SANSIN'S INDUSTRIAL MYCOLOGICAL EXPERIMENTS WITH HYDROGEN PEROXIDE

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SUMMARY

Hydrogen peroxide is an unstable compound (H_2O_2) used especially as an oxidizing agent, antiseptic and propellant. When this compound is added to agar medium, it does not allow small unicellular air-borne contaminants to grow. If a fragment of a fully established multi-cellular organism is placed on the peroxide-containing medium, the mycelium releases peroxide decomposing agents (enzymes) into surrounding area. The enzymes break down the peroxide into water and oxygen, generating a small area of a peroxide-free medium and oxygen-added environment. This will encourage the fungus to spread its hyphae to this area as well as to produce even more enzymes. The process will go on, under slightly increased speed, until a fine, healthy mat of the fungal culture is developed over the whole medium. If a fragment of rotten wood is placed on peroxide protected medium, multi-cellular organisms will be first to grow out.

When hydrogen peroxide is added to heat processed substrate it protects the substrate from the growth of undesirable air-borne contaminants for as long period as it remains in the substrate at sufficient concentration. When peroxide treated substrate is placed in perforated plastic bags and inoculated with a multi-cellular organism (for example a true decay species) the fungus will rapidly grow throughout the substrate, developing a powerful mycellial network. If wood samples (treated or not) are buried into peroxide-protected and inoculated substrate, they will soon be exposed to the intensive bio-degradation process. Such milieu could be used as a mean for testing resistance of wood based materials to decay as well as for determination of the toxic values of fungicides or fungicidal mixtures.

Beer-based culture medium has been used in these tests as an alternative to commercially available media. Heat processed wheat straw was used as a substrate for tests carried out in perforated plastic bags.

More research work is recommended in order to corroborate and supplement the above mentioned hydrogen peroxide potentials.

Key words: Hydrogen peroxide, Antiseptic, Beer-Based culture medium, Liquid culture-medium, Unicellular organisms, Multi-cellular organisms, Heat-processed substrate, Peroxide protected substrate, Perforated plastic bags, Edible mushrooms, Spawn, Decay Tests, Fruit bodies formation.

1. BACKGROUND

Hydrogen peroxide is an unstable compound (H_2O_2) used especially as an oxidizing agent, antiseptic and propellant. The role of peroxide and peroxide degrading enzymes in the bio-degradation processes has been discussed by a number of authors (Tien and Kirk 1983, Rayner and Boddy 1988, Eriksson et al. 1990, Momohara 1998), but not much was published on the use of peroxide as an antiseptic agent in the Wood Protection related experiments.

In the year of 1993 a wicker furniture factory in Yugoslavia asked for a value added solution for its dumped willow bark that was decomposing and releasing toxic gases. We recommended trials on growing edible mushrooms, but pre-tests showed that a temporary antiseptic compound was needed to protect the heat-processed wasted bark from immediate contamination for at least a few hours (Vidovic, 1993). Excellent results were obtained by adding hydrogen peroxide to the substrate (wasted bark) right after the willow shoots were heat-treated and debarked ***(Figure 1)***. Since that time this compound has frequently been used as an antiseptic, especially in the SANSIN'S industrial experiments (Vidovic 1997, 1998,1999, Vidovic 2000). Wayne (1996, 1998, and 1999) used hydrogen peroxide in his tests done for the small-scale mushroom growers. Some potentials for the use of Hydrogen peroxide as an antiseptic in the Wood Protection-related trials are discussed in the text to follow, more to be shown at a time of slide projection.

2. WORK METHOD AND TEST RESULTS

2.1 Hydrogen Peroxide and the Beer-Based Culture Medium

Most of the experiments that are discussed in this chapter have been done using the Beer-based medium whose production is shortly described bellow.

2.1.1. Beer-Based (BB) Agar Culture Medium

Since our commercially acquired medium ($ 150 per a 250g container) absorbed moisture and turned into an unbreakable hard-pack, we searched for a more suitable solution. This led to the development of a medium made from an easy-available and always fresh substance. One hundred milliliters of beer (we use MOLSON CANADIAN LIGHT) is poured into a graduated cylinder to which 300 ml of tap water is added, followed by addition of 1.5 to 3.0 percent of agar (solidifying agent). Upon checking on the pH value, the content is autoclaved (15 minutes at 15 PSI), poured into culture dishes and allowed to cool down. It is then ready for use.

