Biofortification of three different species of *Pleurotus* using Selenium

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Reg.No:19PBO001.

A Dissertation submitted to the
Avinashilingam Institute for Home Science and Higher Education for Women,
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In Partial Fulfillment of the Requirements for the Degree of

DEGREE OF MASTER OF SCIENCE IN BOTANY

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Signature of the
Head of the Department

Signature of supervisor

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Introduction

INTRODUCTION

Globally, agro wastes are produced in huge volume annually, which used to be burnt or dumped. Nowadays these agrowastes are used for organic manure production by composting. Another usage widely seen nowadays is using them as substrate for growing mushroom which has dual usage as it can be used as substrate and the waste produced is used as organic manure. Mushroom plays a vital role in our dietary food nowadays, because they are low in calories and are high in proteins, rich in carbohydrates and it also contains vitamins (thiamine, niacin, riboflavin), calcium and iron (Schmidt et al., 2003). Due to their unique taste, nutritive value and deliciousness mushrooms could be considered as ‘white vegetables’ or ‘boneless vegetarian meat’ (Panjikkaran and Mathew, 2012).

Mushrooms are not only used as food; it also has a wide range of medicinal properties. Among the large resources of fungi, particularly mushrooms coming under higher Basidiomycetes have unlimited sources of remedially useful biologically active agents (Wasser, 2002). Throughout the world, mushrooms have been used as folk medicine from ancient times and are also used as nutritionally functional food and beneficial nontoxic medicines (Wasswe et al., 2003). Among the varied types of mushrooms, the ones that are widely used for consumption is *Agaricus bisporus* (button mushroom). Next to button mushroom *Pleurotus* sps. (Oyster mushroom) are most preferred among edible mushrooms. The shape of the oyster mushrooms are shell shape, oyster shaped and colour of the oyster mushrooms are pink, white, yellow, light-brown and grey.

Genus *Pleurotus* belongs to the family Tricholomataceae and has about 40 well-recognized species, out of which 25 species are commercially cultivated in different parts of country (Singh et al., 2011). It is a lignocellulolytic fungus that grows naturally in the temperate and tropical forests on dead and decaying matter and it is in second grade among the important cultivated mushrooms in the world. *Pleurotus* mushrooms utilize the agro-wastes like coffee pulp, gram husk, sugarcane baggase, paddy straw, wheat straw, saw dust etc., as substrates for their growth. Thus, the cultivation of these mushrooms helps in recycling agro-wastes and alleviates the nutritional gap mainly prevalent among the population of China, India and Africa. Additionally, the spent substrates obtained after the cultivation of mushroom are used as organic manure, animal feed, enzyme production and biogas production (Kakon et al., 2012).

Among the various *Pleurotous* sps varieties such as *Pleurotous florida, Pleurotous ulmarious, Pleurotous eous* are used in cultivation because of its nutritional value, medicinal values, low-cost production and easy to cultivation. Various methods and research activities were successfully carried out to increase the yield and also to increase the nutritional content in them through supplementation of substrates. This growing interest to increase the macromolecules and trace elements are ways to find a cure or treatment or to prevent certain nutritional defective problems in human health.

Selenium is one of the micronutrients essential for the proper functioning of plants and animals, while higher amount of Selenium is toxic to the system. Worldwide, the primary sources of selenium to fungi and vascular plants are soil, sediment, and water and, further to animals that are natural in the human food chain. Soil poor with selenium can be increased by proper soil management and this may support the growth of selenium up-regulated vegetable food. Cultivation of some species of plants and mushrooms or the culturing of yeast with substrate fortified in inorganic salts of selenium (e.g., sodium selenate; Na_2SeO_4) enables production of food that is enriched in this element (e.g., selenium-enriched garlic, which contains even up to > 1000 μg Se/g dw) (Ip and Lisk, 1994, Ogra et al., 2004).

Selenium contribution to body is protecting it from stress-induced oxidative damage to living cells as it is a very good antioxidant agent. Most of the cultivated and edible mushrooms show varied biological properties. However, they are se deficient, which gives a call for Se biofortification of mushrooms. Though selenium biofortification is done in mushrooms already, a comparative study of how it is affecting the growth, yield and the antioxidant capacity was planned to be conducted. The present study was carried out with the following objectives:

1. To study the Selenium accumulation of three *Pleurotus* (*P. ulmarious, P.eous* and *P. florida*) mushroom, after growing them in two different concentrations of inorganic Sodium Selenate in the substrate.
2. To analyse the growth and yield parameters of three *Pleurotus* (*P. ulmarious, P.eous* and *P. florida*) mushrooms.
3. To analyse the carbohydrate present in the three *Pleurotus* (*P. ulmarious, P.eous* and *P. florida*) mushrooms.
4. To evaluate the antioxidant capacity of the three *Pleurotus* (*P. ulmarious, P.eous* and *P. florida*) mushrooms.

Review of Literature

REVIEW OF LITERATURE

In India, every year the average amount of agricultural wastes is calculated to about 1150 million ton of crop residues, which includes cereals, millets, fibre and oilseeds crop residues; rice straw; maize stover; horticultural crops residues like banana, coconut residues and areca nut residues (MoA Report, 2012). Composting of these organic wastes by mushroom fungi is one of the safest methods to eliminate polluting xenobiotic wastes (Ahlawat and Indu Rani, 2003 and Krishnamoorthy *et al*., 2005). In many countries including India, mushroom cultivation has developed into a profitable industry. Mushrooms cultivation not only paves way for production of mushroom, reuse of agrowastes, use of spent substrate as organic manure, it also offers a high potential for rural employment.

Mushrooms have been used as folk medicine from ancient times throughout the world. it is also used mostly as food and food supplements. It is a fleshy, spore bearing fruiting body fungi, produced above the ground on soil or on the substrate on which it grows. Mostly worldwide, people prefer mushroom for various factors or medicinal properties which are hidden in them. They represent an unlimited source of polysaccharides with antitumor and immune-stimulating properties (Jose and Janardhanan, 2000). Most of edible and cultivated mushroom show an array of biological properties such as hypolipidemic, antibacterial, antiviral, antitumor, chemopreventive, and immunomodulatory activities (Olga and Alla 2017; Rodriguez Estrada et al. 2009; Savic et al. 2009).

Oyster mushroom (*Pleurotus* sp.) is popular especially because of its delicious taste. And these are placed 3rd among the world mushroom production after white button mushroom and shiitake (Gyorfi and Hajdu 2007) and second place in edible mushroom production. This mushroom is widely cultivated throughout the world because of quick mycelial growth and fruiting, short life cycle, slightly affected by diseases and high adaptability to varied agroclimatic conditions, as well as low cost of production (Bonatti 2004; Synytsya et al. 2009; Silveira et al. 2014). Pleurotus spp. also has high ability to use a wide variety of lignocellulosic waste (Yildiz et al. 2002)

Pleurotus spp. is increasingly popular because of possessing nutritional and medicinal values (Fernandes et al. 2015). *Pleurotus* sp. is also known having an antioxidant activity. The activity was correlated with the presence of phenolic compounds and other compounds that can scavenge free radicals. Most species of the genus *Pleurotus* are known their healing potential. And the mushrooms are valuable food, which are low in calories, high in vegetable proteins, zinc, chitin, fiber, vitamins and minerals (Alam and Saboohi, 2001). The mineral salt content in mushrooms is superior to that of meat and fish and nearly twice that of the most commonly used vegetables. Mushrooms with their flavour, texture, nutritive value and high productivity per unit area have been identified as an excellent food source to alleviate malnutrition in developing countries.

Pleurotus have an important place among the commercially employed basidiomycetes because they have gastronomic, nutritional and medicinal properties and can be easily cultivated on a large range of substrates (Chang and Hayes,1978; Kumari and Achal, 2008). *Pleurotus* spp. Has a tremendous organoleptic and nutritional appeal (Garcha, 1997). Due to the worldwide awareness regarding the health benefits of consuming mushroom, the demand has increased considerably. Mushrooms are considered as best solution to various health problems like cholesterol, diabetes, hypertension, constipation and also have anti carcinogenic properties. Medicinal properties of mushrooms also includes anticancer, antibiotic, antiviral activities, immunity and blood lipid lowering effects. *Pleurotus* spp. are also rich in medicinal values. *Pleurotus florida* has antioxidant and antitumor activities (Nayana and Janardhanan, 2000; Manpreet et al., 2004). Oyster mushrooms are very effective in reducing the total plasma cholesterol and triglyceride level (Nuhu Alam et al., 2007) and thus reduce the chance of atherosclerosis and other cardiovascular and artery related disorders. It is suitable for people with hypertension (Ebigwai et al. 2012) and obesity and diabetes (Agrawal et al. 2010).

Agro wastes used as substrates for the cultivation of *Pleurotus* sp differs such as straws of cereals, sugarcane baggase, weeds, left overs of the harvested crops, cotton waste, sorghum stalks, soyabeen stalks, maize cobs have been successfully used as substrates for cultivation of oyster mushrooms. But paddy straw is the most widely used substrates for commercial production of oyster mushroom (Mago et al., 2014). Remarkable variations have been observed in the nutritional contents of Oyster Mushroom grown on different lignocellulosic agro wastes as substrates (Shah et al., 2004, Sarker et al., 2007 and Fasidi and Kadiri, 1993).

Substrate enrichment has been practiced a long back either to improve the proteins or macro micro nutrients etc to improve their content in them. Nutritional analysis of several mushroom species of different origins had been carried out in many laboratories in the world. But nutritional values of locally cultivated mushrooms remain speculative. Moreover, nutritional composition is affected by many factors; these include differences among strains, the composition of growth substrate, the method of cultivation, stage of harvesting, specific portion of the fruiting bodies used for analysis (Benjamin, 1995).

Pleurotus sp., is very attractive to the consumers for their excellent flavor and taste. Approximately 70 species of *Pleurotus* have been recorded to date.

