Anti-Growth Factors Associated with *Pleurotus ostreatus* in a Submerged Liquid Fermentation

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ABSTRACT

Aims: Previous studies had revealed that cultivation of *Pleurotus ostreatus* is often met with a lot of challenges ranging from environmental to biological factors which adversely affect the successful cultivation of the mushroom. Hence, a need to determine factors against mycelia colonization of substrate during mushroom's cultivation.

Methodology and Result: Conventional streak method was employed to establish the percentage inhibition as well as intercolony distance between the test organisms obtained from the infected substrate and mycelia of the mushroom during substrate colonization. The test organisms are: a fungus, *Kuttilakesopsis macalpineae* and a bacterium, *Pseudomonas tolaasii*. The effect of pH and temperature on the mycelia growth of *P. ostreatus* was also investigated. There was a gradual increase in the percentage inhibition from 33.3 % at 24 h to 75.0 % at 168 h for *K. macalpineae* and 37.5 % at 24 h to 70.0 % at 168 h for *P. tolaasii*. The inter-colony distance between the antagonists and the mushroom mycelium gradually decreased. Optical density of the mycelium growth was at its optimum at pH 4.5 and temperature of 25 °C respectively. *In vitro* study also showed a significant increase in the optical density from 0.855±0.03 at 24 h to 1.316±0.02 at 168 h in the absence of test antagonist as against 0.812±0.06 and 0.79±0.02 at 24 h to 1.103±0.03 and 0.902±0.03 at 168 h when *K. macalpineae* and *P. tolaasii* were used as test antagonistic respectively.

Conclusion, significance and impact of study: Sterilization of substrate is essential to avoid contamination during mycelia colonization. Also, slightly acidic medium and temperature control is necessary for high yield of fruit bodies.

Keywords: Antigrowth, bio-factors, optical density, submerged fermentation

INTRODUCTION

Over years, the economic production of mushroom has been considered important and the interest for the control and improvement of the culturing technology for the edible ones has gained an unequivocal and considerable attention. Chang (1999) reported *Pleurotus* spp. as one of the valuable and edible mushrooms. It occupies the third place in the world's production of edible mushrooms, after *Agaricus bisporus* and *Lentinula edodes*, however, in recent years; it is reported to be the second most important mushrooms in production in the world of which 25 % of the total world production of cultivated mushrooms are *Pleurotus*. *Pleurotus* as a class of edible mushroom has the capacity to convert nutritionally valueless substances into high protein food and are reputed to have a high saprophytic ability and to grow on a variety of cellulosic wastes (Yildiz *et al.*, 2002). *Pleurotus* sp., a wood-rot fungus, is cultivated by the application of various heat treatments on the substrates to eliminate competitive fungi like *Altemaria* sp., *Aspergillus* sp., *Fusarium* sp., *Monilia* sp., *Mucor* sp., *Rhizopus* sp. e.t.c. to mention but few. Also, the mycelial growth of this specie is fast and various lignocellulosic waste products

can as well be used as culture substrate (Yildiz *et al.*, 2002). *Pleurotus ostreatus*, is among the edible mushrooms consumed in the tropical states of west African and It is used as spice in vegetable soups and also fried to serve as meat (Iwalokun *et al.*, 2007). They have been relished as a delicacy for centuries because of their subtle flavor, nice aroma and physical taste appeal (Sabir *et al.*, 2003). Apart from being famous for its appetizing flavour, it also offers itself as a potential protein source, minerals and vitamins (Wahid *et al.*, 1988). However, *Trichoderma* sp., soil filamentous fungi, are antagonists that can cause extensive losses in mushroom production (Jandaik and Guleria, 1999). These fungi produce several enzymes involved in degradation of the fungal cell walls that may contain chitinases and glucanases (Ait-Lahssen *et al.*, 2001). Therefore the most important step for successful cultivation of *Pleurotus* spp. is the preparation of lignocellulosic material to ward off saprophytic fungi which compete with the mushroom fungus during spawn run (Velazquez-Cedeno, 2008).

