The Effect of Recycling Different Wastes (as a substrate) on Mushroom (*Pleurotus ostreatus*) Fruit Bodies, Morphologically, Genetically and its Metabolites

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Today's some of plant wastes disposal is a great problems for environmental concern. These wastes are rich in nutrient and disposal without pretreatment, which cause a great environmental problem. Recycling of these wastes in cultivation of mushroom will help to remediate the environment and at the same time will produce a safe and highly yield of mushroom. In addition not only are mushrooms a protein-rich food source for humans, but the by-products of mushrooms cultivation unlocking nutrients for other members of the ecological community. Medicinal mushroom was cultivated on three different recycling agricultural wastes. The heavy metals were determined in the three wastes substrates and it was observed that the levels of heavy metals presented in the grown fruit bodies on the three different substrates was low which ranged from 29.85-65.56 µg/g of dry weight for Zn, while Ni was 0.0-1.005 µg/g of dry weight. The low levels of the heavy metals indicated that the obtained fruit bodies were suitable for consumption as a nutritional food. On the other hands the analysis of aspartic amino acids was (2.42, 2.517 and 3.088%) for tea waste, Rice straw and water hyacinth respectively as the highest values. In addition, the morphology of the fruit bodies were different according to the type of substrate used in cultivation and consequently genetic variation was demonstrated as well. The protein contents of the three different fruit bodies are also different. It can conclude that, the residual wastes used in this study affect both of morphology and the fungus cell contents with low levels but without any effect on their human consumption. Rice straw was the best growth of *P. ostreatus* as well as a highly efficient method for disposing waste.

**Keywords:** Mushroom, Agricultural wastes, Heavy metals, Amino acids, RAPD-PCR and SDS-PAGE.

**INTRODUCTION**

A huge amount of ligno-cellulosic residues are annually generated. It is around 200 billion tons per year of organic matter were produced (Tsegaye and Tefera, 2017). These residues are disposed mostly by means of incineration which causes severe pollution. So, it should be discovering an agricultural waste management method which is cost effective and contribute less in environment pollution. Where Mushroom
Recycling of different wastes for mushroom growth

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cultivation on agricultural wastes fulfills these issues (Ritika and Ishita, 2017). World production of Pleurotus mushroom displays an impressive increase during the last few years and is now approaching the threshold of one million metric tons annually which accounts for 25% of the total mushroom production (Chang and Miles, 1991). In addition not only are mushrooms a protein-rich food source for humans, but the by-products of mushrooms cultivation unlocking nutrients for other members of the ecological community. The rapid return of nutrients back into the ecosystem boosts the life cycles of plants, animals, insects (bees), and soil microflora as showing (Paul1994; Ritika and Ishita, 2017). Cultivation of edible mushroom on agricultural and industrial wastes is a valuable added process to convert these materials into human food. It represents one of the most efficient biological ways by which these residues can be recycled. The Pleurotus mushroom is protein-rich palatable food their production in Egypt has been increasing over the past ten years. Cultivation of Pleurotus mushroom on various lignocelluloses materials has been investigated by a number of researchers (Baskaran et al., 1978; Chang et al., 1981; Mira and Ragini, 1984; Tsegaye and Tefera, 2017). Large quantities of rice straw are produced as agricultural by-products in Egypt. Alternative methods of utilizing these agricultural residues are needed to mitigate the environmental pollution problems associated with current disposal methods such as open field burning. In addition, the water hyacinth is affecting water bodies and suppresses growth of other aquatic plants (George, 2008). The plant is the world’s fastest growing water-borne weed with ability to double its biomass in less than two weeks (Lewis, 2002). It is hardly possible to find a water habitat in Egypt, particularly in Delta not menaced by water hyacinth; they completely cover the water surfaces especially in the Damietta branch of the Nile. Because of its exceptionally high evapotranspiration rate, the infestation of Nile system by water hyacinth causes severe water loss, which represents 1/10 of the average yield of the Nile/year. In a country like Egypt that depends on Nile as the main source of water such loss is unacceptable especially during the new current situation of water shortage or water scarcity. This challenge can be turned into an opportunity, for utilization of water hyacinth as substrate for mushroom farming is one way (Nyakundi et al., 2013). While, tea leaves wastes, mushroom cultivation has recently become very popular in the Black Sea Region of Turkey. But, one of the most important problems in mushroom cultivation is to obtain a suitable casing material. Tea plants are commonly grown in the Eastern Black Sea Region of Turkey. Therefore, there is much tea production waste in this region. This waste material might be reused as a new casing in mushroom cultivation (Co &kun and Aysun, 2003; Ritika, 2017). In Egypt, the tealeaves wastes were produced in large quantities as a tea dust from processing & backing the tea.