Table 1 : General Characteristics of *Pleurotus* sp. taken for the study

Pleurotus spp	Colour	Character
Pleurotus ulmarius (CO 2)	White	It grows usually 6 to 8 cm across; Gills are adnate ,stems are 6 to 13cm long & 2to 3cm diameter.
Pleurotus eous (APK 1)	Pink	It grown at pre-treated and untreated rice straw and husk with various combinations of 5,10 & 15% of rice bran and used for commercial purpose
Pleurotus florida	White	It develop into medium size (average 5 cm diameter), funnel shaped fruitbodies and with elongated stems.

Oyster mushroom - Nutraceuticals

Mushrooms are a valuable source of dietary fiber; 100g serving of mushrooms contains 2.5g dietary fiber. It is also rich in essential minerals and trace elements (Rahman et al., 2012). Oyster Mushrooms are valuable health food, as it is a good source of non-starchy carbohydrates which are low in calories, high in vegetable proteins, zinc, chitin, fiber and vitamins (C, D and B-complex) (Caglarirmark, 2007 and Randive 2012). Oyster Mushroom contains 19-35% protein on dry weight as compared to 7.3% in rice 13.2% in wheat and 25.2% in milk (Chang and Miles, 1988). It contains 4.0% fat having good quantity of unsaturated fatty acids which are essential in our diet (Holman *et al.,* 1976). Oyster Mushroom contains cobalt which is required in the synthesis of vitamin B12.

Mushrooms fruit bodies are rich in vitamins, mainly vitamin-B1, vitamin- B2, vitamin-C and vitamin-D2 (Manzi et al. 2004). The vitamin of group B are abundant particularly thamine, riboflavin, pyridoxine, pantotene acid, nicotinic acid, nicotinamid, folic acid and cobalamin as well as other vitamins such as ergosterol, biotin, phytochinon and tocopherols (Mattiala et al. 2001). With respect to thiamine content, mushrooms are a bridge between yeast and other food products of vegetal origin. *P. ostreatus* contains more folacine, vitamin B1, vitamin B3 but less vitamin B12 than other mushroom species (Deepalakshmi and Mirunalini, 2014). The niacin content of oyster mushroom is about ten times higher than any other vegetables. The folic acid present in oyster mushrooms helps to cure anemia. Mushrooms are rare vegan sources of vitamin D and conjugated linoleic acid. Mushrooms have antioxidant property due to presence of compounds like ergothioneine (Weigand-Heller et al. 2012). The nutrient content presen in the mushroom depends on the substrate used for their cultivation (Shashirekha et al. 2005).

The need for exogenous means of antioxidants has prompted many studies to investigate the antioxidant potentials of several natural compounds. In this regard, erothioneine (ERG) contain mainly the amino acids is a natural thiol which is found abundantly in mushroom and is a good source of antioxidant. On this basis, oyster mushroom free radical scavenging principle has been documented (Jayakumar et al., 2007). Pleurotus species are a rich source of vitamin A, C and E, carotenoid, flavanoids and other bioactive phenolic compounds (Ren et al., 2014; Woldegiorgis et al., 2014)

Selenium

Selenium is classified as a metalloid and, is an essential trace element for animals and humans. Consequently, the importance of dietary selenium in human health has received considerable attention in the last several years (De Silva et al. 2012). Dietary selenium has been recognised as an antioxidant, and the deficiency of this element has been associated with numerous chronic degenerative diseases, including multiple types of cancer, cardiomyopathy and endemic osteoarthropathy (Fernandes et al. 2015). This element has many physiological functions, but is most often recognised for its role as a cofactor for the enzyme glutathione peroxidise, which is responsible for the removal of free radicals that reduce oxidative damage in cells (Facchini et al. 2014). In several regions of the world, the content of selenium in the general diet has been estimated to be insufficient to maintain the proper level of activity of protective enzymes (Mishra et al. 2013).

Generally, in Nature Se is taken up in the living cells of microorganisms, plants, animals and humans in several inorganic forms such as selenate, selenite, elemental Se and selenide. These forms are converted to organic forms by biological process, mostly as the two selenoamino acids selenocysteine (SeCys) and selenomethionine (SeMet). The biological systems of plants, animals and humans can fix these amino acids into Se-containing Proteins (Hossain et al., 2021).

Most of the cultivated, edible mushrooms are selenium deficient, as the selenium content is very low (< 1–8.5 μg Se/g dry weight). In some of the wild grown, edible fungi and mushrooms, the Se content ranges from 12-200 μg/g (Costa-Silva et al. 2011; Falandysz 2008). However, most of the wild grown, edible mushrooms are not amenable for large scale cultivation and have negligible culinary value. This mandates the cultivation of selenium fortified edible mushrooms on various substrates enriched with either inorganic or organic forms of Se. As the edible mushrooms are known to accumulate Se from substrate and rich in proteins; their growth on selenium rich/amended substrates results in Se incorporation into selenoproteins and selenoenzymes. The various organo selenium compounds identified in the mushrooms are selenomethionine, selenocysteine, selenomethylselenocysteine, etc. (da Silva et al. 2010; Falandysz 2008; Savic et al. 2009). It is known that the chemical form and dose determines the biological activity of Se (Kora 2018b). The organic, most bioavailable forms of Se such as selenomethionine and selenocysteine show antioxidant, antimutagenic, and anticancer activities (Savic et al. 2009).

Generally, the methods used to enrich fruiting bodies of edible mushrooms with Se are through the addition of either inorganic (SeO2, Na2 SeO3, Na2 SeO4) or organic selenium (selenized yeast, selol) to the organic substrate and irrigation water during cultivation and cultivation on selenium hyperaccumulated agricultural residues such as paddy and wheat straw grown on seleniferous soils (Bhatia et al. 2013; Cremades et al. 2012; Oliveira and Naozuka 2019; van Elteren et al. 1998). A successful enrichment strategy depends on the addition of Se source during substrate production and irrigation; concentration and dose of Se source; species, biological efficiency, and physiology of mushrooms; bioaccessible and bioavailable organic Se levels; protein distribution and selenoprotein content; native antioxidant (ergothioneine) content; bioaccessibility of other essential elements; morphological and chemical characteristics, yield of biomass; and organoleptic properties of the produce (Cremades et al. 2012; Oliveira and Naozuka 2019).

It has been well known that selenium (Se) plays an important role in cellular antioxidant defense system of living tissues (Gerloff, 1992), in which the Se-containing antioxidant enzyme glutathione peroxidase (GSH-Px) is responsible for catalyzing the decomposition of lipid hydroperoxides into less-reactive products (Rotruck et al.,1973; Han et al., 2004). More recently, it has been reported that adequate or supranutritional levels of Se intake could not only decrease incidences of tumors in humans (Clark et al., 1996), but also alleviate risks of many diseases associated with Se (Rayman, 2000). Mushrooms are one of the richest natural sources of selenium, which is vital for human health. Selenium is involved directly in the protection of cell walls from oxidation by free radicals. Selenium also enables thyroid to produce thyroid hormone and helps in lowering the risk of joint inflammation.

Mushrooms have known antioxidant properties provided by different compounds, such as phenolics, ergothioneine, and Se (Beelman & Royse, 2006). The content of Se in mushrooms is species specific (Stijve, 1977). The consumption of food rich in antioxidants plays a protective role for human health, because of the reduction in oxidative damage resulting in enhanced generation of free radicals (Oboh and Ademosun, 2012 and Graversen et al., 2008). Free radicals are unstable and highly reactive, due to having unpaired electrons; they are responsible for oxidative stress, and in consequence, they cause DNA damage, carcinogenesis, oxidation of biomolecules and cellular degradation related to aging, etc. (Hu, 2002, Prasad et al., 2004, Willcox et al., 2004 and Valko et al., 2006). Phenolic compounds are mushroom antioxidants which are strong radical scavengers and free radical inhibitors and phytonutrients (Michalak, 2006 and Newell et al., 2010).

Selenium (Se) is one among the trace elements important for human health (Brenneisen et al., 2005) which is a component of antioxidant enzymes via amino acid (selenocysteine, selenomethionine) and proteins (Tinggi, 2008, Estrada et al., 2009 and Riaz and Mehmood, 2012). These edible mushrooms are known to be Se accumulators. However, the amount of Se is dependent on the species, the stage of maturity, the amount in soil, and the substrates used for the growth of cultivated species (Kalac, 2009). Mushroom species grow and yield on a spectrum of agricultural residues and by-products, such as paddy, wheat, sugarcane bagasse, water hyacinth, rubber wood dust, and tree leaves (Khanna, 2003). The agri-wastes generated in seleniferous region of Punjab contain significant levels of Se accumulated in plant parts, as reported in leaves and straw of wheat, rice, and cereals (Dhillon & Dhillon, 2003), and therefore have potential use as substrates for cultivation of Se 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 Se, but to the best of our knowledge no study has been carried out on mushrooms cultivated on substrates hyper-accumulated with Se through natural processes.

Dietary intake of selenium:

The main source of selenium is meats and seafood because of their high protein contents as well as cereals consumed in large amounts. Vegetables and fruits have relatively low selenium contents, but in some plants that are Se-accumulators, such a broccoli, garlic and onions, its content should be high depending on the level as the proteins of these nuts are rich in sulphur amino acids. As the total selenium content in food strongly reflects the soil conditions, these values vary widely with geographical localization. The yield and chemical composition of mushrooms depend on the substrate used for their cultivation (Shashirekha *et al*. 2005).

