Proceedings of 22nd Agricultural Research Symposium (2024) 231-235

Effect of Biofilm Exudates on Quality and *Trichoderma* Infestation of Oyster Mushroom (*Pleurotus ostreatus*) Cultivated using Different Substrates

Y. G. S. S. GUNATHUNGA[1], M. PREMARATHNA[2], B. L. W. K. BALASOORIYA[1] and G. SENEVIRATNE[2]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), 60170, Sri Lanka*

[2]*Microbial Biotechnology Unit, National Institute of Fundamental Studies, Hantana Road, Kandy, 20000, Sri Lanka*

ABSTRACT

Mushroom cultivation is reported as an economically viable biotechnological process for converting various lignocellulosic wastes. This study investigated the potential of Biofilm Exudates (BFEx) in enhancing Oyster mushroom (*Pleurotus ostreatus*) cultivation, particularly on non-sterilized substrates, and in mitigating *Trichoderma* pathogenic infections. Conventional cultivation methods rely heavily on sterilization and chemical fertilizers, raising environmental and food safety concerns. Our research compared the yield and quality of mushrooms grown on autoclaved and non-autoclaved substrates, with and without BFEx, across three substrate types: one recommended by the Department of Agriculture (DOA) and two cost-effective alternatives, such as chemical fertilizer enriched substrate type and organic fertilizer enriched substrate type. The BFEx was extracted from biofilms developed *in-vitro* using *Aspergillus* sp. and five bacterial species and applied to the substrates. Results showed that autoclaved substrates with BFEx significantly improved mushroom yield, biological efficiency, and protein content. Remarkably, mushrooms grew on non-autoclaved, BFEx-treated substrates despite pathogen presence, indicating BFEx's role in enhancing microbial diversity and natural biocontrol. The FTIR spectroscopy confirmed higher protein content in BFEx-treated mushrooms. These findings suggest that BFEx applications can reduce the need for substrate sterilization, enhance nutrient availability, and improve yield and quality. Further, this pioneering field experiment highlights the effectiveness of BFEx in mushroom cultivation and the need for further research to confirm its impact on pathogens like *Trichoderma*. The study offers valuable insights for sustainable cultivation practices, benefiting both small-scale farmers and commercial cultivators, and informing agricultural policymakers about alternative methods to improve the quality and yield of mushrooms.

KEYWORDS: Biocontrol, Biofilm, FTIR Spectroscopy, Mushroom, *Trichoderma*,

INTRODUCTION

The oyster mushroom (*Pleurotus ostreatus*) is one of the most cultivated mushrooms in the world. The oyster mushrooms are characterized by their distinctive broad, fan, or oyster-shaped white-colored cap (Boddy and Hiscox, 2017). There is a significant body of research on mushroom cultivation methods, but there remains a notable gap in understanding the implications of using non-sterilized substrates and incorporating microbial amelioration specifically concerning *Trichoderma* pathogenic infections (Jayasinghearachchi and Seneviratne, 2004). Existing literature primarily focuses on conventional sterilization techniques, with limited exploration of alternative practices that could potentially reduce reliance on sterilization while enhancing mushroom quality and yield. Addressing this research gap is crucial for advancing sustainable and efficient mushroom cultivation practices, providing valuable insights for both commercial cultivators and agricultural policymakers. Conventional mushroom cultivation relies heavily on chemical fertilizers and sterilization protocols, both raising concerns about environmental sustainability and food safety (Pathmashini *et al*., 2009). Additionally, yield and nutrient content of mushrooms can be variable and susceptible to pathogen outbreaks. Biofilm biochemicals have been reported to increase the diversity and abundance of microbes leading to enhanced natural biocontrol. Moreover, biofilm biochemicals reported to increase crop quality and yield (Jayasinghearachchi and Seneviratne, 2004).

Traditional mushroom cultivation often involves substrate sterilization due to concerns related to *Trichoderma* infestation (Jayasinghearachchi and Seneviratne, 2004). However, this conventional method may disrupt the natural microbial balance within the substrate. In contrast, biofilm microbial ameliorators, exemplified by Biofilm Exudates (BFEx), present a promising alternative, as they have been reported to enhance nitrogen

fixation, ultimately leading to protein enrichment in the cultivated crops (Jayasinghearachchi and Seneviratne, 2004). Here we hypothesized that the same principle could be applied to mushroom cultivation as well. The present study was designed to investigate the potential use of biofilm biochemicals in mushroom cultivation, particularly for small-scale farmers. The current study aimed to study the effect of Biofilm exudates added in mushroom growth media on the mushroom yield, biological efficiency, protein content, and controlling the infestations by pathogens.