This study aimed to compare the effects of different wastes substrate on mushroom production and to show how these substrates can changed the phenotype of mushroom in addition to the morphological and genetic patterns of the resulted fruit bodies.

MATERIALS AND METHODS

The fungus Pleurotus ostreatus (NRRL. 0366) was kindly provided by the Agricultural Research Service (Peoria, USA). The strain was maintained and re grown monthly in Petri dishes containing a sterile solid potato dextrose agar medium. Slants were prepared by cultivation the fungus on Dextrose medium and incubated at 28°C for 4 days (Jodon and Rayse, 1979) and then stored in a refrigerator at 5°C.

Preparation of spawn and cultivation substrates.

Spawn was prepared on about 25 gm. of wheat grains in 250 ml flasks as described by Puri et al., (1981). The flasks contains the grains was autoclaved for 30 min (121°C/1.1 at m) and then kept at room temperature to be cooled. The cooled flasks were inoculated with mycelium of Pleuratus ostreatus on the surface of the medium. The flasks were then incubated at 27°C in the dark room, and leaved for two weeks until mycelium grow.

The substrates and fungus cultivation.

Three different waste substrates were used for fungus cultivation and the first substrate was Rice straw. The straw was ground into small particles with different sizes ranged from 0.5 to 1 cm. The second substrate was Water hyacinth, the plant was collected from the river Nile and dried and then ground into a small particles similar to the Rice straw particles in size and shape. The third substrate is the tea waste. The tea wastes were collected randomly from chimney and ventilator collector of tea factory used as a direct substrate for the fungus. Three batches...
were prepared for production of mushroom fruit body's as follow; sterilized rice straw(I), sterilized water hyacinth+rice straw (II) with 1:1 ratio and sterilized tea waste+sterilized rice straw (III) with 1:1 ratio.

**Mushroom growth and harvesting.**

Each batch was mixed with equal amount of spawn and put into plastic bags; the bags were closed with a plastic ring and a porous sponge plug on the top to prevent possible contamination. Four bags were inoculated for each substrate. The bags were placed in a mushroom growing chamber were the relative humidity was regulated with the aid of mist sprayer at 80%, the light intensity was set at 300-400 lux per day and the temperature maintained at 27 °C. The bags were left closed until the substrate in the bags was fully colonized; the bags were then opened to allow the development of fruit bodies. Mushroom fruit bodies in each bag were picked manually.

**SDS-PAGE and protein profile.**

The total soluble proteins were extracted from the three batches and the obtained proteins were separated on 12% SDS-PAGE according to Lamillae (1970).

**DNA extraction and DNA fingerprinting.**

The total DNA was extracted from the fruit bodies obtained by the three batches. The purified DNA was subjected to DNA fingerprinting using RAPD-PCR. The PCR reaction with volume of 25 µl consisting of 1 µl (0.8 U) of Taq DNA polymerase, 2 µl of dNTPs (2.5m M), 4 µl of each primer, 2.5µl 10x reaction buffer and 1 µl of the fungus DNA. The volume was completed up to 25 µl using sterile H₂O. The PCR reaction conditions were; initial denaturation step at 94°C for 2 min, followed by 45 cycles (94°C for 30 sec, annealing as mentioned with each primer, 72°C for sec) and final extension at 72°C for 10 min. The samples were cooled at 4°C. The amplified DNA fragments were separated on 2.5% agarose gel and stained with ethidium bromide. The primers sequence used were; 20 mer in their length; 5' CAGGCCCTTCCAGCACCCAC3' and 5' GAAACGGGTGGTGATCGCAG'3. The PCR was performed on DNA thermal cycler (Eppendorf).

