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# Production of Oyster Mushroom (*Pleurotus ostreatus*) and Tracking the Lignin Degrading Enzymes on Different Agro-Industrial Residues

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OYSTER MUSHROOM is a promising source of single cell protein because it can consume a wide range of lignocellulosic materials without pre-composting due to its unique oxidative enzyme system. In the current work different agro-industrial residues; Orange peel (OP), Olive mill waste (OMW), Moringa leaves (ML), Corn cobs (CC), Rice straw (RS), Sugar cane bagasse (SCB), jojoba bagasse (JB) were evaluated for their potential to support the growth of oyster mushroom (*Pleurotus ostreatus*) mycelium in submerged static cultures and the fungal oxidative enzymes (Laccase and Peroxidase) were tracked at the end of incubation time. The most potent treatments were used individually or in combinations in the production of oyster fruiting bodies in solid state process. A number of parameters indicating the efficiency of cultivation process were assayed including (Days of completion of spawn running 100%, Days of pinheads formation, Days of fruiting bodies formation, Harvesting time/days, Yield of mushrooms on first and second flush(g), biological efficiencies % and Production rate/day. The highest dry weight in the submerged fermentation experiment was recorded by SCB followed by RS then OMW. Laccase activity recorded its highest values in case of RS, 55.12 $Uml^{-1}$ while the highest peroxidase activity was recorded for SCB 35 Uml-1. The best combination between agro-industrial residues was (50%RS+50% OP) where it gave the highest total yield (866 g) with a yield % 86.58 and biological efficiencies of 144.87%.

**Keywords:** Fungi; Harvesting; Lignocellulosic; Orange peels; Submerged.

## 1. Introduction

The word (mushroom) has been used in many cultures and in a wide geographical and temporal range, and it mostly denotes those large fungi that have a stalk and a hat. **Chang & Miles 1992**. They represent a good source of protein, dietary fibre, vitamins and minerals **Alam et al., 2008; Singh 2017; Hajdú et al., 2022; Törős et al., 2022**.

The global mushroom market size was valued at USD 50.3 billion in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 9.7% from 2022 to 2030. There is no reliable data on the volume of production and trade of mushrooms in Egypt, but based on international databases, the Middle East and Africa region is considered the least productive and consuming region of various types. https://www.grandviewresearch.com/industry-analysis/mushroom-market

Oyster mushroom is related to the Phylum – Basidiomycota and belongs to Family - Pleurotaceae. *Pleurotus ostreatus* is one of the largest species in the genus, Pleurotus, which inhabit and colonize varying agro climatic conditions. It grows at a broad range of temperatures (10-30°C) and pH (6-8) **Yingyue et al 2014**; **Zadrazil and Dube, 1992.** Produces a wide range of oxidative and hydrolytic enzymes that are capable of catalyze the degradation of complex lignocellulosic biomass as a substrates without pre-composting **Lallawmsanga et al., 2019**; **Agba et al.,**

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**2021.** Can be cultivated using minimal environmental control and sterilization; its mycelium can grow and colonize substrates in a short time producing high yield with appropriate nutritional and medicinal values **Kimenju et al., 2009**. The substrate used for its cultivation needs only to be pasteurized by primitive means, however, the fruiting bodies are resistant to infection and contamination, which makes it suitable for home cultivation without long training or complex sterilization or conditioning methods. **Ejigu et al., 2022**; **Fufa et al., 2021; Deepalakshmi and Mirunalini 2014.**

Although almost all agricultural wastes are available for cultivation of *Pleurotus ostreatus* as they contain lignocellulosic biomass, the mushroom growth and development are greatly affected by environmental factors and substrate type **(Agarwal et al., 2016).**

Agricultural residues are biochemically made-up of complex biomolecules, for example, wheat straw is composed of 1 % protein, 13 % lignin, 39 % hemicelluloses and 40 % cellulose. Rice straw is composed of 41 % cellulose, 14 % lignin, 0.8 % total nitrogen, 0.25 % $P_2O_5$, 0.3 % $K_2O$ and 6 % $SiO_2$. Sugarcane bagasse is composed of cellulose (35 – 40 %), hemicellulose (20 – 25 %), lignin (18 - 24 %), ash (1 – 4 %), waxes (<1 %) and nitrogen (0.7 %).

