation. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers (Soler-Rivas *et al*., 2000; Kansci *et al*., 2003). The free radical scavenging potential of mushroom extracts was studied by examining the effect of methanolic extract on scavenging of DPPH. The antioxidants react with the stable free radical DPPH (deep violet colour) and convert it to 1,1-diphenyl-2-picryl hydrazine indicated by discoloration.

Antioxidant activity in terms of free radical scavenging activity of non enriched mushroom extracts is well reported (Kim *et al*., 2008; Puttaraju *et al*., 2006). Compared with those results, the present study showed that non- Se-enriched fruiting of *Pleurotus fossulatus* have higher activity in terms of radical scavenging. On other hand Se-rich extracts (20% methanol) showed significantly higher ($p<0.0001$) scavenging activity (33.6 ± 1.0 %) than non-selenated (21.5 ± 0.4 %) extracts (Table 4). Extracts in 90% methanol also showed higher antioxidant activity but the results were found to be non significant. Our results are supported by the observation of Turlo *et al*. (2010), wherein the free radical scavenging effect of selenated mycelial methanol extracts were almost two times higher than that of non selenated extracts.

Table 4. DPPH scavenging potential of extracts from Se enriched and control *P.fossulatus* fruiting bodies (n=3)

Sample	Scavenging effect (%) of mushroom species on DPPH	
	90% methanol extract	20% methanol extract
Se	40.60 ± 2.87	33.63 ± 1.01
Non-Se	36.03 ± 0.74	21.59 ± 0.40
	ns	***
GA[@]	87.4 ± 0.1	

@ Positive control, *** p<0.0001, ns-non-significant

4.3.3 Lipid Peroxidation

Lipid peroxidation is one of the main manifestations of oxidative damage. Lipid peroxides act on the cell/ cellular components, leading to both structural and functional damage of the biomolecule as well as the cellular structure. Mushrooms are considered to be good phenolic antioxidants to inhibit lipid peroxidation (Yen and Chen, 1995). Malondialdehyde (MDA) is the secondary byproduct, which is released during the lipid peroxidation. A decrease in the production of MDA in turn symbolizes the inhibition of lipid peroxidation. The potential of mushroom extracts in inhibiting lipid peroxidation was studied by TBARS assay (Table 5). The selenium rich extracts (2.1 ± 0.13 μM/g) showed significant decrease (p< 0.0001) in MDA content as compared to control (4.32 ± 0.11 μM/g). Similarly various researchers have defined selenium role in inducing antioxidant capacity in terms of decrease in lipid peroxidation (Fatma and Demerdash, 2004; Yan and Chang, 2012).

Table 5. Lipid peroxidation activity (TBARS) of extracts from Se enriched and control *P.fossulatus* fruiting bodies (n=3) *** p< 0.0001

Lipid peroxidation (μM MDA/g) Extraction in phosphate buffer		
Se		Non-Se
2.1 ± 0.13	***	4.32 ± 0.11

The current results explain the importance of selenium in preventing lipid per oxidation and in protecting the integrity and functioning of tissues and cells as selenium is one of the necessary trace element, which has the ability to counteract free radicals and protect the structure and function of proteins, DNA and chromosomes against the injury of oxidation (Yuan and Tang, 1999).

3.4 Metal chelating activity

Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in lipid peroxidation process (Dodig and Cepelak, 2004). Since ferrous ions are the most effective prooxidants in food systems (Yamaguchi *et al*., 1988), the enhanced chelating effects of methanolic extracts from Se-enriched mushrooms would be more desirable over those obtained in control mushrooms. Chelating effect of selenium rich mushrooms from both extractions (20 and 90 % methanol) was found to be significantly higher ($p< 0.05$) than non-enriched mushrooms. Extracts in 20 % methanol from Se-enriched and control showed the chelating ability of 56.5 ± 2.07 % and 44.7 ± 3.5 % respectively, likewise in 90 % methanol Se –enriched and control mushrooms showed chelating ability of 53.6 ± 3.3 % and 45.8 ± 0.48% respectively (Table 6). It has been reported that four selenium compounds (methyl-selenocysteine, selenocystamine, 3,3 diselenobispropionic acid, and 3,3-selenobispropionic acid) helps in inhibition of iron mediated DNA damage and methyl-selenocysteine alone is known for protection against both copper and iron mediated DNA damage (Battin and Brumaghim, 2008). Metal-selenium complexation may afford cellular protection against oxidative DNA damage by altering the redox potential of the metal ion and preventing reduction of H_2O_2 and thus oxidative DNA damage.