We add more agar when working with decay fungi, especially when evaluating infected material that is often sent to the Sansin R. & D. Center for analysis. Rotten wood often harbors numerous contaminants including yeast and bacteria. Increased agar content in the medium will produce less condensation on the inside of the dish lids and there will be

no free water to run over the medium. This will reduce the chance for contaminants to rapidly spread over the medium.

The Beer-based medium has been evaluated for fungal growth and cultural appearance in comparison with a commercial medium. The results indicated that in no one occasion the Beer-based agar medium was inferior ***(Figure 2)***.

2.1. 2. Beer-Based Liquid Medium (BBL)

Storage of fungal strains in distilled water is a known method that has been widely practiced for the last decade or two. It is believed that the strain will last indefinitely as mycelium goes dormant (Wayne 1999). Similarly to the results of Wayne, our experience with water stored strains was not absolutely positive. For some reason, most of water stored strains showed vitality for 12 to 18 months.

Therefore, we carried out the trials with our Beer-based medium; to which no agar was added (liquid medium). Cultures of *Pleurotus ostreatus* (Jacquin ex Fries) Kummer and a *Morshella sp.* were stored at ambient temperature (15 to 25 °C) in 100 ML Liquid Beer-based medium. Re-inoculation trials have been done periodically, by cultural transfer of stored mycelium to the Beer-based agar medium and by monitoring the cultural growth. Within the two-year storage period, both species always showed high vitality, producing fine, healthy fungal mats.

2. 2. Hydrogen Peroxide Treated Beer-Based Agar Medium

2.2.1. Inoculation with Unicellular Organisms

Two drops of commercially acquired 35% hydrogen peroxide solution were added to 100 ML of autoclaved Beer-based agar medium at the temperature of about 45 °C and the medium was dispensed into culture plates. The two drops weighed 0.094 g making the final peroxide concentration of 0.033 %. The plates were then used for testing the ability of some unicellular test organisms to germinate and produce the new strains. The following organisms were used in the test:

- *Bacteria*: two different unidentified species, each used as a separate test organisms.
- *Trichoderma sp.,* the SANSIN strain, isolated from infected wood.
- *Pleurotus ostreatus* (Jackuin:Fries) Kummer, The Oyster mushroom, spores were taken from a fruit body in a mushroom farm.
- *Lentinula edodes* (Berkeley) Pegler, Shi-take mushroom, spores were taken from a fruit body in a mushroom farm.
- *Trametes versicolor* (Lloyd ex. Fries) Lloyd, The Turkey Tale, strain CV-97. This strain was isolated from rotten architectural wood taken from a Toronto monument in 1997. Its pure culture was inoculated into hydrogen peroxide containing substrate and was later induced to form a fruit body that helped the fungal identification ***(Figure 8)***. The spores of this fungus were used in the test.

-*Polyporus squamosus* (Hudson:Fries), The Dryad's Saddle, spores were taken in September 2000 from a fruit body growing on a Maple tree stump in Strathroy.

The plates (containing 0.033% hydrogen peroxide) were separately inoculated with spore suspension (prepared in distilled water) of the above organisms and left on a quiet dark place at room temperature. When used as spore suspension the organisms can be considered as separated unicellular units. Growth of bacteria over the medium and spore germination was observed over the next three weeks. The same organisms were also placed on peroxide free medium for comparison.

Both bacteria species were successfully growing over the peroxide free medium, but no growth was registered on peroxide protected medium ***(Figure 3)***.

Sixty to ninety five percent of the fungal spores were germinating on the peroxide-free medium (light microscopy observation technique), but no one spore unit was germinating on the peroxide protected medium. This experiment proved that unicellular fungal and bacterial organisms are not capable of establishing their cultures when placed in contact with peroxide-containing medium.

Wayne (2000) states that peroxide in culture medium may not always be absolutely effective, especially if a "concentrated dot of millions of spores is added to the surface of the peroxide medium". In our trials spores of *Pleurotus ostreatus* (mushroom grower's strain) and *Trametes versicolor* were able to germinate when they were transferred in groups to the medium, without being prepared in water diluted spore suspensions.

2.2.2 Inoculation with Multi-cellular Organisms

Fragments of agar medium holding live mycelium of a multi-cellular organism (white or brown rot species, blue stain fungi or mold fungi) were inoculated on the peroxide containing Beer-based medium and observed for fungal growth.