About 50% of the total orange fruit weight is discharged during juice production; these residues can be used in biotechnological applications (**Inácio et al., 2015)**

The lignin-degrading enzymes are the secret behind the strength of oyster mushrooms and their ability to consume plant materials without previous treatments. (**Thurston, 1994).**The most famous of these enzymes are laccases and peroxidases, so their measurement gives valuable information on the feasibility of using a particular vegetable material in the production of mushrooms.

Hence, this work was carried out to evaluate the feasibility of using available agro-industrial residues commonly produced in Egypt as substrate for the cultivation of oyster mushroom (*Pleurotus ostreatus*) by using the screening in submerged culture followed by solid state fermentation in polythene bags method of cultivation to determine their relative biological efficiency and their variation in laccase and peroxidase enzymes production. The purpose is also to optimize the mixtures of different lignocellulosic wastes as substrate for the cultivation of oyster mushroom and pave way for effective utilization of these wastes and reduction of environmental pollution caused by these wastes.

## 2. Experimental design

### Oyster mushroom strains

*Pleurotus ostreatus* (NRRL–2366) was provided from Northern Regional Research Laboratory, Illinois, Peoria, USA. The microorganisms were sub cultured every 30 days.

### Agricultural residues

Rice straw (RS) was obtained from the experimental farm of Agricultural Research Centre, Giza, Egypt. SCB and OP were obtained from the local juice shop, Giza, Egypt. OMW and JB were obtained from nearby oil mile factory. Agricultural residues were air-dried and stored for further use.

### Screening the potential of collected agriculture residues to support the growth of *Pleurotus ostreatus* (NRRL–2366) in submerged culture

Agricultural residues were dried at 70C° overnight then grounded and sieved through 40-mesh screen. 100g of each material was suspended in 1000 ml (production medium described by **Fujian et al., (2001)**. This medium contained the following (g/l): yeast extract (0.5); $(NH_4)_2SO_4$ (1.0) and tween 80 (1.0) in 2L capacity glass jars then jars nozzles were sealed by cotton plugs and sterilized in autoclave at 121 °C for 30 min. after cooling jars were inoculated with 5mm diameter disc obtained from a growing edge of *Pleurotus ostreatus* (NRRL–2366) grown on Potato Dextrose Agar plate (PDA) then incubated statically at 28 °C until fruiting bodies were formed, the liquid level was kept constant during incubation period by adding sterilized distilled water when needed. At the end of incubation period, growth parameters and lignin degradation enzymes (laccase and peroxidase) were recorded.

### Solid state cultivation of *Pleurotus ostreatus* (NRRL–2366)

The straw parts of dry agricultural residues (Rice and caraway straw ) were shredded into tiny bits of about 1.5 cm to 2 cm and soaked overnight in tap water then pasteurized at 100 °C for two hours and transferred to perforated plastic bags to get red excess water and reduce moisture content. Other agricultural residues were filled into polypropylene bags and pasteurized at 121°C for 1 hour and allowed to cool overnight. The moisture content of at 70% then used in oyster cultivation.

### Spawn preparation

Sorghum grains were soaked overnight in water then, A mixture of pre-soaked sorghum grain, wheat bran,

and calcium sulfate (gypsum) in the ratio of 88:10:2 respectively was prepared. The mixture was distributed in 1000 ml glass bottles and autoclaved at 121°C for 45 minutes. After cooling, each bottle was inoculated with 20 sorghum grain and soaked overnight in a sufficient amount of water sorghum grain was weighed and soaked overnight in a sufficient amount of water 28°C until the substrate were fully colonized by mushroom.