Selenium enrichment:

The Se- enrichment of mushrooms using agriculture residue has been an alternative for accumulation of Se and production of organic Se (da Silva et al., 2012; Falandysz, 2008; Fang et al., 2018; Hu et al., 2018; Nunes et al., 2012; Solovyev al., 2018). Furthermore, mushrooms are much appreciated food, which contain protein, essential aminoacids, fibers, fattyacids and minerals (Manzi et al., 1999). As selenium deficiency remains a problem in many human populations, Se- enriched foods and supplements have been proposed to circumvent this problem (Ivory and Nicoletti 2017; Wan,Zhang, and Adhikari 2018).Some technologies for designing

The yield and chemical composition can be enhanced by adding micronutrients such as Selenium (Se) to the substrate (Zhao et al. 2004). Se is an essential micronutrient required for the biosynthesis of biologically significant selenoenzymes and selenoprotiens. Its optimal intake could potentially prevent various types of cancer and diseases like diabetes, age-related immunosuppression and even problems related to fertility (Sharma et al. 2018). Se-enriched functional foods have been recently discussed (Adadi et al. 2019).

Materials and Methods

Materials and Methods

The present preliminary study was conducted to find the standardize amount of organic sodium selenate to enrich the selenium in mushroom

3.1 Collection of materials

1. ***Substrate*** : Paddy straw which is used as the substrate is collected from TNAU, Wetland, Poosaripalayam, Coimbatore
2. ***Spawn***: Spawn of the *Hypsizygus ulmarius* var.CO2 & *Pleurotus eous* var.APK1 were collected from Mushroom Cultivation centre, TNAU, Coimbatore

3.2 Substrate preparation

Paddy straw were shred, and soaked in water for 2 hrs. The soaked straws were water drained and sterilized. After sterilization they are bagged in heat resistant polythene bags after reaching room temperature.

Pasteurization of substrate for cultivation:

The pasteurization of substrates viz., boiling, streaming and autoclaving pre-soaked paddy straw for 30 mins. At 15 psi. Three replications were maintained for each treatment and the observations namely, number of days taken for spawn run, button formation, total yield and biological efficiency were recorded.

3.3 Cultivation and Harvesting of Mushroom

Mushrooms were cultivated from the mushroom hut, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore with selenium treatment (Table 2).

3.3.1 Sanitation of incubation and cropping room:

The incubation and cropping rooms were cleaned and disinfected by spraying 0.2 % carbendazim. After spraying, the room was kept airtight for 24 hours and then opened for air fresh air circulation.

3.3.2 Cultivation

- Sterile chopped paddy straw was bagged in the transparent polythene bags of 60x30cm size with a thickness of 100 gauge.

Table:2 Treatments for Selenium enrichment

Without Selenium	Control
2.5% Se	2.5% inorganic Sodium selenate Per 500gm of dry weight of substrate
5% Se	5% inorganic Sodium selenate Per 500gm of dry weight of substrate

- Cylindrical beds were prepared basis following layer spawning method as described by Sivaprakasan (1980).
- A layer of paddy straw was placed at the bottom of the polythene bag, over this, a twenty gm of spawns were sprinkled.
- Five layers like the above-mentioned way was bagged and then bag was tied at the top (modified cylindrical bed method).
- Eight holes of 1 cm diameter were made at random in the polythene bags.
- The mushroom beds were hanged from the bamboo by means of ropes (‘uri’method).
- After spawn running stage, the temperature was maintained at 23 to 28° C and relative humidity at 80to 90 %.
- Water was sprinkled regularly as in the std cylindrical bed preparation method.
- The moisture of the mushroom hut was maintained at 80-85% relative humidity by spraying water three times per day.
- The temperature of the mushroom hut was maintained between 22 and 25° C while primordia starts to emerge.
- The number of primodia (pinhead-like appearance) was counted and recorded.
- Oyster mushrooms achieved maturity within two or three days after primordial initiation.

3.3.3 Harvesting

- The matured fruiting body was identified by the curve margin of the cap, as described by Amin *et al.*, (2007).
- Mushrooms were harvested by twisting to uproot from the base.

Plate 1: *Pleurotus sp.* used in this study

Pleurotus ulmarius **CO2**

Pleurotus eous **APK1**

Pleurotus florida

- Fully developed fruiting bodies were counted to determine the number of effective ones; tiny and deformed fruiting bodies were discarded at the time of counting.
- To obtain the average weight of individual fruiting bodies, the weight of each flush was taken and divided by the number of fruiting bodies.
- The experiment was a completely randomized design, with replicates.
- The data were subjected to analysis of variance and mean were compared by Tukey's test ($p<0.05$)

3.4 Analysis of Mushrooms

Mushrooms grown from the spawn were collected packet wise and all the wastes and dusts were removed from the fruiting body. The proximate analysis of the mushroom of total experiment was conducted with the determination of moisture, total carbohydrate, protein and DPPH analysis.

3.4.1 Biological efficiency

The biological efficiency (BE) was calculated according to Wang, Sakoda, and Suzuki (2001):

$$\text{BE} = \frac{\text{fresh weight of harvested mushroom}}{\text{dry weight of the substrate}} \text{x } 100$$

3.4.2 Determination of moisture and dry matter

Moisture amount was determined by keeping weighed quantity of sample in a thermostat-controlled oven at 105ºC for 6 hours.23-24 The dry weight of each sample was taken on an electric balance. The percentage of the moisture content and dry mater was then calculated by the following formula:

$$\text{Moisture (\%)} = \frac{\text{Initial Wt} - \text{Final Wt}}{\text{Original weight of sample}} \text{x } 100$$

$$\text{Dry matter (\%)} = 100 - \text{Moisture (\%)}$$

3.4.3 Ethanol extract preparation (Kokate, 1994)

- ✓ About 5 g of powdered material was weighed and macerated with 100 ml of 90% ethanol in a closed flask for 24 hours shaking frequently during the first 6 hours and kept undisturbed for 18 hours.

- ✓ Thereafter, it was filtered rapidly taking precautions against loss of the solvent.

3.4.4 Quantitative test

The biochemical parameters analyzed were

1. Carbohydrates
2. Proteins

3.4.4.1 Biochemicals parameters

3.4.4.1.1 Total Carbohydrates: (Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone, a green coloured product with an absorption maximum at 630nm.

Materials

- ➢ 2.5N HCl
- ➢ Anthrone reagent: Dissolve 200 mg anthrone in 100 ml of ice cold 95 % H_2SO_4 prepared fresh before use.

Standard glucose (Stock): Dissolved 100 mg in 100 ml of water.

Working standard – 10 ml of a stock solution was diluted to 100 ml distilled water. After adding few drops of toluenetored in refrigerator after adding a few drops of toluene.

Procedure

- ✓ About 100 mg of the sample was taken in a boiling tube and it was hydrolysed by keeping it in a boiling water bath for three hours with 5ml of 2.5N HCl and cooled to room temperature.
- ✓ Then it was neutralized with solid sodium carbonate until the effervescence created.
- ✓ The volume was made up to 100 ml and centrifuged.
- ✓ The supernatant was collected and 0.5 and 1 ml aliquots were taken for analysis.
- ✓ The standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and '0' served as blank.

- The volume was made up to 1 ml in all the tubes including the sample test tubes by adding distilled water.
- Then, 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath.
- Then it was cooled rapidly and the green colour developed was read at 630 nm.
- A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
- From the graph, the amount of carbohydrates present in the sample tube was calculated.

Calculation

Amount of carbohydrates present in 100mg of the sample is calculated by

$$\frac{\text{mg of glucose}}{\text{volume of test sample}} \times 100$$

3.4.1.1.2 Estimation of protein (Lowry *et al.*, 1951)

Principle

The blue colour developed by phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

Materials:

- Reagent A - 2 % sodium carbonate in 0.1 sodium hydroxide
- Reagent B - 0.5 % copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate.
- Reagent C - Alkaline copper solution : Mixed 50 ml of A and 1 ml of B prior to use.
- Reagent D - Folin-Ciocalteau Reagent.

Stock standard : 50 mg of bovine serum albumin (Fraction V) was weighed and dissolved in distilled water and the volume was made up to 50 ml in a standard flask.

Working standard: About 10ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contain 200 µg proteins.

Procedure

Extraction of protein from sample

Extraction is usually carried out with buffers used for the enzyme assay.

About 50mg of the weighed sample was ground well with pestle and mortar in 5- 10 ml of the buffered centrifuged. The sample was used for protein estimation.

Estimation of protein

- ✓ About 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard were pipette into a series of test tubes and 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
- ✓ The volume was made up to 1 ml in all test tubes. A tube with 1 ml of water severed as the blank.
- ✓ About 5 ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10 minutes.
- ✓ Then 0.5 ml of reagent D was added. Mixed well and incubated at room temperature in the dark for 30 min. Blue colour developed was read at 660 nm.
- ✓ A standard graph was drawn and the amount of protein present in the sample was calculated.

Calculation

The amount of protein present in the sample was expressed in

$$\mathrm{mg/g\ or100g} = \frac{\text{mg of protein}}{\text{volume of the test standard}} \times \text{concentrationof the standard}$$

3.4.4.2 Free radical scavenging activity

3.4.4.2.1. DPPH radical scavenging activity (Mensor *et al.*,2001)

Principle

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenylpicryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

Reagents

1. DPPH – 2,2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

Procedure

The extracts (20µl) were added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer.

Calculation

The radical scavenging activity was calculated as follows

$$\text{Scavenging activity \%} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

3.4.4.3 Elemental Analysis using EDAX

EDAX analysis were performed for 2g og the powdered leaf samples in DR. C.N.Rao Lab, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore

Results & Discussion

Results and Discussion

Mushroom cultivation not only decreases the environmental pollution but also gives value added product, which are good for health and sometimes used for medicinal purposes. Agrowastes used as substrate for cultivation of mushroom are also used as organic fertilizer after completion of full harvesting. Mushroom usually contains more of dietary fibre, protein and other minerals which are good for human health.