Table 6. Metal chelating activity of extracts from Se enriched and control *P.fossulatus* fruiting bodies (n=3)

Sample	Metal chelating activity (%)	
	90% extract	20% Extract
Se	53.60 ± 3.33	56.52 ± 2.07
Non-Se	45.86 ± 0.48 *	44.74 ± 3.59 *
EDTA[@]	85.40 ± 0.70	

*p< 0.05

The results suggested that moderate ferrous-ion chelating ability showed by Se-enriched mushroom extracts could be beneficial to health. Iron can stimulate lipid peroxidation by the fenton reaction and can also accelerate peroxidation by decomposing lipid hydroperoxide into peroxide and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain of lipid peroxidation (Halliwell, 1999). The high metal chelating activity of Se-enriched mushroom is therefore assumed to be due to specific metal-selenium compound interactions that greatly affect antioxidant activity based on the type of metal and the specific features of the selenium compound.

4.3.5 Total Antioxidant content

Total anti-oxidant content of the methanol extracts of mushrooms were measured spectrophotometrically through phosphomolybdenum assay taking BHT as standard and were expressed in terms of BHT equivalents. The total antioxidant content of the methanol extracts of Se-enriched mushrooms was significantly higher than control (Table 7). Se-enriched mushroom (41.9 ± 1.15 mg BHT /g dw) extracted in 90 % methanol showed higher ($p<0.0001$) antioxidant content than non enriched extracts (34.7 ± 0.65 mg BHT/g dw). Similar results ($p<0.05$) were obtained in case of extracts prepared in 20 % methanol, however the antioxidant content obtained was lesser than 90 % methanolic extracts. Rios *et al*. (2008) reported increase in various antioxidants in broccoli as a result of selenium application in the form of selenate.

Table 7. Total anti-oxidant content of extracts from Se enriched and control *P.fossulatus* fruiting bodies (n=3)

Sample	Total Antioxidant content	
	90% extract	20% extract
Se	41.93 ± 1.15	32.8 ± 0.30
Non-Se	34.76 ± 0.65	31.0 ± 1.00
	***	*

***p< 0.0001,* p< 0.05

4.3.6 Reducing power

The reducing capability of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*., 1995). Reducing properties of a substance are generally associated with the presence of reductones (Duh, 1998), which have been reported to exert antioxidant action by breaking the free radical chain through donation of a hydrogen atom (Gordon, 1990). In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (Barros *et al*., 2007). By measuring the formation and intensity of Perl's Prussian blue at 700 nm, it is possible to determine the Fe^{2+}concentration.

Se-enriched mushrooms showed higher reducing power in both 20 % (0.32 ± 0.03) and 90 % methanol (0.33 ± 0.01) extractions as compared to control (0.23± 0.015 and 0.29 ± 0.049) mushrooms, although the extent of increase in activity was not statistically significant (Table 8). Reducing power of BHT at 0.1 mg/ml was found to be 0.85. Similar study has been conducted in *Lentinus*, where selenium rich mycelia extracts exhibited 30% higher reducing power than the control mushrooms extracts (Turlo *et al*., 2010). The marginally high reducing power of extracts from Se-rich samples might be due to higher expression of glutathione peroxidase (protect against oxidative damage) in presence of selenium, as it is an integral part of the enzyme and is required for its proper functioning (Rotruck *et al*., 1973).

A trace element of fundamental importance to human health, selenium (Se), has been shown to possess an analogous antioxidant function with respect to the immune system (Hoffman and McConnel, 1987; Rayman, 2000).

Table 8. Reducing power activity of extracts from Se enriched and control *P.fossulatus* fruiting bodies (n=3)

Sample	Reducing power	
	90% extract	20% extract
Se	0.33 ± 0.01	0.32 ± 0.03
Non-Se	0.29 ± 0.04	0.22 ± 0.02
	ns	ns
BHT[@]	0.85 ± 0.33	

ns- non significant

Selenium contributes to antioxidant enzymes that protect cells against the effects of free radicals (de Burbure *et al*., 2007). The results in the present study revealed that methanolic extracts of se-enriched mushroom acted as free radical scavengers, acting possibly as primary antioxidants.

Conclusion

The present study demonstrates the use of Se-rich agricultural residues as substrates for cultivation of Se-enriched *Pleurotus fossulatus* (oyster mushrooms), which hitherto has been reported with only exogenous selenium supplementation. *P.fossulatus* indicated notable selenium accumulation and corresponding antioxidant activities on cultivation using substrates naturally enriched with selenium. Se-enriched mushrooms with enhanced antioxidant content can therefore serve as effective dietary supplements or neutraceuticals. The present study thus proposes the use of Se-rich agricultural residues as substrates for mushroom cultivation for human and livestock Se supplementation.

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