All the test fungi (*Trametes versicolor*, *Lentinula edodes*, *Pleurotus ostreatus*, *Gloeophylum trabeum*, *Polyporus Squamosus*, *Trichoderma sp.*, *Aspergilus niger*, *Aureobasidium pullulans*, *Stachybotrys sp.*, *Fusaruim sp.*, and a number of unidentified fungal organisms) were capable of expanding their hyphae to the medium and developing the new healthy colonies. In comparison to peroxide free medium the speed of the hyphal development initially appeared slower, but later became normal and even accelerated, so that no difference in time was noticed at the end of cultural mat formation. It was also noticed that fungal mycelium does not show growth reduction if first grown on peroxide treated medium and transferred to the same – peroxide treated medium.

The experiments described above suggest that on peroxide containing agar medium air-borne contaminants will not grow but established multi-cellular organism will grow and re-establish its colony.

2. 3. Wood Decay Tests in Peroxide Protected Substrate

As mentioned earlier, when heat processed wasted bark was treated with hydrogen peroxide, the substrate was protected from air-borne contaminants for the time needed to perform inoculation with concentrated mushroom mycelium (spawn). Spawned substrate was then packed in 20-kg plastic bags (Vidovic 1993). Due to the fungal enzymatic activity in the bags, hydrogen peroxide was successively decomposed, creating the environment enriched with water and oxygen. Oxygen-added environment stimulated fungal colonization, creating intensive substrate disintegration. However, this created high temperatures (close to 30°C) causing fungal death in the central portions of the bags. To obtain better aeration and heat release we used perforated bags. Around 400 small needle-like perforations were machine-made around the whole bag circumference. No contamination through the holes was noticed through the time of the test duration because the substrate was protected by peroxide. With perforated bags even denser mycelial concentration was registered in the substrate, and the mushroom yields approached 30% in comparison to the wet weight of the substrate.

Having in mind such a strong decomposing activity in the bags, at SANSIN we decided to set up a small wood decay test under similar conditions. For this purpose 25x12x7mm Alder wood blocks were buried into spawn-inoculated substrate. The fungal spawn was prepared according to the originally developed procedure: wheat grain was microwave-heated, peroxide protected and inoculated with the CV-97 - "The Turkey Tale" test fungus (*Trametes versicolor*). More information regarding the fungal spawn production were published by Stamets (1983 and 1993) and Wayne (1996, 1998, and 1999). An alternative spawn substrate for brown rot strains is shown in ***(Figure 4)***.

As Alder blocks were prepared from kiln dried wood it was assumed that they were free from interior contaminants and any peroxide decomposing agents. The blocks were conditioned for one week in dried atmosphere (closed container at about 65% RH) and weighed. The surface contaminants were killed by simple immersion of the blocks into 0.5% peroxide for 1 to 2 seconds. There was no need to adjust the moisture content in the wood as the specimen absorb enough of moisture from the substrate as soon as the test begins. The blocks were placed in the milk-size needle-perforated bags together with peroxide protected and inoculated wheat straw based substrate ***(Figure 5)***. A parallel Agar Block Decay test in 150mm diameter dishes (similar to the EN 113 - 1988), was carried out with the same size wood blocks for comparison.

Five weeks later the blocks were taken out for evaluation. They were overgrown with a thick layer of white mycelium, appeared rotten and had an average weight loss of 23%. The blocks that were simultaneously tested according to the EN-113 method were similarly overgrown, but the weight loss averaged 15.5%.

Using the same perforated-bag method the test was repeated on a bigger scale using 50 cm high x 25 cm (diameter), commercially acquired pre-perforated bags (Vidovic 1999). For this purpose test specimen were cut from 20-mm thick agri-fiber composite board

material (42x8x20mm) and buried into substrate inoculated with the test fungus *Pleurotus ostreatus*. Twelve weeks later the weight loss averaged 41 %, while the moisture content increased from 14 to 96% ***(Figure 6).***

The above described method was easy to set up and to maintain as there was no need to care for moisture content, air exchange or contamination. Apart from this, extraordinary convenient conditions encouraged the test fungus for increased activity in decomposing the test blocks

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Peroxide treated wheat straw has also been evaluated as a substrate for mycological tests with brown rot fungi ***(Figure 7)***.