In general, the bran and straw of the family of gramineae (rice bran, wheat bran, wheat straw, sorghum straw, maize straw) and cotton wastes have been in use for the

cultivation of *P. ostreatus* in Nigeria. The low availability of these materials for the cultivation of this mushroom in recent years however has led to the search for an alternative source of raw materials as substrates for the cultivation of this specie of mushroom. Attention has therefore been turned into the sawdusts of tropical trees from our saw-mills which of course have been yielding tremendous results. Also, there is a dearth of information on the fundamental processes governing the growth of *P. ostreatus* on sawdust of the tropical trees as substrates. The objective of this study has therefore focused mainly on the investigation of the anti-growth factors of the mycelium of *P. ostreatus* particularly in a submerged liquid fermentation and also the possible and practical steps to take on how it could be surmounted.

MATERIALS AND METHODS

Medium preparation

Two media were used for the experiment: extracts from *Spondias mombin* sawdust and *Pycnanthus angolensis* sawdust. One hundred gram of sawdust of each was screened and soaked with 500 mL of deionized water. The sawdust was sterilized with the water and filtered immediately after sterilization using a sterile muslin cloth. The media were prepared according to the modified method of Gbolagade (2004) by dissolving the following chemicals in a 250 mL of deionized water: glucose, 10 g; ammonium citrate, 0.75 g; KH_2PO_4 , 0.25 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75 mg; To the solution was added a mixture of vitamins containing thiamine, niacin, riboflavin, biotin, pyridoxine, and folic acid, each at the rate of 50 µg/L.

Submerged cultured of mycelium

The mycelium of *P. ostreatus* used in the investigation was obtained from the Microbiology Research Laboratory of the Federal university of Technology, Akure, Nigeria. The mycelium was resuscitated by inoculating an actively growing mycelial disc into a flask containing 100ml of extract from the sawdust. Culture was incubated in a rotary shaker (WSZ-100A Model) at 25 °C, pH 5.0 and agitation speed of 150 rpm for 72 h. This was used as stock medium.

Source of inoculums

A fungus *Kuttilakesopsis macalpineae* and a bacterium *Pseudomonas tolaasii* both isolated from infected substrate during solid state fermentation were used.

Effect of temperature on the mycelial growth of *P. ostreatus* in a submerged culture

Ten millimeter of double strength Potato Dextrose Broth was added to equal volume of buffer solution in test tubes to give 20 mL. Five mL of stock medium was inoculated after sterilization. The test tubes were incubated at

varying temperatures of 21 °C, 23 °C, 25 °C, 27 °C, 29 °C, 31 °C, 33 °C, and 35 °C. Growth in terms of turbidity was measured on daily basis for 168 h at 540nm wavelength using spectrophotometer (Spectrumlab 22pc model).

Effect of pH on the mycelial growth of *P. ostreatus* in a submerged culture

Citrate-Phosphate buffer solution of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 were prepared (Gbolagade, 2004). Ten millimeter of double strength PDB was added to equal volume of buffer solution in test tubes to give 20ml. Five millimeter of stock medium was inoculated after sterilization. The test tubes were incubated and growth in terms of turbidity was measured on daily basis for 168 h at 540 nm wavelength using spectrophotometer (Spectrumlab 22pc model).

Detection of antagonistic activity

Conventional streak method described by Fakoya *et al*, (2009) was used for this assay. With the use of a sterile cork borer of 7 mm diameter, an actively growing edge of a 4-day old mycelial culture of *P. ostreatus* was aseptically cut and placed at the center of a PDA plate. Forty millimeter streak of test organisms (*P. tolaasii* and *K. macalpineae*) was made from a 24 h and 72 h old culture of *P. tolaasii* and *K. macalpineae* respectively, 23 mm away from the center of the Petri-dish. The plates were incubated for 168 h at 25 °C for the fungus and 37 °C for the bacterium respectively. The percentage inhibition and inter-colony distance were measured on daily basis.

Inhibitory activities of test antagonists on the mycelium growth of *P. ostreatus* in a submerged culture

Ten millimeters of PDB was dispensed into a screw capped test tubes and were sterilized at 121 °C for 15 min and allowed to cool before inoculation. Five millimeters of stock medium (mycelium in broth culture) and the test organisms (antagonists) each were inoculated into the test tubes. The treatment was carried out in triplicate while the control tube was left without any inoculum. Growth in terms of turbidity was measured on daily basis for 168 h at 540 nm wavelength using spectrophotometer (Spectrumlab 22pc model).