**Scoring and analysis of RAPDs.**

DNA bands were scored for their presence (1) or absence (0) in the RAPD profile of the mushroom among castes. The index of similarity between the mushroom cultivation on different substrate the number of common fragments observed in individuals and b, was calculated using the formula: \( Bab = 2Nab/(Na+Nb) \), where \( Na \) and \( Nb \) are the total number of fragments scored in a b respectively (Lynch,1990). The BS values were calculated for each primer separately and averaged for all primers huge comparison. A dendrogram was constructed using the Average Linkage between Groups related as described by Sneath and Sokal (1973).

**Heavy metals analysis.**

The heavy metal content was analyzed in fruit bodies resulted from the three batches of the cultivated mushroom. Mushroom samples were dried in an oven at 60°C after that the fruit bodies were placed in a porcelain dish and ignited at 55°C in a muffle furnace, then cooled and dissolved in HCl (5ml 2N HCl) followed by filtration through an acid washed filter paper, then dilution to 25 ml and analyzed for detection of Cu, Zn, Pb and Cd using atomic absorption spectrophotometer (Apha and Wpcf, 1985).

**Amino acids analysis.**

Each of the defatted samples of three batches was weighed (200 mg) into a glass ampoule, 5 ml of 6N HCl/L was added to the ampoule, and the contents were hydrolyzed in an oven preset at 105°C for 22 h. Oxygen was expelled in the ampoule by passing nitrogen gas into it. Amino acid analysis was done by (SYKAM S 433 Amino Acid Analyzer). The analysis was carried out with a gas flow rate of 0.5ml/min at 60°C, and the reproducibility was 3%. The amino acid composition was calculated from the areas of standards obtained from the integrator and expressed as percentages of the total protein. (Spackman et al., 1958).

**RESULTS**

The study revealed that the growth of the fruits bodies was faster on the rice straw compared with the other two substrates. In addition, the amount of mushroom fruits bodies produced is influenced by the type of substrate. Large fruit bodies were obtained with the rice straw and the amino acids contents were also high in this substrate compared with the other two substrates as shown in Table (1). The highest amino acid is aspartic acid (3%) and the lowest amino acid was Methionine (0.2%).

The results shown in Figure (1) showed that the three different substrates affect the
morphological characters of the resultant fruit bodies. These morphological changes were tested on the molecular level to discover, if the changes resulted from genetic variation in the three grown fungus cells. The RAPD-PCR results presented in figure (2A) revealed that there are different band patterns was observed between the three examined genomic DNA of the fungus grown on the three different substrates. In case of the Rice straw the DNA band pattern was completely different on the others two profiles.

Whenever, the DNA band patterns of water hyacinth and tea are closely similar. When the phylogenetic tree (2B) was constructed based on the obtained DNA band pattern it was observed that, the effect of the water hyacinth and tea on the mushroom genome is low in compared with the effect initiated by the Rice straw.

Table (1): Essential amino acids percentage in mushroom fruit bodies cultivated in different wastes.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Rice straw</th>
<th>Water hyacinth</th>
<th>Tea waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>2.517</td>
<td>3.088</td>
<td>2.42</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.698</td>
<td>0.919</td>
<td>0.753</td>
</tr>
<tr>
<td>Serine</td>
<td>0.565</td>
<td>0.098</td>
<td>0.747</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.352</td>
<td>0.0355</td>
<td>2.708</td>
</tr>
<tr>
<td>Proline</td>
<td>0.685</td>
<td>0.857</td>
<td>0.539</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.566</td>
<td>0.883</td>
<td>0.549</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.737</td>
<td>1.009</td>
<td>1.026</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.0124</td>
<td>0.0372</td>
<td>0.0144</td>
</tr>
<tr>
<td>Valine</td>
<td>0.759</td>
<td>0.92</td>
<td>0.759</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.223</td>
<td>0.34</td>
<td>0.27</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.568</td>
<td>0.776</td>
<td>0.552</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.798</td>
<td>1.1026</td>
<td>1.037</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.327</td>
<td>0.461</td>
<td>0.3912</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.602</td>
<td>0.772</td>
<td>0.656</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.385</td>
<td>0.54</td>
<td>0.409</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.541</td>
<td>0.843</td>
<td>0.649</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.754</td>
<td>0.97</td>
<td>0.856</td>
</tr>
</tbody>
</table>

Figure 1: The fruit bodies of Shetakii mushroom grown on different waste substrates. A; Rice straw, B; Water hyacinth, C; Tea waste.
Figure 2: RAPD-PCR for the three fruit bodies grown on three different substrates. M: phax 174 DNA marker lanes; 1: rice straw, 2: water hyacinth, 3: Tea. Protein band patterns for the three types of fruit bodies.