METHODOLOGY

Study Location

The experiment was conducted at the National Institute of Fundamental Studies in Hantana, Kandy (07˚ 26′ N, 80˚ 63′ E; 1145 masl). The average annual temperature of 23-25 °C and 2000 mm rainfall prevailed in the area were ideal conditions for mushroom cultivation (Department of Agriculture Sri Lanka, 2024).

Treatments and Experimental Design

Twelve treatments (T_1-T_{12}) were used in the study with three mushroom substrate types, autoclaved/ not autoclaved and added/ not added biofilm exudates as detailed in the below section (Figure 1).

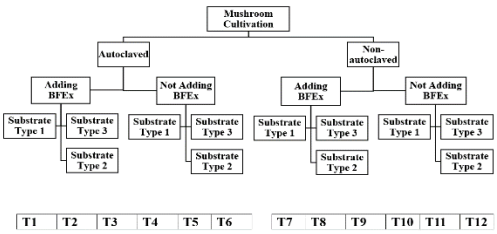

Figure 1. Treatment combinations used in the study *(The general understanding is that T_1 to T_{12} follows from left to right. For instance, T_1 is the furthest left-cornered one representing type 1 while T_2 is the middle representing type 2, and T_3 represents type 3 substrate as go on until T_{12})*

Preparation of Mushroom Substrates

Three types of substrates were used for mushroom growth: Type 1 (DOA recommended substrate), Type 2 (sawdust, urea, $CaCO_3$, $(NH_4)_2SO_4$, and $MgSO_4$) and Type 3 (sawdust, rice bran, eggshells, soya flour, and dolomite). For each substrate, 200 gauge polypropylene bags with dimensions of 7" in width and 14" in height, standard size mushroom bags were prepared and half of the bags were autoclaved while the rest were not sterilized (Department of Agriculture, Sri Lanka, 2024).

Adding Biofilm Exudates and Incubation

Biofilm exudates (BFEx) were extracted from a biofilm, developed *in-vitro* by using *Aspergillus* sp. with five bacterial species (*Azorhizobium* sp, R*hizobium* sp, *Acetobacter* sp, *Azotobactor* sp, and *Azospirillum* sp). The biofilm exudates consist of proteins, polysaccharides, lipids, DNA/RNA, and quorum-sensing molecules. 1mL of micro-filtered BFEx using a 0.45µm filter, was mixed with 50 mL of distilled water and then applied to one mushroom bag by injecting method in a laminar flow cabinet (Jayasinghearachchi and Seneviratne, 2004). Mushroom bags spawned with oyster mushrooms were kept for 20-30 days in an incubation room. (consistent temperature of 24-27 ^oC, humidity 90-100%, good air circulation, and total dark condition (Department of Agriculture, Sri Lanka, 2024). After the completion of spawn running, they were transferred to the cropping house. Oyster mushrooms were chosen for this study because they are the most commercially utilized mushroom variety in the world.

Harvesting and Collecting Yield Data

After the incubation period, the presence of primordia was identified. At the harvesting time, the fresh weight of all mushrooms harvested was measured. The samples were dried under sunlight and air for further analysis. The biological efficiency and dry matter content were calculated using the total fresh weight and dry weight of the mushroom harvest and substrate (Birhanu *et al.*, 2016).

Determination of Phosphorous Content

The colorimetric method was used to determine the total phosphorous (%). Acid-digested samples were reacted with molybdate reagent, forming a blue complex (Pulliainen and Wallin, 1994). The color intensity was measured spectrometrically. Phosphorous concentrations were determined by comparing absorbance to a standard calibration curve.

Protein Analysis using FTIR

Protein content in samples were analyzed using FTIR spectroscopy. Samples were prepared and dried, and FTIR pellets were prepared by mixing the samples with KBr in a 1:20 ratio for subsequent FTIR spectroscopy analysis, then spectra were obtained. Absorbance peaks corresponding to protein bonds were identified and analyzed. Amide I band was observed in the 1600-1700 cm^{-1}

range of the IR spectrum (Sadhana and Malini, 2018). Differences in peak intensities were compared across treatments, allowing the relative protein content to be assessed and contrasted.

C, H and N Analysis using CHNS/O Analyzer

Total C and N content was determined with a CHNS/O analyzer (Model: 2400 Series II UV-VIS). Samples were weighed and placed in the analyzer's combustion chamber. High-temperature combustion was performed, converting the sample elements into gases (Kristensen and Andersen, 1987). These gases were then passed through detectors specific to each element. The detector signals were calibrated using known standards and contents were quantified by comparing the sample signals to these calibration curves.