RESULTS AND DISCUSSION

Inhibitory effect of test antagonists against the mycelial growth of *P. ostreatus*

A gradual increase in the percentage inhibition of *K. macalpineae* and *P. tolaasii* against mycelium of *P. ostreatus* in the submerged fermentation process was observed as shown in Figures 1 and 2. Also, the percentage inhibition and the inter-colony distances between the antagonists and the mycelium *in vitro* were

shown in Figures 3 and 4 respectively.

Effect of temperature on the mycelial growth of *P. ostreatus*

Figure 5 showed how temperature was varied during the fermentation processes in order to ascertain the optimum temperature that best enhanced mycelial growth of the mushroom. This was observed to be 25 °C.

Effect of pH on the mycelial growth of the mushroom

Table 1 showed the effect of varying pH during the course of the fermentation processes as the optimum pH was recorded at 4.5 for the mycelial growth of the mushroom.

Figure 1: Antagonistic ability of *K. macalpineae* on the mycelial growth of *P. ostreatus* in a submerged culture

Figure 2: Antagonistic ability of *P. tolaasii* on the mycelial growth of *P. ostreatus* in a submerged culture

Figure 3: Antagonistic activities of *K. macalpineae* on mycelial growth of *P. ostreatus*. (Photograph taken 7 days after inoculation)

Plate A - Control

Plate B - Conventional streak method

Figure 4: Antagonistic activities of *P. tolaasii* on mycelial growth of *P. ostreatus*. (Photograph taken 7 days after inoculation)

Plate A - Control

Plate B - Conventional streak method

Inhibitory effect of *K. macalpineae* and *P. tolaasii* on the mycelial growth

The pronounced effect of the presence of the test antagonists on the optical density of the mycelial growth of *P. ostreatus* was shown in Figure 6 as the growth was greatly impaired during submerged fermentation process due to the presence of the test antagonists.

The ability of *K. macalpineae* and *P. tolaasii* to inhibit mycelium growth of *Pleurotus* is a great threat to the cultivation of *P. ostreatus*. However, **Figure 1** and **2** show the gradual increase of the percentage inhibition of *K. macalpineae* against mycelium of *P. ostreatus* from 33.3 % at 24 h to 75.0 % at 168 h in the conventional streak method. Also the percentage of inhibition of *P. tolaasii* against the mycelium of *P. ostreatus* increased gradually

Figure 5: Effect of temperature on the mycelial growth of *P. ostreatus* in a submerged culture (absorbance at 540nm)

Table 1: Effect of pH on the optical density of mycelial growth of *P. ostreatus* in a submerged culture (absorbance at 540nm)

pH values	Hours of incubation						
	24	48	72	96	120	144	168
4.0	0.612±0.02 ^{d,e}	0.654±0.00 ^e	0.698±0.01 ^e	0.718±0.02 ^f	0.732±0.01 ^f	0.766±0.00 ^{e,f}	0.794±0.00 ^{e,f}
4.5	0.756±0.01 ^{e,f}	0.787±0.01 ^{e,f}	0.819±0.00 ^f	0.841±0.01 ^f	0.866±0.00 ^f	0.967±0.01 ^g	0.998±0.01 ^g
5.0	0.644±0.00 ^e	0.664±0.0 ^e	0.687±0.02 ^e	0.699±0.01 ^e	0.720±0.01 ^f	0.753±0.02 ^{e,f}	0.788±0.01 ^{e,f}
5.5	0.567±0.02 ^d	0.587±0.01 ^d	0.603±0.01 ^{d,e}	0.619±0.00 ^{d,e}	0.646±0.02 ^e	0.658±0.01 ^e	0.671±0.00 ^e
6.0	0.502±0.01 ^{c,d}	0.519±0.00 ^{c,d}	0.532±0.02 ^{c,d}	0.554±0.01 ^{c,d}	0.579±0.00 ^d	0.598±0.01 ^d	0.602±0.01 ^d
6.5	0.468±0.01 ^c	0.479±0.01 ^c	0.491±0.00 ^c	0.504±0.02 ^{c,d}	0.518±0.01 ^{c,d}	0.534±0.00 ^{c,d}	0.542±0.00 ^c
7.0	0.405±0.00 ^{b,c}	0.422±0.01 ^{b,c}	0.438±0.01 ^c	0.453±0.01 ^c	0.469±0.01 ^c	0.482±0.01 ^c	0.495±0.01 ^c
7.5	0.323±0.01 ^b	0.337±0.00 ^b	0.349±0.01 ^b	0.358±0.01 ^b	0.370±0.00 ^{b,c}	0.382±0.02 ^{b,c}	0.393±0.01 ^{b,c}
8.0	0.272±0.00 ^a	0.285±0.01 ^a	0.298±0.02 ^a	0.306±0.00 ^{a,b}	0.318±0.01 ^{a,b}	0.328±0.01 ^b	0.337±0.00 ^b