Figure 3: SDS-PAGE for the total protein extracted from the three fruit bodies grown on different waste substrate.

Figure 4: Comparison of heavy metals concentration of fruit bodies of edible mushroom on different substrates.
Table (2): Concentration of heavy metal (µg/g) in fruit bodies of edible mushroom on different substrates:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Zn</th>
<th>Cu</th>
<th>Pb</th>
<th>Cd</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice straw</td>
<td>65.56</td>
<td>10.47</td>
<td>5.49</td>
<td>0.265</td>
<td>0.0</td>
</tr>
<tr>
<td>Water Hyacinth</td>
<td>29.85</td>
<td>0.17</td>
<td>1.04</td>
<td>1.45</td>
<td>0.0</td>
</tr>
<tr>
<td>Tea waste</td>
<td>59.46</td>
<td>3.356</td>
<td>2.200</td>
<td>1.357</td>
<td>1.005</td>
</tr>
</tbody>
</table>

The extracted protein of the three fruit bodies were separated and the protein band patterns presented in figure 3 revealed that, the protein pattern of the fruit bodies grown on tea differed on the other two profiles of the two substrates. Whereas, the protein pattern of the fruit bodies of the fungus grown on either rice straw or water hyacinth are closely similar. The phylogenetic tree constructed based on the obtained protein patterns showed that the similarity between the water hyacinth and rice straw was 98% but between the fruit bodies grown on the tea and the other two types was 96%.

**Heavy metals.**

The heavy metal concentration as (µg/g) of the edible mushroom samples is given in Table 2. The data presented in Table 2 revealed that mushrooms cultivated on Rice straw show the highest zinc content (65.5 µg/g) followed by mushroom cultivated on tea waste (59.5 µg/g) followed by mushrooms cultivated on water hyacinth (30 µg/g). The levels of copper (Cu) in the three fruit bodies ranged from 0.17 to 10.5 µg/g. The highest copper conc. (10.5µg/g) was in mushrooms harvested from rice straw substance, followed by tea and water hyacinth 3.4 and 0.2 µg/g respectively. Lead (Pb) concentration in the three types of fruit bodies were 5.5, 2, and 1 µg/g in rice straw, tea and water hyacinth in respective manner. On the other hand, Cadmium (Cd) was presented in high concentration in the fruit bodies grown on water hyacinth, followed by that grown on tea and lastly these grown on the rice straw waste; 1.5, 1.3 and 0.3 µg/g, respectively. Nickel (Ni) contents of the mushrooms from three different substrates from Table 1 was not detected expect for mushrooms collected from tea wastes substrate was 1. The heavy metal concentration as (µg/g) of the edible mushroom samples is given in Table 2. From Table 2, in the mushrooms cultivated on Rice straw, the highest zinc content was 65.6 µg/g followed by mushroom cultivated on tea waste, where zinc concentration was 59.5 µg/g followed by mushrooms cultivated on water hyacinth, was 29.85 µg/g. The levels of copper (Cu) in the samples obtained from different mushrooms fruits bodies from table (2) ranged from 0.2 to 10.5 µg/g. The highest value was to be 10.5 µg/g in mushrooms harvested from rice straw substrate, followed by 3.4 µg/g for tea waste substrate, then 0.2 µg/g for water hyacinth substrate.

In the mushroom collected from rice straw substrate e the highest lead content was found as 5.5 µg/g followed by 2.200 µg/g for mushroom which collected from tea waste substrate, then the lowest value was 1.04 µg/g for mushroom collected from water hyacinth substrate. The highest values for Cadmium (Cd) were in fruits of mushroom cultivated on water hyacinth substrate followed in fruits of mushroom cultivated on tea wastes substrate, then fruits of mushroom cultivated on rice straw (1.5, 1.4 and 0.3 µg/g) respectively. Nickel was detected only in the mushrooms fruit bodies grown in tea (1 µg/g) but not detected on the other two types.