Selenium is one such compound which has a good antioxidant property. Low amount has good antioxidant property, while the high amount leads to serious health problems. The fungal mycelium of mushroom can absorb and store certain elements when are given in the substrate. In this present study selenium enrichment is done by the addition of inorganic Sodium selenate into the substrate at 2 different concentrations. Three different kind of *Pleurotus* (*ulmarius* CO2, *eous* APK1 and *florida*) are used for biofortification with selenium.

4. 1 Moisture content and Dry matter

Moisture content was found to be more in all the three species in the 5% selenium treated mushrooms than non-biofortified mushrooms. Comparing the three species *P. eous* APK1 was found to have the highest moisture content while the lowest in *P. florida* of 57 % and 49 % respectively in non-biofortified mushrooms. While, in 5 % selenium fortified mushrooms highest and lowest moisture content were observed in *P. ulmarius* CO2 (73 %) and *P. florida* (70 %) respectively.

Table.2: Moisture content and dry matter of the three *Pleurotus* sp.

Pleurotus species	**Treatments**	**Moisture %**	**Dry Matter %**
P. ulmarius CO2	Control	53	49
	Se 2.5 %	61	31
	Se 5 %	73	27
P. eous APK1	Control	57	43
	Se 2.5 %	64	36
	Se 5 %	71	29
P. florida	Control	49	51
	Se 2.5 %	67	33
	Se 5 %	70	30

Dry matter of the mushroom was analysed and the results shows that highest and lowest value were found in *P. florida* (51 %) and *P. eous* APK1 (43 %) respectively in non-biofortified mushrooms. Among the selenium fortified mushrooms in all the species 2.5 % of selenium treated substrate showed the highest when compared to 5 % selenium treated mushrooms. In the 2.5 % selenium fortified mushrooms, the highest and lowest value were recorded in *P. eous* APK1 (36 %) and *P. ulmarius* CO2 (31 %) respectively

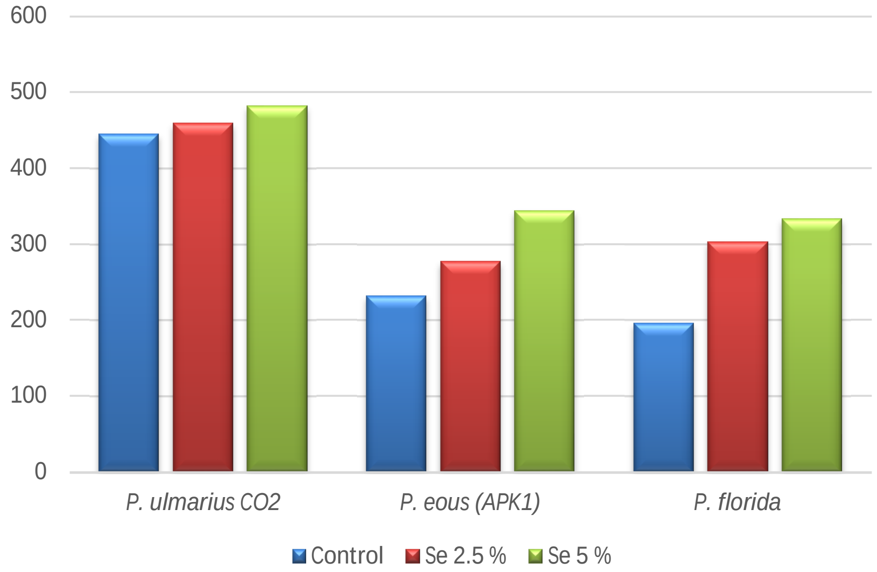
4.2 Biological Efficiency (BE)

The highest biological efficiency in non-biofortified selenium was found to be maximum in *P. ulmarius* CO2 (46.4 %) and minimum in *P. florida* (24.8 %). In selenium fortified mushrooms maximum and minimum was found in *P. ulmarius* CO2 (55.8 %) and minimum in *P. florida* (29.6 %) respectively. According to Murugan and Kannan, (2019) the yield was high in *P. ulmarius* CO2 (296.83 g) and low in *P. florida* (261.16 g) and BE was found to be high in *P. ulmarius* CO2 (59.4 %) and low in *P. florida* (52.2 %), which was similar to the results that we have observed.

Table.3: evaluation of the three *Pleurotus* sp for biological efficiency

Pleurotous species	**Treatments**	**No. of Primordias**	**No. of Fruiting bodies**	**Biological**	**Economical**	**BE (%)**
P. ulmarius CO2	Control	29	147	232	202	46.4
	Se 2.5 %	32	159	255	229	51
	Se 5 %	35	172	279	243	55.8
P. eous (APK1)	Control	22	125	163	124	32.6
	Se 2.5 %	18	122	156	132	31.2
	Se 5 %	20	137	168	152	33.6
P. florida	Control	13	17	124	117	24.8
	Se 2.5 %	17	20	167	146	33.4
	Se 5 %	19	27	148	131	29.6

Fig. 1: Graphical representation of Biological Efficiency in the three *Pleurotus* sp



4.3 Total Carbohydrate

Carbohydrates in mushrooms are mainly involved in the structural composition except for sugar-free components, essential in maintaining the high osmotic concentration and serving for the energy release intact with the fast metabolism rate. The Pleurotus mushrooms contain large amounts of carbohydrates ranging between 24.95 and 75.88% (Patil et al., 2010 and Koutrotsios et al., 2014).

Total carbohydrate in non-biofortified mushroom were recorded maximum in *P. ulmarius* CO2 and minimum in *P. florida* with 74.7 mg/100g and 60.2 mg/100 g respectively. In biofortified selenium treatments the highest value was found to be high in selenium at 5 % in all the mushrooms. In 5 % selenium biofortified maximum was found in *P. florida* and minimum in *P. eous* APK1 with 85.5 mg/100g and 76.34 mg/100 g respectively.

Alam and his workers carried out a nutritional analysis in which they figured out that the carbohydrate present in dried mushroom is 42.83 mg/ 100g in *P. florida.*

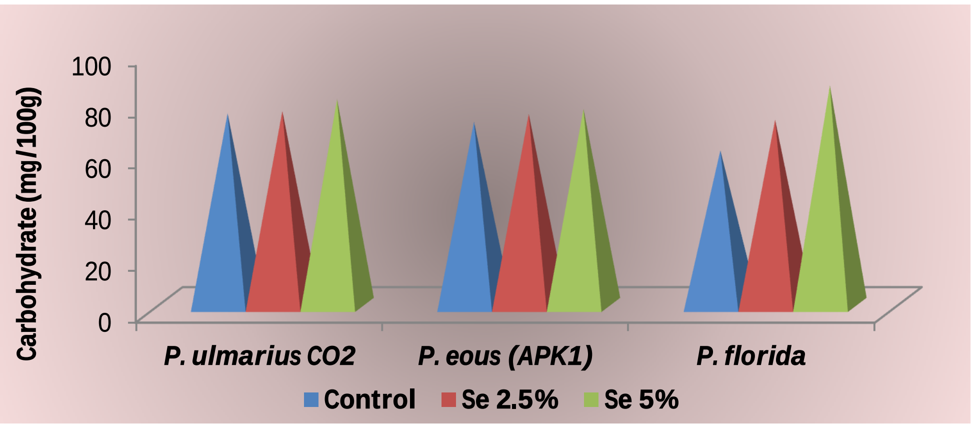
Table. 4: Total carbohydrate present in methanolic extract of *P. ulmarius* CO2, *P. eous* APK 1 and *P. florida* in control and Se bifortified mushrooms

Pleurotus sp.	**Total Carbohydrate (mg / 100 g)**		
	Control	**Se (2.5%)**	**Se (5%)**
P. ulmarius CO2	74.7 ± 0.06	75.57 ±0.07	80.15 ± 0.12
P. eous APK1	71.4 ± 0.08	74.43 ± 0.09	76.34 ± 0.10
P. florida	60.2 ± 0.09	72.14 ± 0.10	85.5 ± 0.11
SEd	**0.086**		
CD (p<0.05)	**0.177**		

Values are mean ± SD of three samples in each group

* - Significant at 5% level (p<0.05)

Fig 2: Comparision of Carbohydrate in methanolic extract of *P. ulmarius* CO2, *P. eous* APK 1 and *P. florida* in control and S bifortified mushrooms



4.4 Protein

Genus *Pleurotus* can be considered as a good source of palatable proteins. Non-protein nitrogen compounds are in the form of amino acids, chitin, and nucleic acids. *Pleurotus* species also contain high amounts of c-aminobutyric acid (GABA) and ornithine. GABA is a nonessential amino acid required for brain functioning and mental activity (Raman et al., 2021).

Protein was observed to be high in Se 5% bio-fortified than non-biofortified and 2.5% Se biofortified samples. In Se 5 % bio-fortified mushrooms maximum protein content was recorded in *P.florida* (38.21 mg/100g) and minimum in *P. ulmarius* CO2 (27.78 mg/100g).