**Treatments design for solid state cultivations**

The most promising four agricultural materials obtained from the results of submerged experiment were used individually or in combinations as follow:

| Code | Treatment |
|---|---|
| T1 | RS |
| T2 | 50% RS+50% OMW |
| T3 | SCB |
| T4 | 50% SCB +50% OMW |
| T5 | 50% RS+50% OP |
| T6 | 50% SCB +50% OP |
| T7 | 50% ONW+50% OP |

**Spawning and Spawn Running**

The substrates were transferred to transparent polyethylene cultivation bags (65cm length and 45cm width). Substrate with about 70% moisture content was mixed with spawn in a ratio of 10:1 respectively (dry weight/wet weight basis) under laminar flow hood and the inoculated bags were then tightly tied with suitable rubber ring. Pin holes were made with sterilized needle through bags for drainages and aerations. The inoculated bags were incubated at room temperature (23-25°C) in the dark until primordial was formed.

**Biological Efficiency and production rate**

Biological efficiency defined as the ability of fungi to convert a substrate into fruiting bodies. (BE) and the production rate (PR) was assessed according to equations 1 and 2.Total weight of the fruiting bodies harvested from the substrates within first and second flush was measured as total yield of the mushroom. The biological efficiency and production rate (yield of mushroom per kg substrate on dry weight basis) was calculated as the formula proposed by Chang *et al*. (1993).

1- BE% = FWM/DWS_x100

Where, BE=Biological Efficiencies, FWM=Fresh weight of mushroom and DWS=Dry weight of substrates.

2- PR % = BE/ PT x100

Where, PT=Production time.

**Enzymatic activities assay in submerged culture**

**Peroxidase assay**

Peroxidase enzyme was assayed by Methylene Blue method according to Magalhaes et al (1996). Briefly, 2.2 ml of the supernatant and 0.1 ml of methylene blue (1.2 mM) were added to 0.6 ml of Tris-HCl buffer (0.5 M, pH 8.0). Then 0.1 ml of 2.7 mM $H_2O_2$ was added to the mixture to start the reaction. The decrease in absorbance at 664 nm was tracked using Specol UV/VIS spectrophotometer and the results were recorded as the change in absorbance per minute (mM methylene Blue)

**Laccase assay**

Laccase enzyme activity was assayed according to Palmieri et al (1997) by determining the rate of [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] ABTS oxidation. The reaction mixture contained 1.0 ml of 0.03% ABTS solution and 1.0 ml of 50 mM, pH 8.0 Tris-HCl buffer in addition to 1 ml of diluted enzyme then the mixture was incubated at 37 ◦C for 10 min. and the absorbance was recorded at 420 nm using Specol UV/VIS spectrophotometer. One unit of laccase enzyme activity was defined as the amount of enzyme essential to oxidize 1.0 μmol of ABTS per min.

**Determination of protein**

Protein content was determined according to Lowry et al. (1951) for soluble protein determination was used.

## 3. Results

In an a trial to track the production of lignin-degrading enzymes during oyster mushroom development, the strain was grown in submerged culture containing the lignocellulosic materials as a sole carbon source, the growth in submerged culture comfortably permit the estimation of required enzymes. SCB recorded the highest fruit bodies dry weight at the end of incubation beriod (13.4% increase compared with OMW while RS came in second place after SCB. Table (1). In the case of mushrooms grown in a culture containing RS, the time required to complete the production of fruiting bodies was observed to be only 43 days, compared to the culture containing SCB, which came in second place by 48 days, while the culture containing OMW came in the last place by 56 days. The protein content relatively followed the same trend observed in dry weight where SCB gave the highest protein content. For lignin-degrading enzymes, a large discrepancy was observed between treatments. This may be due to the difference in the content of each substrate of

lignin, and the highest values were recorded for the enzymes laccase and peroxidase in the case of the culture containing RS and SCB, respectively. It was also found that the culture consisting of CC contains a high percentage of laccase (49.64 U/ml) Table (1).