**DISCUSSION**

The results of cultivation of mushroom *Pleurotus ostreatus* on different recyclable wastes as a substrates indicates that rice straw is the most suitable substrate , this may be due to the availability of the cellulose in the straw which is utilized by mushroom mycelium. According to (Lbaschagne et al., 2000; Ritika and Ishita, 2017) it is possible to distinguish two separate phases in the degradation of straw by *P. ostreatus*. Phase one involves degradation of small water-soluble materials and high activity of cellulose. Phase two is mainly lignin degradation. Data obtained from both protein pattern and RAPD-PCR analysis was confirmed each other. These results suggest that the above mentioned RAPD primers can be used to differentiate within the same species if the growth condition had been changed .It means that, the morphological changes found in the resultant fruits back to genotypic changes on the molecular levels, i.e. one or some of genes may be induced by the used substrate. These genes, which so called conditional genes, expressed on
themselves and gave protein bands with different molecular weights. The appearance of those bands only with the fungus grown on the tea waste, these gave an indication that there are a substance make induction to that genes founded in substrate. The average heavy metals concentration of edible mushroom species are given in Table (2) , according to (Kalac et al., 1991) they reported that the amounts of heavy metal contents are related to species of mushroom, collected site of sample, age of fruiting bodies and mycelium and source of pollution. On the other hand heavy metals concentrations in the mushroom are hardly affected by pH or organic matter content of the substrate which agree with (Demirbas, 2002; Sesli and Tuzen, 1999; Gast et al., 1988). The trace element content of the species depends on the ability of the species to extract element from the substrates and on the selective uptake and decomposition of elements in tissues as reportedby (Demirbas, 2001; Sesli and Tuzen, 1999). The uptake of heavy metal ions in mushrooms is higher than in plants. For this reason, the concentration variations of heavy metals could be considered due to mushrooms species and their ecosystems (Isildak, 2004). In addition, toxic metals are present in high concentrations in the fruiting bodies of both edible fungi from an area or substrates greatly favored by mushroom pickers is of particles importance in relation to the FAO/WHO standards (1976) and EU (European commission, 2001) for lead and cadmium as toxic metals. The maximum permissible dose for as adult is 3 mg lead and 0.5 mg cadmium per week, but the recommended doses are only one fifteenth of those quantities (Demirbas, 1999). Gast et al., (1988) investigated the uptake of cadmium copper, lead and zinc in mushrooms and their relationship with results presented in Table (2). In general, zinc, copper, lead and cadmium concentration accumulation by the mushrooms were low; any higher concentrations of those metals show a pythotoxical effect and consequently lower the yield (Demirbas, 2000). Lower concentrations of copper were accumulated to some extent by the mushrooms; on the other hand, higher concentration of copper reached equilibrium and remained constant which agree with the obtained results presented in Table (2). According to Stijve and Besson (1976), the mechanism by which some heavy metals are accumulated is somewhat obscure although it seems to be associated with a chelating reaction with the sulfhydryl groups of protein and especially with methionine. However, the same authors found very low levels of lead and cadmium in samples cultivated with a high content of methionine in relation to other species (Demirbas, 2000).In general, from the previous results, showed that the lowest values of heavy metals were recorded in mushroom cultivated on WH substrates. These observations agree with Sinkala et al. (2002) who stated that production of mushroom on water hyacinth or vegetables grown on manure or substrate performed better than those grown using commercial fertilizers.

CONCLUSION

The cultivation of Pleurotus ostreatus in Egypt and other subtropical countries seems promising. The most significant aspect of mushroom cultivation, if managed properly, is to create zero emission of wastes materials. The rice straw yielded better mushroom production and grew faster than other substrates. Mushroom cultivation proves to be a highly efficient method for disposing of agricultural wastes, as well as producing human food. Heavy metal (Zn, Cu, Pb, Cd and Ni) concentration of three mushrooms from three different substrates (Rice straw, water hyacinth and tea leaves wastes) collected from different region, in Alexandria was investigated. Levels of heavy metals are considerably low, and the fruit bodies were suitable for consumption as a nutritional food.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest”.

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The author would thank all participants.

AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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