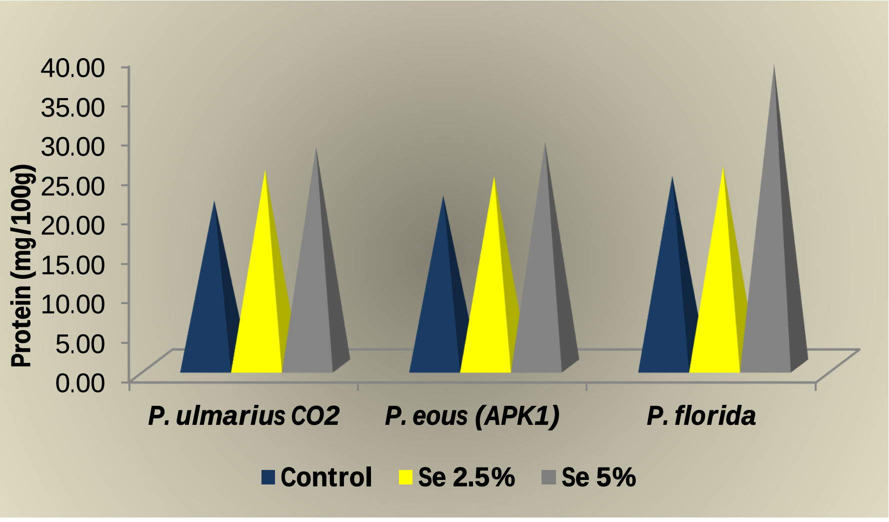
Table. 5: Protein present in methanolic extract of *P. ulmarius* CO2, *P. eous* APK 1 and *P. florida* in control and Selenium bifortified mushrooms

Pleurotus sp.	Total Protein (mg / 100 g)		
	Control	Se (2.5%)	Se (5%)
P. ulmarius CO2	20.99 ± 0.09	24.94 ± 0.13	27.78 ± 0.10
P. eous APK1	21.60 ± 0.11	24.07 ± 0.07	28.40 ± 0.09
P. florida	24.07 ± 0.10	25.31 ± 0.09	38.21 ± 0.11
SEd	**0.08819**		
CD (p<0.05)	**0.18202**		

Values are mean ± SD of three samples in each group

* - Significant at 5% level (p<0.05)

Fig 3: Comparision of Protein in methanolic extract of *P. ulmarius* CO2, *P. eous* APK 1 and *P. florida* in control and Selenium bifortified mushrooms



Protein content increase was recorded by Bhatia and his co-workers (2014) in Se biofortified *P. fossulatus* than non-biofortificated samples. According to Raman and his co-workers the protein content in *P. florida* was 20.56 g/100g. FAO report showed that the *Pleurotus* varieties had about 30.4 % protein content. (Lee et al., 2018 and FAO, 1973). The mushroom protein assimilability depends mainly on the species, ranging from 9.29 to 37.4 g/100 g of fruit bodies dry weight (Ritota and Manzi, 2019). Protein content in mushrooms runs a wide range based on inherent and agro-climatic factors.

4.5 DPPH scavenging activity

DPPH scavenging potential in methanolic extracts of Se biofortified and non-biofortified samples of *P. ulmarius* CO2 and *P. florida* were analysed. In this analysis the IC_{50} values of *P. ulmarius* CO2 ranged from 27.32 to 39.68 mg/ml, while in *P.florida* it ranged from 28.89 to 40.62 mg/ml.

DPPH scavenging potential of Se-rich *P. fossulatus* was recorded by Bhattia and his co-workers (2014), as 40.60 % while non-enriched mushroom was recorded as 36.03 %.

Table. 6. DPPH scavenging potential in methanolic extract of various selenium treated *Pleurotus ulmarius* CO2 (mg/ml)

Conc	Control	Se - 2.5%	Se - 5%	L-Ascorbic
10	27.22 ± 1.26	28.66 ± 0.96	29.02 ± 1.44	30.82 ± 3.32
20	29.92 ± 1.77	38.36 ± 1.73	37.29 ± 1.38	41.60 ± 2.95
30	45.01 ± 1.56	50.49 ± 1.92	54.90 ± 2.29	59.57 ± 2.51
40	50.40 ± 0.93	56.15 ± 0.66	60.92 ± 2.35	63.79 ± 4.32
50	64.96 ± 4.29	63.07 ± 0.91	68.37 ± 3.27	71.70 ± 2.73
60	73.32 ± 5.21	71.88 ± 3.23	70.17 ± 1.88	76.82 ± 2.56
SEd **CD ($p<0.05$)**	2.48 5.25	1.41 3.23	1.97 4.41	2.21 5.10
IC_{50}	**39.68**	**29.71**	**27.32**	**25.18**

Table. 6. DPPH scavenging activity in methanolic extract of various selenium treated *Pleurotus florida* (mg/ml)

Conc	Control	Se 2.5%	Se 5%	L-Ascorbic
10	22.91 ± 3.36	27.22 ± 1.75	25.43 ± 2.04	30.82 ± 3.32
20	34.68 ± 0.99	36.21 ± 2.07	33.15 ± 2.32	41.60 ± 2.95
30	41.60 ± 2.80	46.00 ± 2.01	44.74 ± 1.65	59.57 ± 2.51
40	55.35 ± 3.27	54.09 ± 3.57	57.05 ± 2.10	63.79 ± 4.32
50	64.24 ± 3.92	64.24 ±1.95	65.77 ± 4.32	71.70 ± 2.73
60	71.25 ± 1.02	73.05 ± 0.97	73.94 ± 1.30	76.82 ± 2.56
SEd **CD ($p<0.05$)**	2.38 5.05	1.81 3.93	2.01 4.79	2.21 5.10
IC_{50}	**40.62**	**29.40**	**28.89**	**25.18**

Fig. 4 Comparison of DPPH free radical scavenging ability of Selenium treated fruiting bodies of *Pleurotus ulmarius* CO2

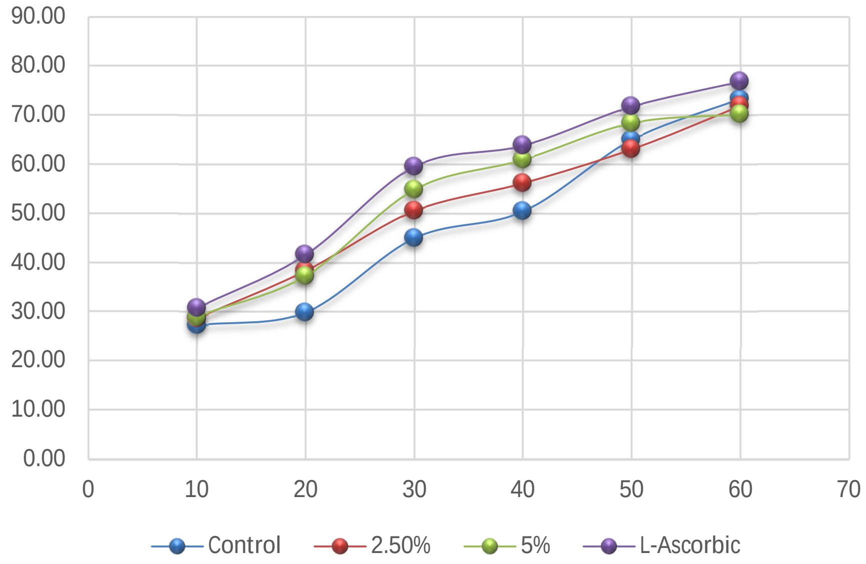
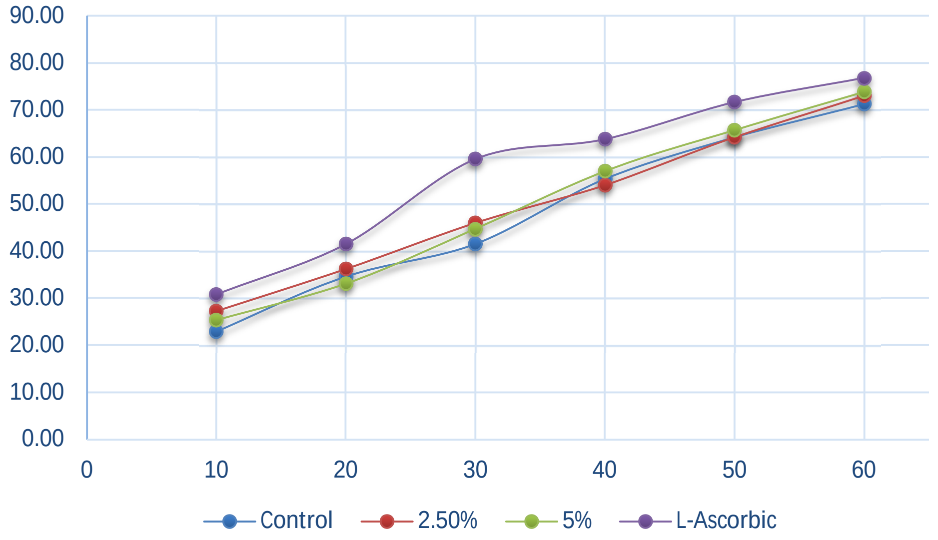


Fig. 5 Comparison of DPPH free radical scavenging ability of Selenium treated fruiting bodies of *Pleurotus florida*

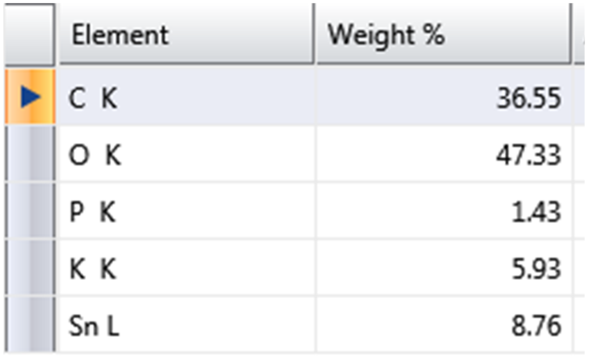


4.5 Energy dispersive X-ray (EDAX) analysis

EDAX analysis was performed to observe whether enrichment of Selenium has happened, as EDAX is mostly performed for qualitative elemental analysis and not quantitative. The current results states that increase in selenium in the substrate should be done.