Determination of Biological Efficiency

The total fresh weight of the mushroom harvest and the substrate dry weight for each sample were recorded. Biological efficiency was measured according to the following equation (Birhanu *et al.*, 2016).

$$\text{BE (\%)} = \frac{\text{Total fresh weight of yield (g)}}{\text{Substrate dry weight (g)}} \times 100$$

Statistical Analysis

The data from visual observations were analyzed using Microsoft Excel, while quantitative data underwent ANOVA and Tukey HSD tests in R Studio (Version 4.1.3). The FTIR spectroscopic data were analyzed and visualized using ORIGIN Pro (Version 8.5.1). All statistical tests were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Mushroom Quality Parameters

The results of preliminary analysis indicate that the biofilm exudates applications in different substrate types have significantly affected the nutrient availability in harvested mushroom and the substrate. Specifically yield parameters such as mushroom dry matter content, mushroom biological efficiency, mushroom phosphorous, mushroom nitrogen, mushroom carbon, and substrate physiochemical parameters of substrate potassium, substrate phosphorus, and substrate nitrogen were significantly higher with biofilm exudates application compared to the other treatments. The primordia were observed earlier in the BFEx-added autoclaved substrate types than BFEx not-added autoclaved and non-autoclaved substrate types.

Growth of Mushroom in Substrates

The growth of mushrooms was observed only in 6 treatments (T_1, T_3, T_4, T_6, T_7 and T_9) (Figure 2). Notably, the mushroom was not grown in substrate type 2.

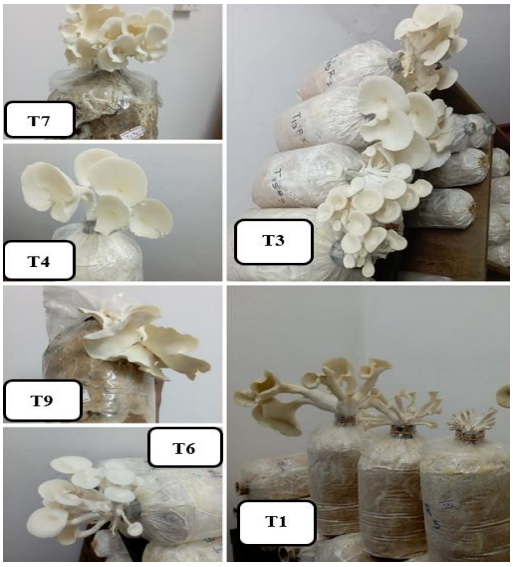

Figure 2. Mushroom growth in different treatments *(T1) Autoclaved BFEx added DOA, (T3) Autoclaved BFEx added Alternative 2, (T4) Autoclaved BFEx not added DOA, (T6) Autoclaved BFEx not added alternative type 2, (T7) BFEx added not autoclaved DOA and (T9) BFEx added not autoclaved alternative type 2*

Dry Matter Content of Mushrooms (MDM)

The MDM value was highest in the BFEx-added autoclaved treatments, T_1 and T_3. Numerically, the average values of T_4 and T_9 were higher than those of T_6 and T_7, but the differences were not statistically significant, ($p < 0.001$, Figure 3).

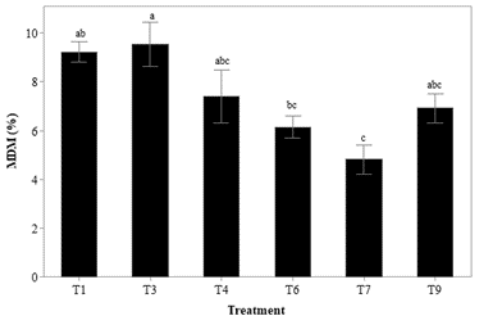

Figure 3. Dry matter contents of mushroom harvest in different treatments

Table 1. Quality parameters of mushroom harvest and physiochemical parameters of substrates under the different treatments