2.4. Isolation of Decay Fungi

As mentioned earlier, rotten wood is often inhabited by many organisms, which make it difficult to isolate those - most responsible for decay development. If unicellular organisms, such for example bacteria, begin growing over the culture medium first, the other fungi have little chance to develop. A few tests done at SANSIN showed that on peroxide treated agar medium, multi-cellular organisms were growing out first, while bacterial growth was delayed. This suggests that peroxide in agar medium can facilitate isolation of decay fungi from rotten wood.

2. 5. Fruit Bodies Formation

Occasional fruit body formation inside or outside the bags confirmed intensive degradation processes. Some fruit bodies, for example the CV-97 strain (*Trametes versicolor*) developed fruit bodies only upon the bags were removed ***(Figure 8)***. This gives an idea of setting up the trial on inducing fungal fructification for the purpose of visual identification.

3. CONCLUSIONS

When hydrogen peroxide is added to agar medium, it protects the medium from small preferably unicellular air borne contaminants for as long time as it remains in the medium at sufficient concentration. If a fragment of healthy, fully established multi-cellular fungal organism is placed on the peroxide containing medium, the mycelium will commence releasing peroxide decomposing agents (enzymes) into surrounding area. The enzymes will break down the peroxide into water and oxygen, generating a small area of a peroxide free medium. This will encourage the fungus to expand its mycelium to this area as well as to produce even more enzymes. The process will go on, under slightly increased speed, until a fine, healthy mat of the fungal culture is developed over the whole medium. At that time there will probably be no more peroxide left in the medium.

If hydrogen peroxide is added to heat processed substrate it will protect the substrate from the growth of undesirable air-borne contaminants for as long period as it remains in the substrate at sufficient concentration. When peroxide treated substrate is placed in perforated plastic bags and inoculated with a multi-cellular organism (for example a true decay species) the fungus will rapidly grow throughout the substrate, developing a powerful hyphal network. If wood samples (treated or not) are buried into peroxide protected and inoculated substrate, they will also be exposed to the intensive bio-degradation process. Such milieu could be used as a mean for testing the resistance of wood based materials to decay as well as for determination of the toxic values of wood treating substances.

The potentials for the use of hydrogen peroxide in the wood protection related trials could be as follows:
- Better maintenance of dispensed culture media.
- Help in purifying fungal cultures.
- Facilitated isolation of multi-cellular organisms from decayed wood by delaying the growth of the unicellular organisms.
- Easy way for setting up the tests on natural durability of wood or wood-composites.
- Easy way for setting up the tests on toxic values determination for fungicides and fungicidal mixtures.
- Support in inducing fungal fructification for the purpose of visual identification.
- Better composting away of wood wastes.

This paper expresses the personal experience of the author, gained from practical industrial tests carried out with a limited number of replicates. Perhaps it could be used as a basis for more intensive research work, so to corroborate and supplement the above-discussed results.

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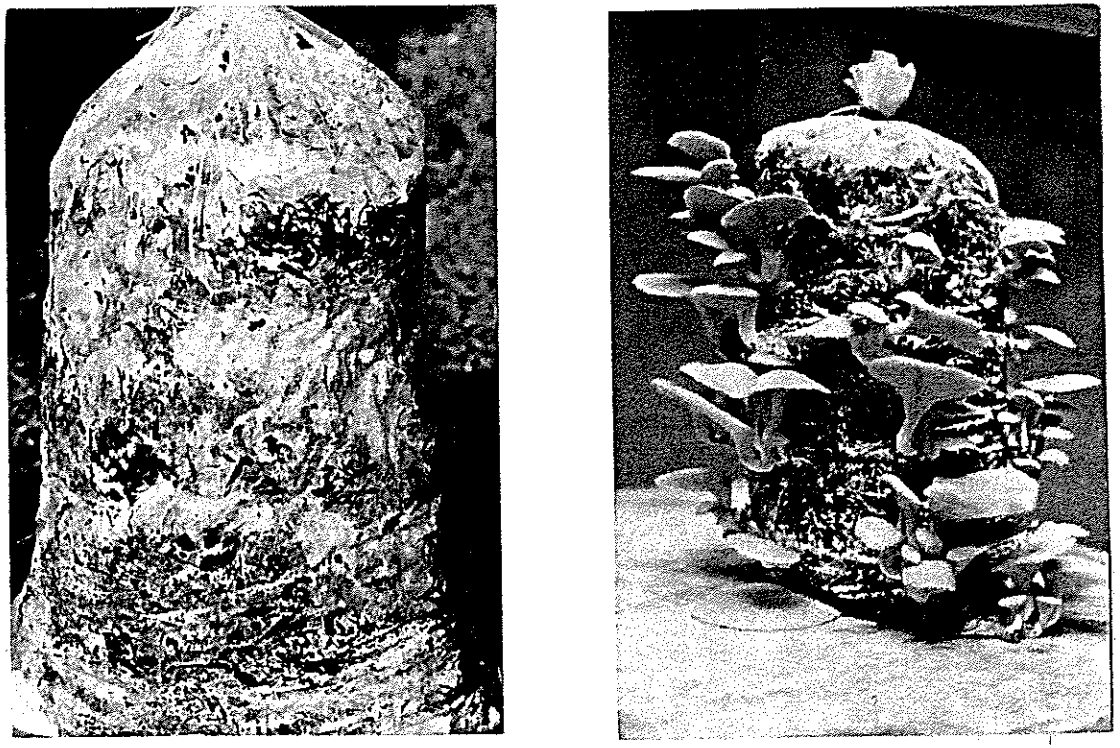