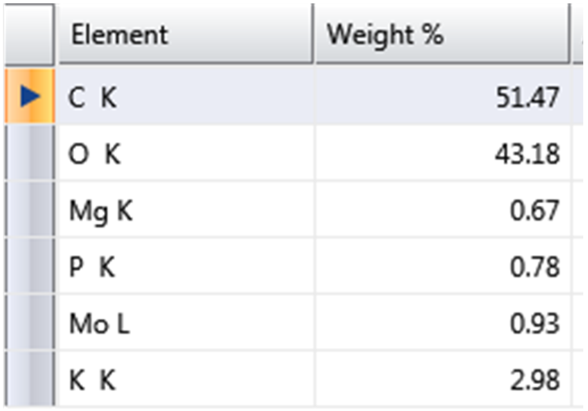
Table 7: EDAX elemental composition of *Pleurotus ulmarius* CO2

Control



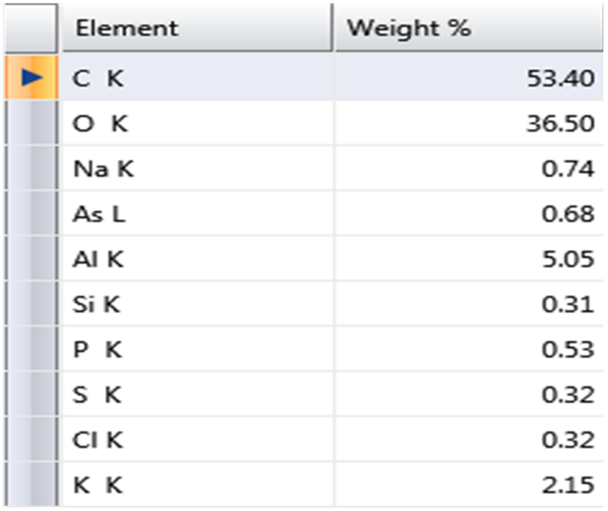
Element	Weight %
C K	36.55
O K	47.33
P K	1.43
K K	5.93
Sn L	8.76

Se 2.5 %



Element	Weight %
C K	51.47
O K	43.18
Mg K	0.67
P K	0.78
Mo L	0.93
K K	2.98

Se 5 %



Element	Weight %
C K	53.40
O K	36.50
Na K	0.74
As L	0.68
Al K	5.05
Si K	0.31
P K	0.53
S K	0.32
Cl K	0.32
K K	2.15

Fig.6: Pie chart representation of elements present in *Pleurotus ulmarius* CO2

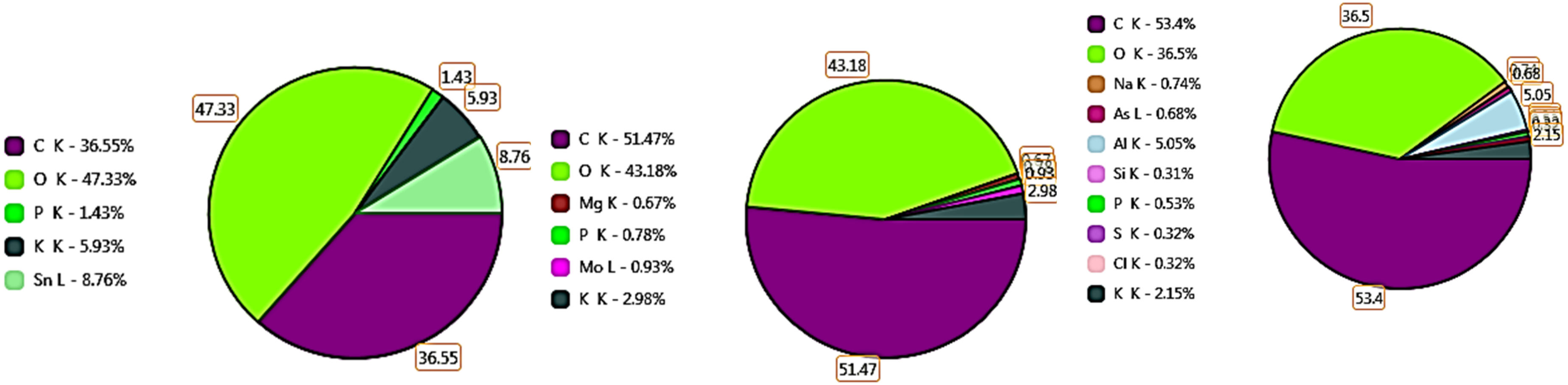
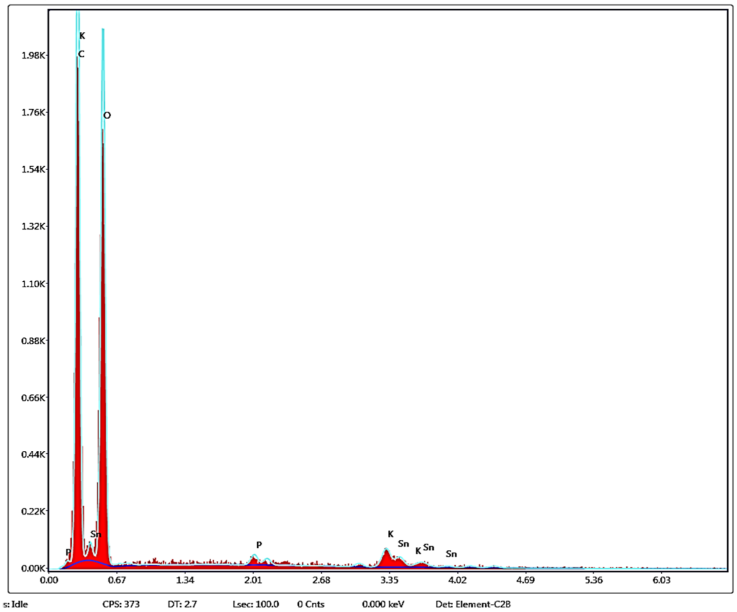
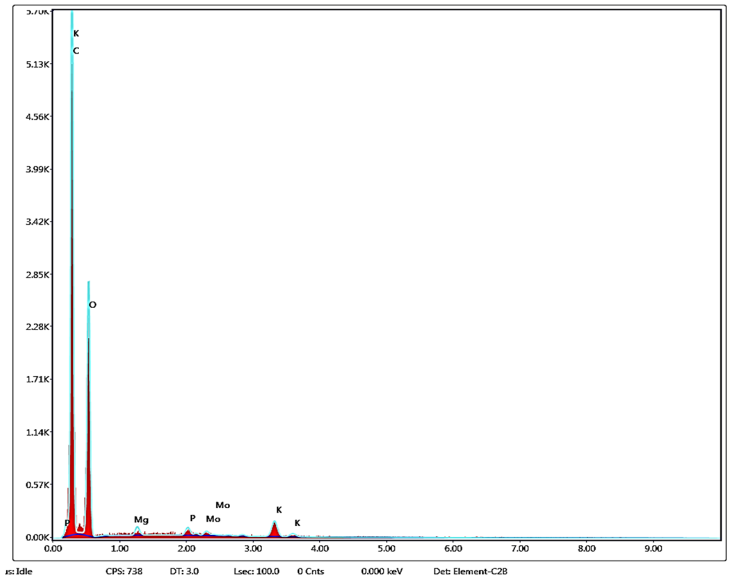


Fig.7: EDAX Spectrum Results of dry powdered *Pleurotus ulmarius* CO2

Control



Se 2.5 %



Se 5 %

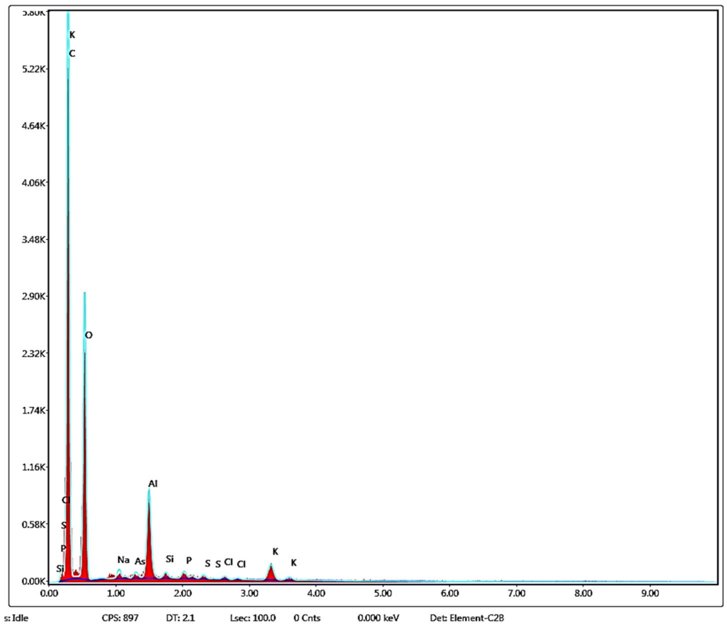
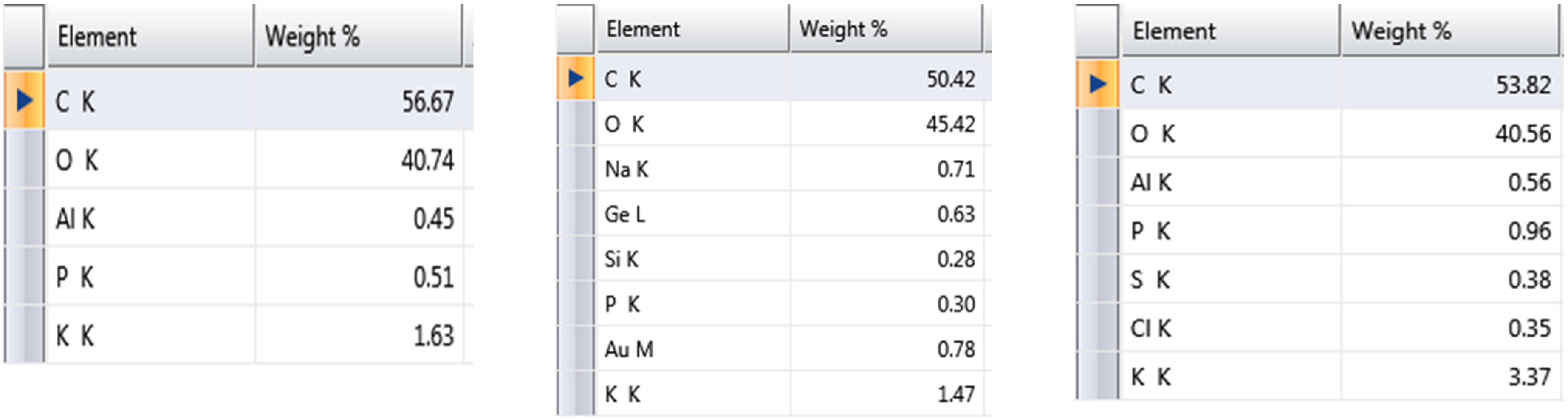