Values are means of triplicates ± SD, Samples carrying the same superscripts are not significantly different at (p>0.05)

from 37.5 % at 24 h to 70.7 % at 168 h in the conventional streak method. The gradual increase in the percentage inhibition and decrease in the inter-colony distances between the antagonists and the mycelium *in vitro* (Figures 3 and 4) is a clear indication of their ability to implicate good yield production of the mushroom during solid state fermentation. The inter-colony distance between the mycelium and the antagonist streak and the degree of growth rate inhibition as observed in this study supported the view that antagonism results from the active production of a diffusible antimicrobial compound by the microorganisms. These results are in agreement with other studies where the inhibitory effects of some bacteria having antifungal properties were the main factor affecting the potential of *Pleurotus* spp. in ecosystem colonization (De Boer *et al.*, 1998; Savoie *et al.*, 2001). Also some bacterial strains are capable of inhibiting through the production of volatile organic compounds (Mackie and

Wheatley 1999) or by releasing antibiotics (Nielsen *et al.*, 2000). These compounds can provoke cell perturbations at the membrane structure level and *Pseudomonas* spp. have been identified as one of such antagonists (Ellis *et al.*, 2000). However, colonization of the mycelium of *P. ostreatus* was strongly established in this work to be affected adversely by the presence of some bacteria and fungi in its cultivation substrate when cultivated on pasteurized or unsterilized substrate as compared to that sterilized. Therefore, an uninterrupted colonization of mycelium could be achieved by proper sterilization of the substrate(s) or media used for the mycelium propagation of the mushroom. As a matter of fact, an ideal sterility should also be maintained during spawn inoculation as well as the during mycelium colonization of the substrate.

The effect of temperature was greatly pronounced on the growth of mycelium of *P. ostreatus* as the optimal growth

Figure 6: Inhibitory effects of *K. macalpineae* and *P. tolaasii* on the mycelial growth of *P. ostreatus* in a submerged culture.

was observed at 25 °C (**Figure 5**). This suggests that substrate colonization is at its best at this temperature. **Table 1** also show the effect of pH on the mycelium growth as the optimal proliferation of the mycelium growth of *P. ostreatus* was observed at 4.5 and this suggests that colonization of mycelium of mushroom performs best at aslightly acidic medium. This confirms the findings of Bello and Akinyele (2007) that increase in fungal count throughout the period of fermentation may be due to the optimum pH obtained because fungi are only tolerant to acid.

The inhibitory effect of *K. macalpineae* and *P. tolaasii* on the mycelium growth was shown in **Figure 6**. It was observed that the test antagonists had significant effects on the growth and colonization of the mycelium *in vitro* in a submerged culture as the optical density increased appreciably in the absence of the test antagonists. This however suggests that the organisms could presents itself as an antigrowth bio-factor even in a solid state fermentation.

Furthermore, this study has shown the role of the microflora of the culture substrate in mushroom cultivation, therefore it is necessary to improve the technology in substrates treatment in order to eliminate possible antagonist that could inhibit the growth and the development of edible mushroom, thus producing better

yields. In further studies, the fungus and the bacterium involved in the antagonistic phenomenon in this research work could be screened and characterized for their biocontrol activities particularly against poisonous and pathogenic micro and macro-fungi as well as pathogenic and harmful bacteria.

CONCLUSION

Some of the observed important bio-factors during the course of this study that can adversely affect the growth of mycelium and invariably implicate successful cultivation of *P. ostreatus* are the temperature, pH and improper sterilization of the substrates used in the cultivation. Therefore, if adequate and proper care is taken during the course of *P. ostreatus* cultivation, all these biological banes could be averted.

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