Figure 1: Peroxide protected willow bark fully colonized by the strain of *Pleurotus Ostreatus* (left). Oxygen added environment in the plastic bag stimulated intensive fungal activity, which turned the residue into beneficial (edible) product. Photo by courtesy of the *INSTITUTE FOR TESTING OF MATERIALS (IMS)*, Belgrade, Yugoslavia (Vidovic 1993).

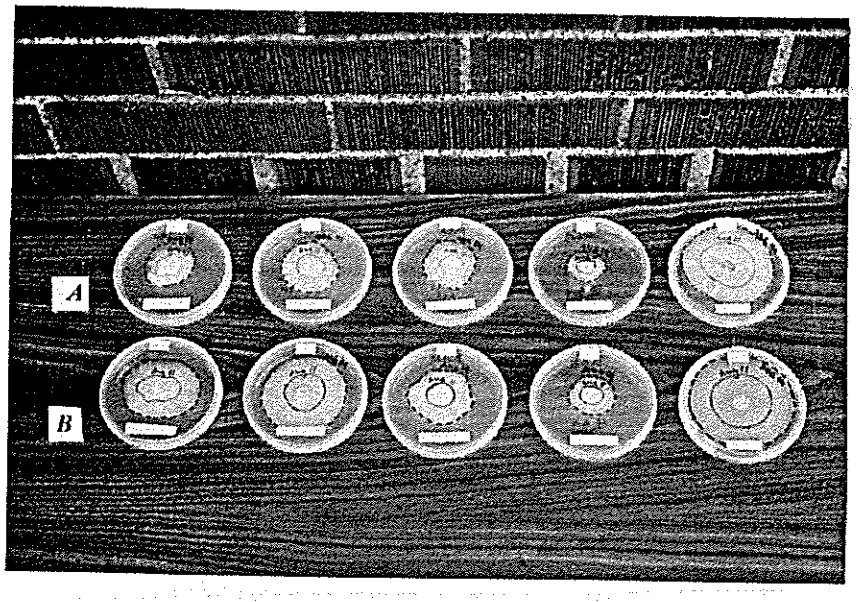

Figure 2: Growth of fungal mats over a commercial agar medium (line A) and the Beer Based agar medium (line B). The species employed in the test are: *Pleurotus Ostreatus* (Jacquin ex Fries) Kummer; *Pleurotus Citrinopileatus*, Singer; *Hypsizygus ulmarius* (Bulliard : Fries) Redhead; *Lentinula edodes* (Berkley) Pegler and *Morchella sp.* Photo: *THE SANSIN CORPORATION.*

Figure 3: As long as peroxide remains in the medium at sufficient concentration, airborne contaminants have no chance to grow. *On left:* beer-based agar medium inoculated with bacteria previously isolated from rotten wood. *On right:* peroxide protected beer-based agar medium inoculated with the same strain of bacteria. Photo: *THE SANSIN CORPORATION.*