Table 8: EDAX elemental composition of *Pleurotus eous* APK1



Control

Element	Weight %
C K	56.67
O K	40.74
Al K	0.45
P K	0.51
K K	1.63

Se 2.5 %

Element	Weight %
C K	50.42
O K	45.42
Na K	0.71
Ge L	0.63
Si K	0.28
P K	0.30
Au M	0.78
K K	1.47

Se 5 %

Element	Weight %
C K	53.82
O K	40.56
Al K	0.56
P K	0.96
S K	0.38
Cl K	0.35
K K	3.37

Fig.8: Pie chart representation of elements present in *Pleurotus eous* APK1

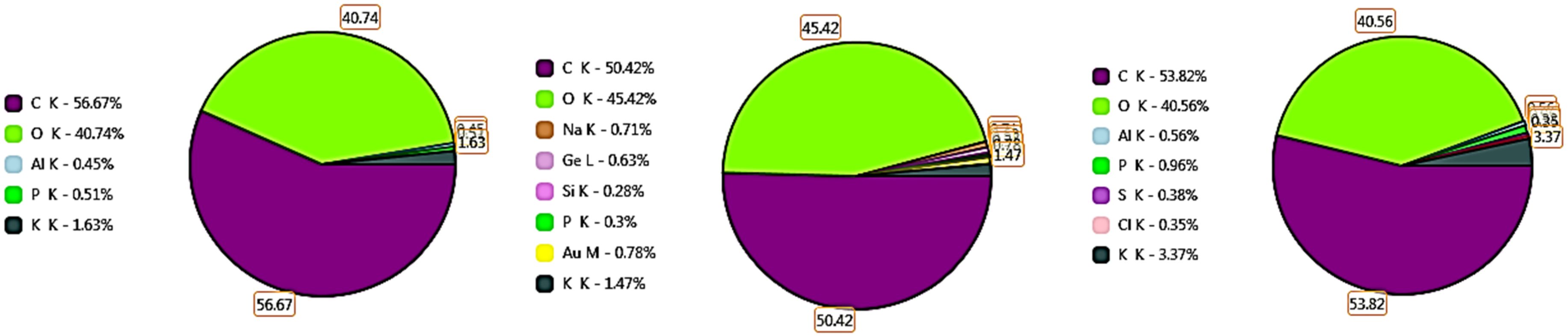
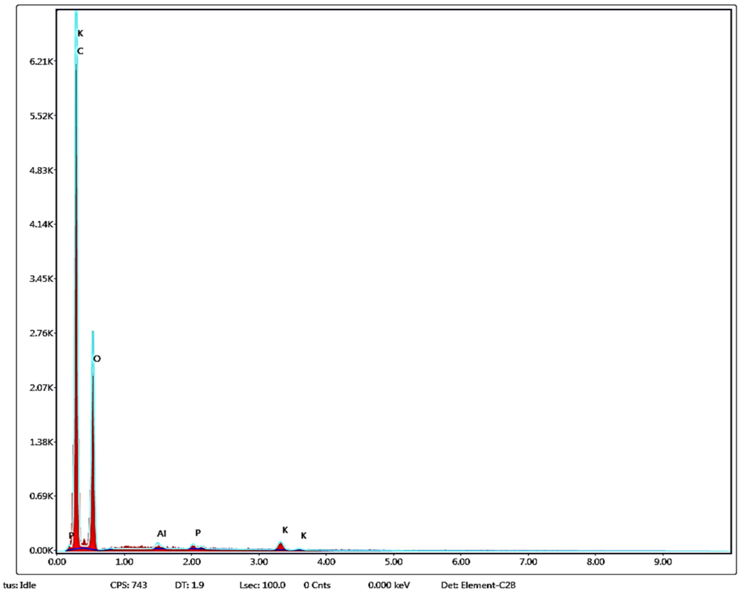
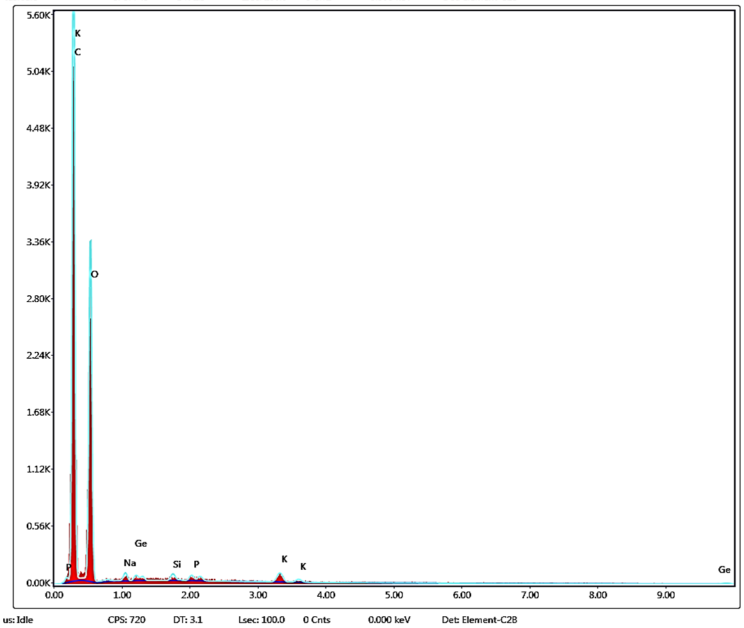


Fig.9: EDAX Spectrum Results of dry powdered *Pleurotus eous* APK1

Control



Se 2.5 %



Se 5 %

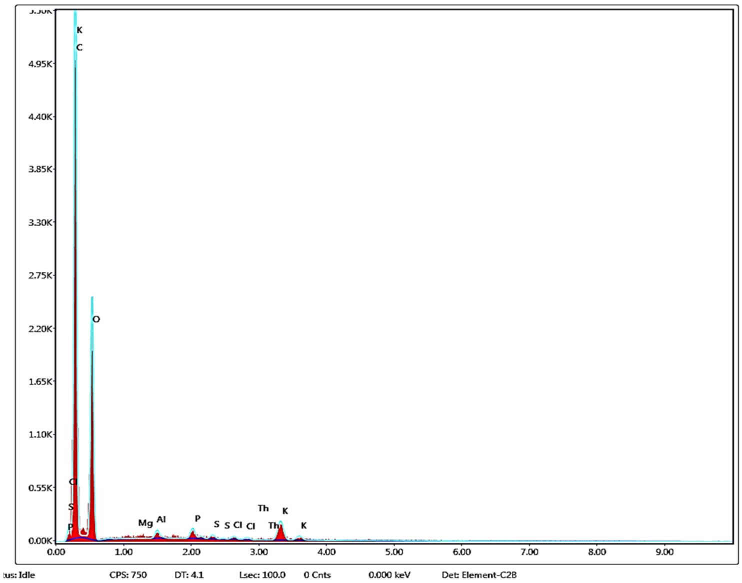
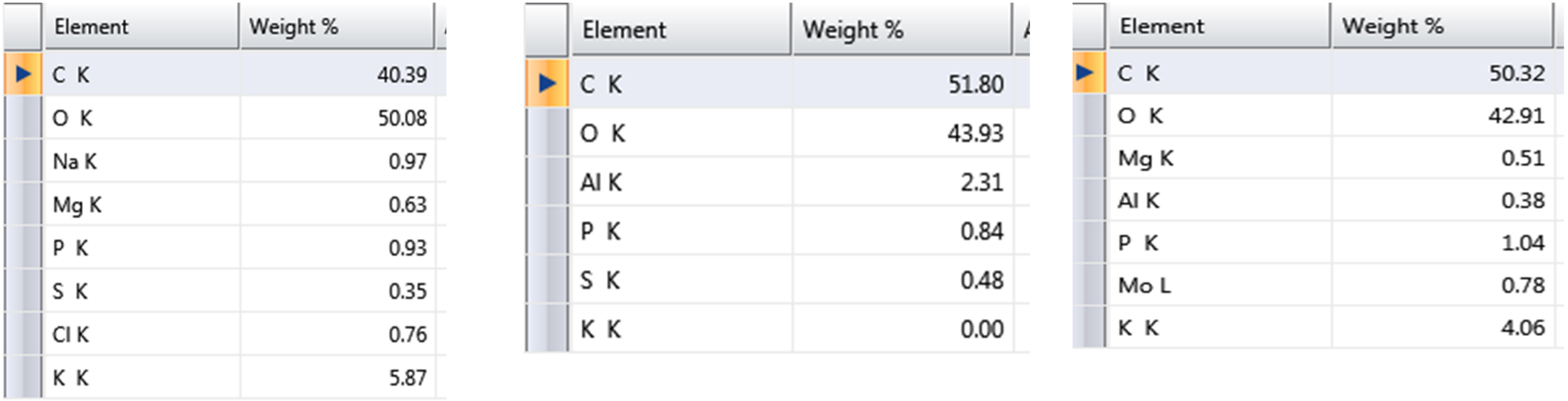


Table 9: EDAX elemental composition of *Pleurotus florid*



Control

Element	Weight %
C K	40.39
O K	50.08
Na K	0.97
Mg K	0.63
P K	0.93
S K	0.35
Cl K	0.76
K K	5.87