The difference in the content of lignocellulosic materials from major and minor elements makes mixing them up in mushroom cultivation a promising idea. In case of RS, the combination with OP reduced the time required for completion of spawn running and fruiting bodies formation by an average of one day. While the same treatment increased the time required to pinheads formation by an average of one day compared with the individual treatment containing only rice straw. The situation was relatively the same in case of sugar can bagasse where mixing with OP led to the reduction in time required for completion fruiting bodies formation and harvesting time. It is also worth noting that the treatments containing OMW took the longest time in the time required for completion of spawn running, pinheads formation and harvesting time Table (2).

The yield of the first flush recorded an increase of 10-34 % compared to the second flush in all treatments, and the largest difference was recorded in the treatments containing SCB and RS with a percentage of 34 and 23, respectively. As for the total yield represented by the sum of the two flushes, the treatment containing a mixture of RS and OP achieved the highest productivity, followed by the treatment containing SCB and OP while the lowest yield was recorded for the treatment depended on SCB as a substrate (fig1).

The previous observations were reflected also on the biological efficiency and production rate, where the highest biological efficiency was recorded for the treatment containing RS + OP as a substrate followed by SCB +OP Table (3).

The product of the most promising treatment (50% RS+50% OP) was subjected to analysis for its nutritional values on the basis of dry weight. The ratio between carbohydrates: protein: fats were 21: 13: 1 in addition it contains a considerable amount of fibres (5.5%) Table (4).

**Table 1. Dry weight, harvesting time, Protein and laccase and peroxidase activity (U/ml), during the growth of *Pleurotus ostreatus* on different substrates under static submerged conditions.**

| Agro-industrial residue | Dry weight (g/l) | Harvesting Time/day | Protein % | Laccase | Peroxidase |
|---|---|---|---|---|---|
| Orange peel (OP) | 6.30 | 54 | 26.58 | 21.75 | 16.3 |
| Olive mill waste (OMW) | 6.50 | 56 | 27.34 | 23.12 | 20.3 |
| Moringa leaves (ML) | 5.30 | 49 | 17.78 | 20.57 | 21.7 |
| corn cobs (CC) | 6.00 | 55 | 22.21 | 49.64 | 26.7 |
| Rice straw (RS) | 6.50 | 43 | 28.25 | 55.12 | 32.2 |
| Sugar cane bagasse (SCB) | 7.37 | 48 | 31.77 | 25.85 | 35.3 |
| Jojoba bagasse (JB) | 5.32 | 55 | 19.66 | 29.56 | 25.7 |
| **LSD 0.01** | 1.22** | 10.51** | 2.02** | 2.32* | 2.21** |

**Table 2. Effect of different substrates on the growth parameter of mushroom under submerged fermentation.**

| Agro-industrial residue | Days of completion of spawn running 100% | Days of pinheads formation | Days of fruiting bodies formation | Harvesting time/days |
|---|---|---|---|---|
| RS | 19 | 25 | 5 | 2 |
| 50% RS+50% OMW | 20 | 27 | 4 | 2 |
| SCB | 19 | 26 | 6 | 4 |
| 50% SCB+50%OMW | 20 | 27 | 6 | 3 |
| 50%RS+50% OP | 18 | 26 | 4 | 2 |
| 50% SCB+50% OP | 19 | 27 | 5 | 3 |
| 50%OMW+50% OP | 21 | 28 | 4 | 4 |
| **LSD 0.01** | 3.17* | 3.03** | 1.98** | 1.87** |