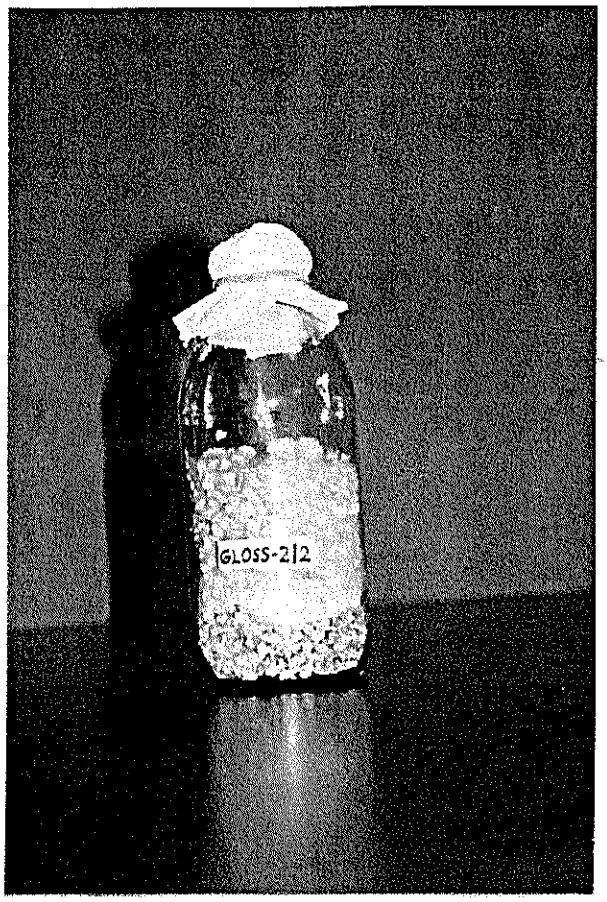

Figure 4: SPAWN PRODUCTION: Brown rot test fungus (47D), *Gloeophyllum trabeum* (Pers. ex Fries) Murrill, colonizing the microwave heated peroxide protected ground corncobs. Fungal mycelium will aggregate (concentrate) in the corn kernels being ready to blowout as soon as the spawn is transferred to the substrate (inoculation phase). Photo: *THE SANSIN CORPORATION.*

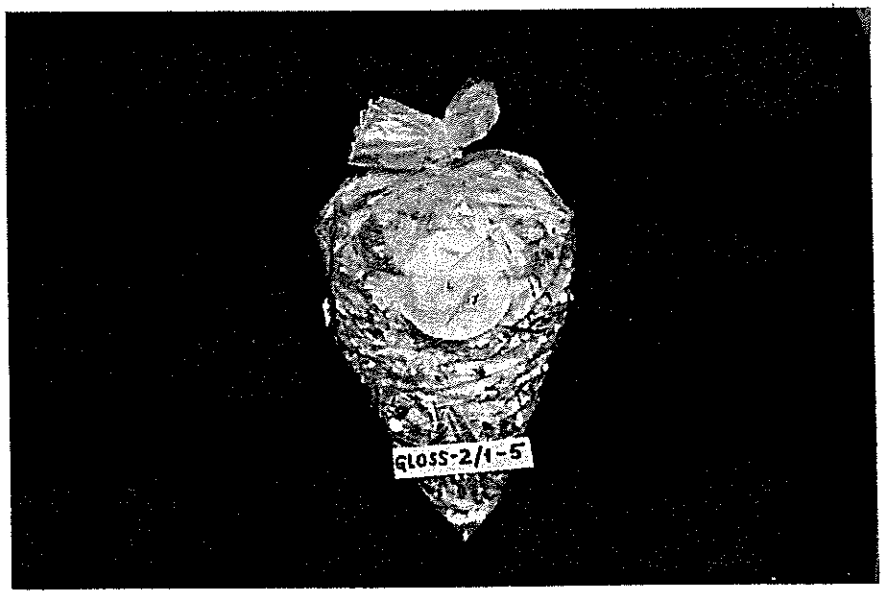

Figure 5: The CV-97 test fungus *Trametes versicolor*, colonizing the wood block 3 days after insertion. For better viewing this block was not buried in the center, but was placed right behind the bag wall. The block was placed in perforated plastic tray to test fungal ability to crawl through the perforations and attack the wood block. Perforated plastic trays (or wraps) are used to bury the blocks treated with leachable wood preservatives. Multiple perforations on the bag enable metabolic products (including carbon dioxide) to leave the bag. The substrate and the wood specimen are exposed to an extraordinary strong decomposing process. "Hungry" mycelium is searching for more food by extending its extremities through the bag perforations. Photo: *THE SANSIN CORPORATION*.