Se 2.5 %

Element	Weight %
C K	51.80
O K	43.93
Al K	2.31
P K	0.84
S K	0.48
K K	0.00

Se 5 %

Element	Weight %
C K	50.32
O K	42.91
Mg K	0.51
Al K	0.38
P K	1.04
Mo L	0.78
K K	4.06

Fig.10: Pie chart representation of elements present in *Pleurotus florida*

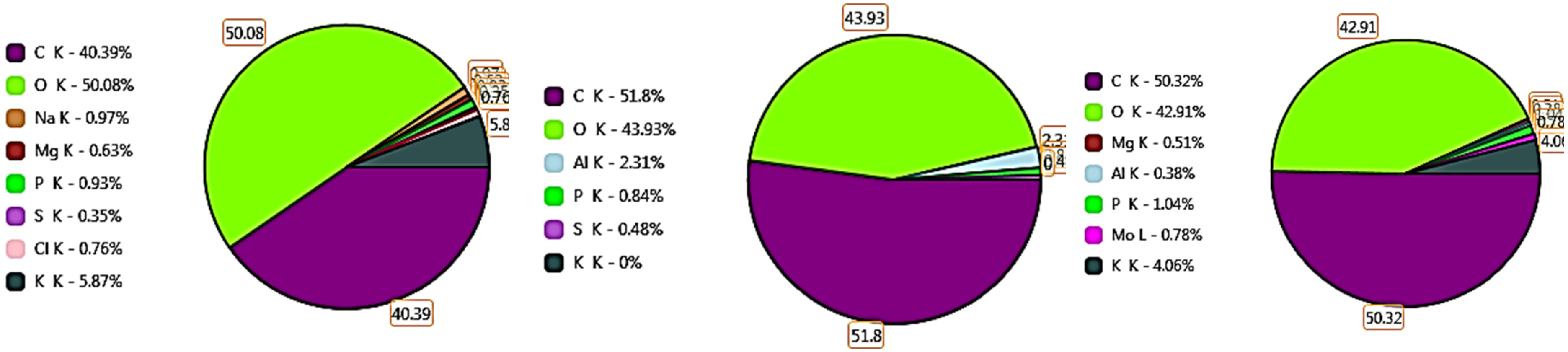
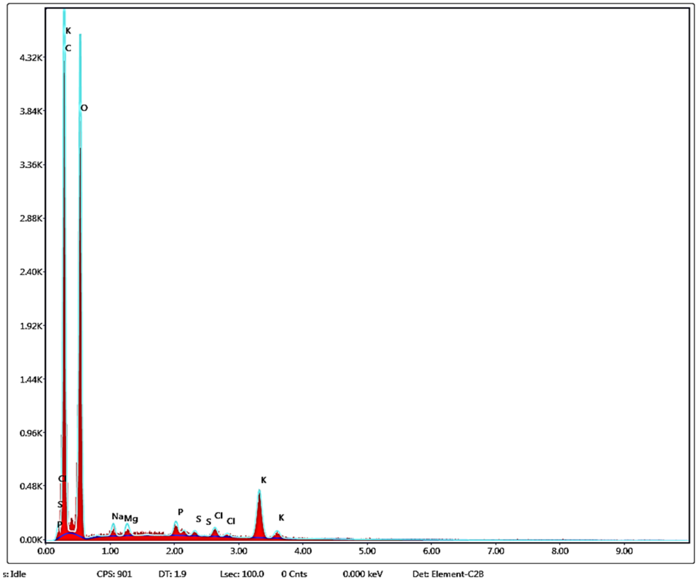
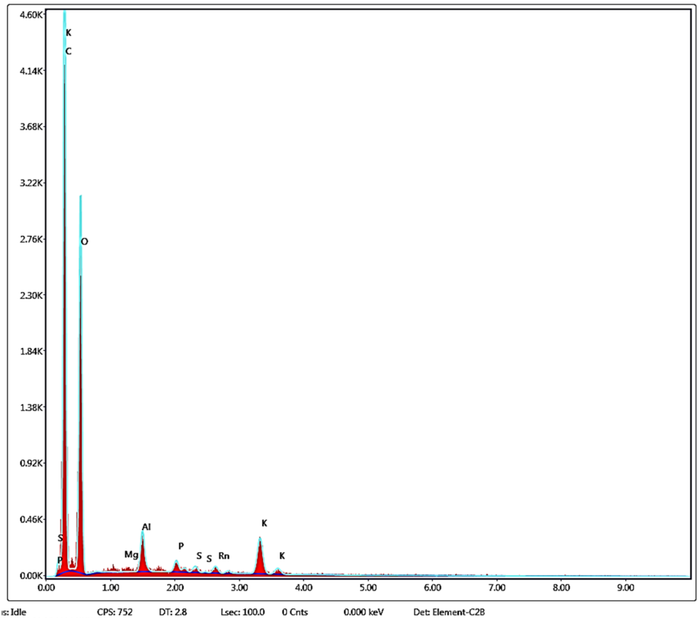


Fig.11: EDAX Spectrum Results of dry powdered *Pleurotus florid*

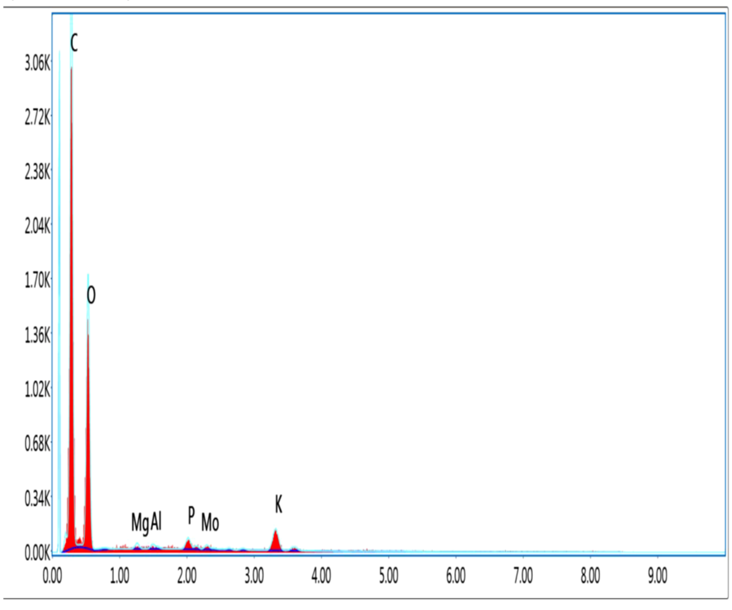
Control



Se 2.5 %



Se 5 %



Carbon is found in a decreasing trend as the selenium concentration increase in the treatment in substrate. Phosphorus is also found to be higher in 5% biofortified samples than non-biofortified and 2.5 % biofortified samples in *P. eous* APK1 and *P. florida.* Selenium was not detected in any of the selenium biofortified samples. Studies carried out earlier, indicates that the element distribution in biological materials is not uniform (McCully et al., 2010), and the random selection of powdery specimens might be the cause of no detection in selenium.

Summary & Conclusion

Summary and Conclusion

Mushroom is good in taste and healthier too. It is a good source of proteins, vitamins and minerals. Biofortification of vegetables or mushrooms are done to improve the content in them, which helps in production of enriched component. In this present study selenium is enriched in the mushroom by addition of inorganic Sodium Selenate at two different concentrations.

Moisture content of the three non-biofortified *Pleurotus* species were compared and found that *P. eous* APK1 to have the highest moisture content and the lowest in *P. florida* of 57 % and 49 % respectively. While, in 5 % selenium fortified mushrooms showed highest moisture content than the other treatments. The highest and lowest moisture content in 5 % selenium fortified mushrooms were observed in *P. ulmarius* CO2 (73 %) and *P. florida* (70 %) respectively.

Dry matter of the three *Pleurotus* sp. were analysed and in non-biofortified mushrooms the highest and lowest dry matter were found in *P. florida* (51 %) and *P. eous* APK1 (43 %) respectively. Among the selenium fortified mushrooms in all the species 2.5 % of selenium treated substrate showed the highest when compared to 5 % selenium treated mushrooms. In the 2.5 % selenium fortified mushrooms, the highest and lowest value were recorded in *P. eous* APK1 (36 %) and *P. ulmarius* CO2 (31 %) respectively.

Biological efficiency of the three *Pleurotus* sp, were calculated. In non-biofortified mushrooms maximum and minimum BE were found in *P. ulmarius* CO2 (46.4 %) and minimum in *P. florida* (24.8 %). In selenium fortified mushrooms maximum and minimum was found in *P. ulmarius* CO2 (55.8 %) and minimum in *P. florida* (29.6 %) respectively.

Total carbohydrate in non-biofortified mushroom were recorded maximum in *P. ulmarius* CO2 and minimum in *P. florida* with 74.7 mg/100g and 60.2 mg/100 g respectively. In biofortified selenium treatments the highest value was found to be high in selenium at 5 % in all the mushrooms. In 5 % selenium biofortified maximum was found in *P. florida* and minimum in *P. eous* APK1 with 85.5 mg/100g and 76.34 mg/100 g respectively.

Protein was observed to be high in Se 5% bio-fortified than non-biofortified and 2.5% Se biofortified samples. In Se 5 % bio-fortified mushrooms maximum protein content was recorded in *P.florida* (38.21 mg/100g) and minimum in *P. ulmarius* CO2 (27.78 mg/100g).

DPPH scavenging potential in methanolic extracts of Se biofortified and non- biofortified samples of *P. ulmarius* CO2 and *P. florida* were analysed. In this analysis the IC_{50} values of *P. ulmarius* CO2 ranged from 27.32 to 39.68 mg/ml, while in *P. florida* it ranged from 28.89 to 40.62 mg/ml. All of the above parameters show that the selenium biofortification has improved. But in EDAX elemental analysis Se was not identified in both the concentrations of Se biofortification. This preliminary study reveals that the se content was not improved, so the inorganic sodium selenate has to be added more to the substrate in future studies.

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