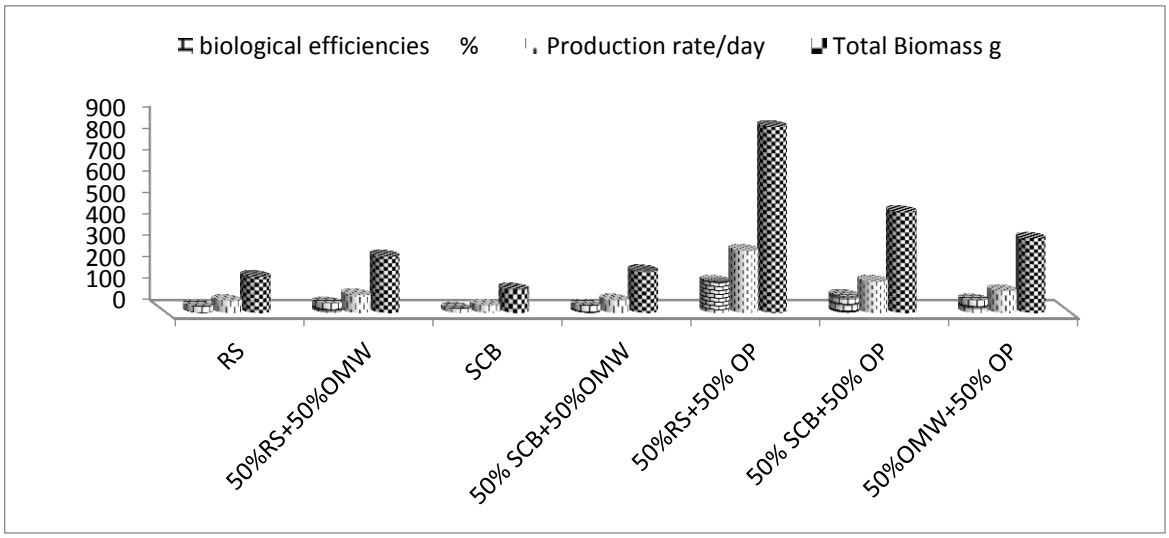



**Fig. 1. Effect of different Agricultural residues combinations on the yield components of mushroom.**

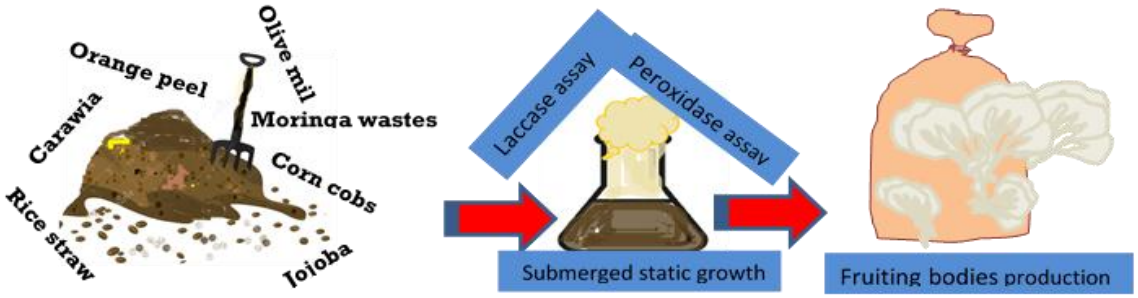
**Table 3. effect of different substrate of agro-industrial waste on biological efficiencies and total biomass of *Pleurotus ostreatus* mushroom under solid state cultivation.**

| Substrate | biological efficiencies % | Production rate/day | Total Biomass |
|---|---|---|---|
| Rice straw | 27.81 | 54.52 | 166.84 |
| 50%rice straw+50%olive mile waste | 43.60 | 82.26415 | 261.62 |
| Sugar cane bagasse | 18.04 | 32.8 | 108.27 |
| 50% Bagass+50%Olive mile waste | 32.20 | 57.5 | 193.16 |
| 50%Rice straw+50% Orange peel | 144.30 | 288.6 | 865.75 |
| 50% Bagass+50% Orange peel | 78.23 | 144.8704 | 469.40 |
| 50%Olive mile waste+50% Orange peel | 57.46 | 100.807 | 344.77 |
| **LSD 0.01** | $2.26^{**}$ | $2.4^{*}$ | $2.9^{**}$ |

**Table 4. Nutritional values of oyster mushroom fruit bodies from mixture waste (50%Rice straw+50% Orange peel) cultivation.**

| Chemical composition | Oyster fruit bodies for 100 g (Dry weight) |
|---|---|
| Dry matter | 12.5 |
| Carbohydrate | 43.69 |
| Total protein | 26.5 |
| Fat | 2.09 |
| Lipids | 3.5 |
| Fiber | 24.6 |
| Ash | 5.5 |
| Moisture | 85 |
| Energy value (Kcal/100g dry w) | 299.57 |