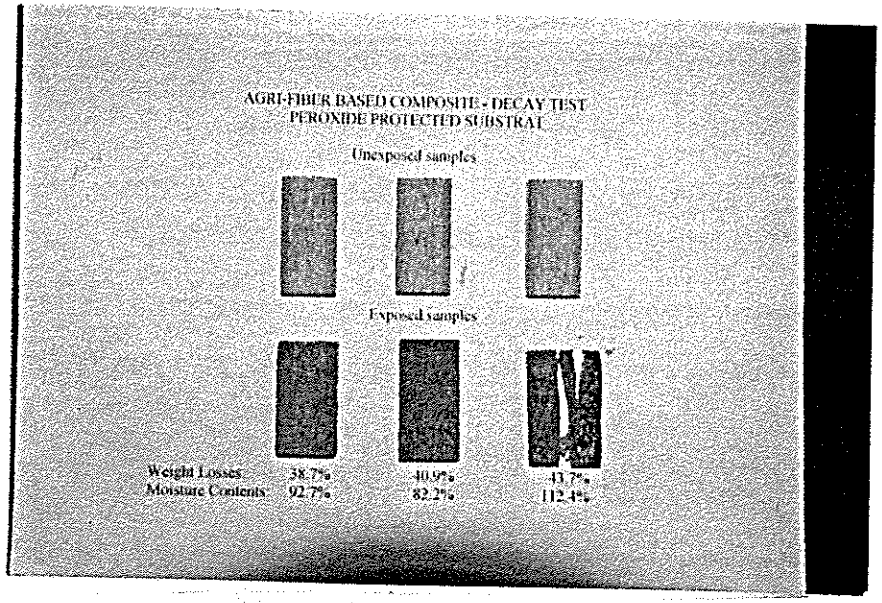

Figure 6: Agri-fiber based composite test blocks after twelve weeks exposure to the white rot fungus (*Pleurotus ostreatus*). Photo: *THE SANSIN CORPORATION.*

Figure 7: The brown rot fungus *Gloeophyllum trabeum* in search for more food: powerful mycelial mass that was grown on peroxide protected wheat straw proliferates through bag perforations. This part of the test was implemented using the U of T Forestry Faculty facilities. Photo: by courtesy of the *U of T, FORESTRY FACULTY*, Toronto.

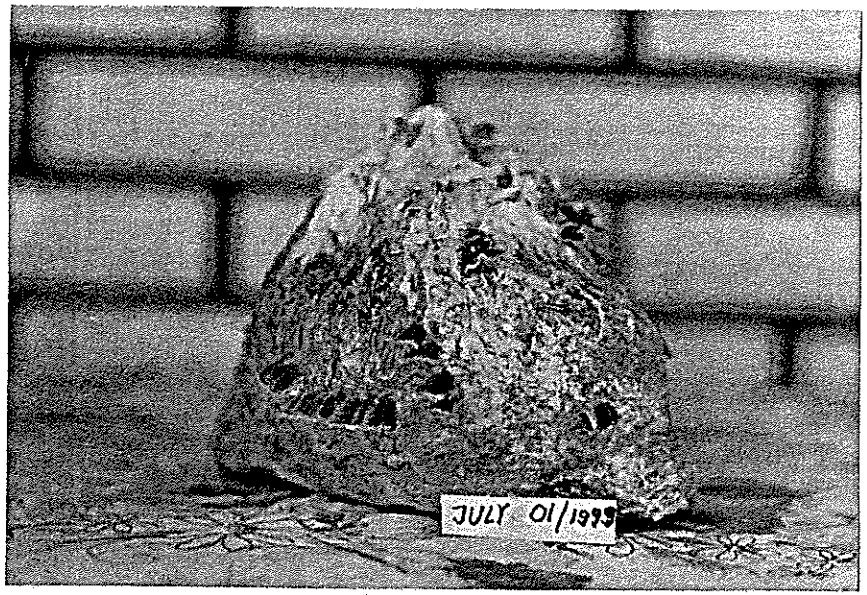

Figure 8: Fructifications of the "Turkey Tale " (CV-97) test fungus *(Trametes Versicolor)*, grown on nutritionally supplemented peroxide-protected wood fuel pallets prepared as a substrate. The method used was similar to that as described by Wayne (1998,1999), except that perforated plastic bags were used as containers, and the spawn was made using original microwave heating method. Photo: *THE SANSIN CORPORATION.*