Treatment	Mushroom quality						Substrate	
	MC %	MH %	MN %	MP %	MBE %	MDM %	SN %	SK %
T_1 (n=30)	$40.89^{a} \pm 0.253$	$7.08^{a} \pm 0.0431$	$5.42^{ab} \pm 0.166$	$5.51^{ab} \pm 0.834$	$57.29^{a} \pm 2.19$	$9.22^{ab} \pm 0.419$	$80.60^{a} \pm 3.23$	$132.00^{a} \pm 3.92$
T_3 (n=30)	$40.42^{ab} \pm 0.158$	$6.98^{a} \pm 0.0864$	$5.15^{b} \pm 0.120$	$7.75^{ab} \pm 1.58$	$53.66^{ab} \pm 1.72$	$9.54^{a} \pm 0.907$	$62.00^{bc} \pm 4.30$	$116.80^{ab} \pm 9.90$
T_4 (n=30)	$38.85^{ab} \pm 0.599$	$6.696^{ab} \pm 0.197$	$4.69^{b} \pm 0.193$	$4.009^{b} \pm 1.49$	$48.044^{b} \pm 2.68$	$7.404^{abc} \pm 1.07$	$46.80^{c} \pm 7.12$	$84.40^{c} \pm 6.61$
T_6 (n=30)	$38.58^{b} \pm 0.0813$	$6.56^{ab} \pm 0.192$	$4.85^{b} \pm 0.145$	$9.096^{a} \pm 1.40$	$50.61^{ab} \pm 3.561$	$6.139^{bc} \pm 0.450$	$68.60^{ab} \pm 3.91$	$102.20^{bc} \pm 4.44$
T_7 (n=30)	$38.52^{b} \pm 0.840$	$6.49^{ab} \pm 0.155$	$6.206^{a} \pm 0.243$	$3.84^{b} \pm 0.453$	$17.898^{c} \pm 0.408$	$4.83^{c} \pm 0.597$	$17.00^{d} \pm 1.52$	$48.20^{d} \pm 5.30$
T_9 (n=30)	$38.95^{ab} \pm 0.582$	$6.296^{b} \pm 0.0939$	$5.30^{ab} \pm 0.337$	$7.67^{ab} \pm 0.334$	$14.33^{c} \pm 0.604$	$6.92^{abc} \pm 0.604$	$11.00^{d} \pm 1.45$	$31.40^{d} \pm 3.78$
P Value	0.012	0.005	0.001	0.013	< 0.001	0.001	< 0.001	< 0.001

Mean ± SE in each column. Within columns, values with different letters differ significantly ($p < 0.05$, Tukey's HSD test). (MC)Mushroom Carbon content, (MH) Mushroom Hydrogen content, (MN) Mushroom Nitrogen, (MP) Mushroom Phosphorous, (MBE) Mushroom Biological Efficiency, (MDM) Mushroom Dry Matter content, (SN) Substrate Nitrogen content, (SK) Substrate Potassium content

Trichoderma Infestation in Oyster Mushroom

Treatments T_2, T_5, T_8, and T_{11}, with autoclaved substrates, showed no pathogenic infections. Non-autoclaved bags, except T8 and T_{11}, were heavily contaminated by *Trichoderma* and *Aspergillus*. Despite pathogen presence, treatments T_7 and T_9 still yielded mushrooms. After the first harvest, T_4 faced a pathogen attack but produced well. *Trichoderma* identification was confirmed through microscopic observations and culture methods, highlighting the importance of sterilization in preventing infections to ensure successful mushroom cultivation (Błaszczyk *et al*., 2014).

Table 2. Detection of *Trichoderma* infestation under different treatments

Treatment	*Trichoderma* Infestation	Time (Days)
T_1	No	-
T_2	No	-
T_3	No	-
T_4	Yes	46
T_5	No	-
T_6	No	-
T_7	Yes	20
T_8	No	-
T_9	Yes	21
T_{10}	Yes	19
T_{11}	No	-
T_{12}	Yes	18

Biological Efficiency of Mushrooms (MBE)

The highest MBE value was observed in the BFEx-added DOA recommended substrate type (T_1). Numerically, the T_3 value was higher than T_4 and T_6, but the differences were not statistically significant. However, the lowest MBE values were observed in the BFEx-added, non-autoclaved treatments T_7 and T_9 ($p < 0.001$, Figure 4).

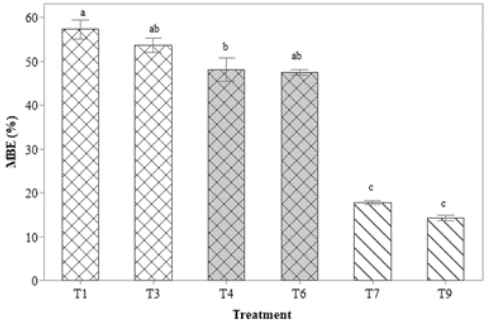

Figure 4. Biological efficiency of mushroom harvest under the different treatments

Protein Content in Mushrooms

The FTIR analysis revealed that the treatments with autoclaved substrates and biofilm exudates (T_1 and T_3) resulted in significantly higher protein content, as evidenced by the pronounced absorbance values in their FTIR spectra compared to treatments without biofilm exudates (Sadhana and Malini, 2018). Conversely, substrates that did not receive biofilm exudates showed the lowest protein content, with statistical significance ($p < 0.001$). These findings suggest that the application of biofilm exudates markedly enhances protein accumulation in mushrooms, highlighting the potential benefits of biofilm exudates in mushroom cultivation, ($p < 0.001$, Figure 5).