Total available carbohydrate (%) = [100 – (moisture + total ash + fiber + protein + fat)] -
Energy (kcal/100g) = [(protein × 4) + (carbohydrate × 4) + (fat × 9)]




**Graphical Abstract**

**Discussion**

Lignocellulosic biomass represent an ideal substrate for the production of oyster mushroom (*Pleurotus ostreatus*), and almost all types of agricultural residues can be consumed by mushroom. **Diana et al., (2006)**

The potential of different agricultural residues as a substrate for mushroom production depends on many factors including mushroom species. The substrate type can affect one or more of mushroom production parameters. For example, **Sherief et al., (2010)** stated that the fruiting was earlier when a commercial strain of oyster mushroom cultivated on a substrate composed of rice straw compared with sawdust. **Obodai et al., (2003)** reported that rice straw was the best choice between eight lignocellulosic residues for cultivation of *Pleurotus ostreatus*. Therefore, the recommendation of any substrate for commercial mushroom production should be based on the scientific evaluation of this substrate.

One of the most important parameters in commercial mushroom production is spawn Running Phase. In the current study, the spawn running phase took between 18 to 21 days. Similarly**, Buah et al., 2010,** reported that the complete mycelium colonization takes 2-3 weeks after inoculation on corncob and sawdust. Also, **Girmay et al., 2016** found that the mycelium running phase took about 16 days after inoculation. Other researchers reported longer spawn Running Phase **Taskirawati et al.**, **(2020)** The variation in the time consumed for full mycelia colonization of production substrates may be due to growth conditions like humidity, temperature, $CO_2$, and the substrate type (C :N ratio, mineral content, presence of agrochemical residues) or mushroom species or strain **Zhou et al., (2012)**.

About 91 to 96% of the total yield is obtained in the first three consecutive flushes **Ejigu et al., 2022.** Therefore, only the first three flushes were adopted in our study. The pattern of gradual decline in mean yield per flush remains constant for cultivated oyster mushroom nevertheless of the mushroom species or strains and of the substrate composition (chemical or biological) used to grow mushrooms **Tsegaye and Tefera, 2017.** It is well known between mushroom producers that first flush gave more yield than the second and third flushes because the quantity of harvested fruiting bodies in each flush is directly proportional to the nutrients consumed from the substrate **Dissasa 2022**.

Also the substrates with a lower C/N ratio straw provide the fastest pinhead formation, but the lowest in their mushroom yield **Ejigu et al, 2022**

The ability of any microbial species to consume particular substrate depends on the enzymatic system necessary to degrade that substrate **Rajarathnam et al. 1998.** The species and strains of mushroom differs in their ability to produce the enzymes necessary for the decomposition of lignocellulosic materials. This difference may be attributed to the genetic nature of each strain or mushroom species, and also depends on the growth conditions and substrate type. The ability of mushrooms to produce enzymes is also reflected in the production stages of mushroom fruits. The most important of these enzymes are the lignin-degrading enzymes, which enable the fungus to reach the carbohydrates represented in cellulose and hemicellulose **Atikpo et al., 2008**. Production of laccase by mushroom has been carried out in both submerged and solid state fermentation **Daassi et al 2016**. As in the current study, Previous reports indicated that high concentration of laccase has been obtained under static conditions **Chowdhury et al., 2014**.

## 4. Conclusion

In this study, the possibility of using rice straw mixed with orange peel as substrate for cultivation of oyster mushroom was evaluated. The results indicated that the best substitute for conventional rice straw was a substrate that contained 50% rice straw mixed with 50% orange peel. Overall, results from this study suggested that the mixing of different types of agricultural residues may optimize the growth ad yield of oyster mushroom.

## 5. Conflicts of interest

There are no conflicts to declare.

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