Table 3. Mean values of absorbance at 1650 cm^{-1}

Treatment	Mean (Absorbance)
T_1	$0.12990^{a} \pm 0.00133$
T_3	$0.08760^{b} \pm 0.00304$
T_4	$0.05480^{c} \pm 0.00313$
T_6	$0.03391^{d} \pm 0.00111$
T_7	$0.027567^{d} \pm 0.000786$
T_9	$0.00695^{e} \pm 0.00104$

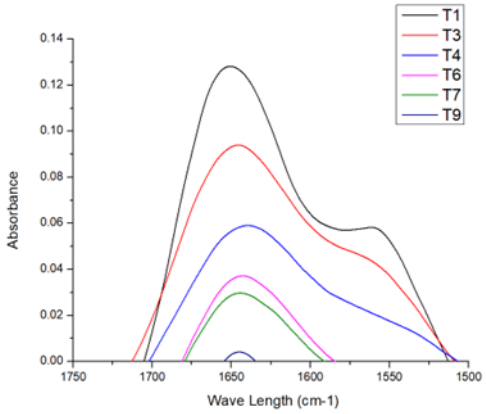

Figure 5. FTIR absorbance spectra of the protein analysis of mushroom harvest in different treatments

CONCLUSIONS

This study revealed the positive effects of Biofilm Exudates in mushroom cultivation. BFEx-added substrates yielded mushroom harvests with higher protein and dry matter contents under both autoclaved and non-autoclaved conditions, emphasizing the crucial role of BFEx in mushroom cultivation. This pioneering field experiment confirms that adding BFEx is highly effective for mushroom harvests within the limitation of the study and emphasizes the necessity for further research to verify BFEx's impact on *Trichoderma* and other pathogens. The promising results from this study pave the way for innovative approaches in sustainable mushroom farming, potentially revolutionizing pathogen management and yield optimization in the industry.

ACKNOWLEDGEMENTS

All the staff members and colleagues at the Microbial Biotechnology Unit of the National Institute of Fundamental Studies and Department of Biotechnology, Wayamba University of Sri Lanka are acknowledged for their support during this research study. The research study was conducted at and fully funded by the NIFS. For correspondence, please contact the corresponding author at mahesh.pr@nifs.ac.lk.

REFERENCES

Birhanu, G., Girmay Z., Gorems. W. and Zewdie, S. (2016). Growth and yield performance of *Pleurotus ostreatus* (Jacq. Fr.) Kumm (Oyster Mushroom) on different substrates. *Springeropen*, **32**, 6-87.

Błaszczyk, L. (2014) 'Trichoderma spp. - Application and prospects for use in organic farming and industry, *Journal of Plant Protection Research*, **54**(4), 309–317.

Boddy, L. and Hiscox, J. (2017). Fungal ecology: Principles and mechanisms of colonization and competition by saprotrophic fungi. *The Fungal Kingdom*, (**31**), 293-308.

Department of Agriculture, Sri Lanka, Oyster Mushrooms (American oyster/ Abalone/ Pink oyster) (Accessed on 20.05.2024). Available at <https://doa.gov.lk/hordi-crop-mushroom/>

Jayasinghearachchi, H. S. and Seneviratne, G. (2004). Can mushrooms fix atmospheric nitrogen? *Journal of Biosciences*, **29**(3), 293-296.

Kristensen, E. and Andersen, F. (1987) Determination of organic carbon in marine sediments: a comparison of two CHN-analyzer methods. *Journal of Experimental Marine Biology and Ecology*, **109**(1), 15-23.

Pathmashini, L., Arulnandhy, V. and Wijeratnam, R.W. (2009) Cultivation of oyster mushroom (*Pleurotus ostreatus*) on sawdust. *Ceylon Journal of Science (Biological Sciences)*, **37**(2), 177-182.

Pulliainen, T. K. and Wallin, H. C. (1994) Determination of total phosphorus in foods by colorimetric measurement of phosphorus as molybdenum blue after dry-ashing: NMKL interlaboratory study, *Journal of AOAC International*, **77**(6), 1557-1561.

Sadhana, B. and Malini, R. H. (2018) FTIR analysis for harvested oyster mushroom, *Tejas. Tcarts*, **3**(2), 49-61.