Soaking of substrate in alkaline water as a pretreatment for the cultivation of *Pleurotus ostreatus*

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SUMMARY
Substrate for cultivating *Pleurotus ostreatus* was prepared by soaking it in water containing 0.5% lime with no further thermal treatment. Five substrates were used: grass (*Digitaria decumbens*), corn cob, corn straw and two mixtures of corn straw or grass with coffee pulp. Immersion time varied between 0-48 h. Mushroom yield measured as biological efficiency varied between 37.3 and 126%. No fungal contamination was found, however certain group of bacteria like pseudomonads, bacilli and coliforms were detected. This technique seems to have potential; however good control of the moisture of the substrate must be maintained to limit proliferation of substrate bacteria.

Avoidance of substrate contamination is an important part of the cultivation of edible mushrooms. Competitor bacteria and undesirable fungi in the substrate drain nutrients as well as limiting mushroom growth and fruiting, thus reducing yield and quality.

Various treatments are used for the preparation of the substrate used to cultivate the oyster mushroom *Pleurotus* spp.- steam pasteurization, submersion in hot water, composting, chemical sterilization, and lactic fermentation. (Ferri, 1985; Kurtzman, 1979; Leiley and Janssen, 1993; Muez and Pardo, 2002; Soto Velasco et al., 1989; Vijai and Sohi, 1987; Zadrzal and Schneider, 1972). Each of these methods has advantages and disadvantages depending on the prevailing technological situation. (Laborde, 1989; Pani and Mohanty, 1998; Rajararhnam and Bano, 1987; Royse and Schisler, 1987).

In the state of Chiapas, Mexico, growers of *Pleurotus* commonly prepare the substrate by simply soaking lignocellulosic residues in alkaline water for various times. This is a low output, empirical technique used by very small scale growers. A farm at Jomanichim, in the municipality of Tenejapa, which has a capacity of two tonnes of mushrooms per month and grows *P. ostreatus* on corn straw, represents the largest and best known of these small operations. This farm has operated fairly continuously for two years, apparently without contamination problems and with biological efficiencies (BE) averaging 40-50% (Sanchez, 2001). The above-mentioned soaking technique has replaced submersion in hot water over the last few years in Chiapas because of the contamination problems observed (Guzman et al., 1993; Lopez et al., 1996).

Philippoussis et al. (2001) reported that soaking the substrate in water with 2% calcium carbonate to a pH of 6-7 followed by sterilization was an efficient method for preparation for cultivating *Pleurotus* spp. This method had the additional advantage of a shorter colonization time than with composting.

The use of a more alkaline base than calcium carbonate to avoid the need to sterilize or heat pasteurize was suggested by Hernandez et al. (2003). These authors adjusted the initial pH values of the substrate with lime to 8.4-8.5 and found that even without composting it was possible to achieve values of Biological Efficiency (BE) around 80% in two harvests.

Few reports are available on the cost of pasteurizing a kilogram of substrate for *Pleurotus* cultivation. However, some estimates can be drawn from the work of Verma (2002) on total production cost. Although there are no exact figures for the difference in the cost of pasteurization by heat v pasteurization by soaking, it is clear that the cost of soaking is much lower because it requires no energy outlay. This fact has significance in rural communities where simple, flexible technology is required, and where there is a need to identify substrates which may contribute to energy savings and increased production efficiency.

The object of this study was to evaluate the technical viability of alkaline water soaking as a substrate preparation technique for *P. ostreatus* cultivation. The BEs obtained using this method with five alternative substrates (or mixtures) was also determined.

MATERIALS AND METHODS

**Strain and spawn**

*Pleurotus ostreatus* strain ECS-0152 from the ECOSUR mycological collection was used for this study. The substrate was inoculated at a rate of 5% with spawn prepared on sorghum seeds (Quimio, 2002).

**Substrate preparation**: About 10 kg of raw materials were broken into 3-4 cm pieces using a mill hammer (Sanke and Hunkel, Model A-1052) and then soaked in a 75 l plastic container containing 20 l tap water (temperature of 25 ± 2°C) with 0.5% added lime (commercial lime from Calhidra Balun, Canan Inc, Mexico with 81.5-82% calcium hydroxide content, 0.75-0.9% calcium oxide, and 45.2-44.7 calcium content). Soaking time varied from 0 to 48 h depending on the experiment. (Soaking for 5 min was considered “0” time.) After the soaking period, the...
substrate was placed in plastic trays to permit drainage and the moisture content was adjusted to approximately 70%. Six substrates were used, as shown in Table I. A control was prepared as per Hernández et al. (2003). After soaking and draining, 950 g of substrate, mixed with 50 g spawn, were placed in 2 kg capacity polyethylene bags.

Spawn run and fruiting.

Spawn run was completed in darkness for 15 d at 26°C. Fruiting was induced at 23°C, 70% moisture, with continuous ventilation to maintain low CO₂ (less than 800 ppm) in the air. Fruiting bodies were collected and weighed for three flushes.

Parameters evaluated

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Grass (%)</th>
<th>Corn straw (%)</th>
<th>Corn cob (%)</th>
<th>Coffee pulp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>6*</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>30</td>
</tr>
</tbody>
</table>

* - Control (Grass 70%-Coffee Pulp 30%, composted)

Bacterial groups studied were: total coliforms, *Pseudomonas* spp and *Bacillus* spp. The specific media ingredients used for each culture were:

**Total coliforms:** Brilliant green bile broth (Bioxon, Beckton Dickenson de Mexico, Inc.) The final pH of the medium was adjusted to 6.9-7.1, and 2% agar added. *Pseudomonas group:* A combination of Media #1, #54 and #81 (DSM, 1993). Per litre, 5 g peptone of casein (Difco), 5 g dextrose (Sigma), 0.1 g yeast extract (Difco), 0.3 g NiCl₂ (J.T. Baker), 0.3 g K₂PO₄ (Reproquifin), 0.3 g CaCl₂ x 2 H₂O (Omnichem), 0.2 g (J.T. Baker), 0.1 g NaCl (Merck), and 0.1 g MgCl₂ x 6H₂O (Omnichem). The medium was supplemented with a trace elements solution based on the SL-10 trace element solution (DSM, 1993) as follows: 10 ml HCl; 1.5 g Fe Cl₂ x 4H₂O; 70 mg ZnCl₂; 80 mg MnCl₂ x 2 H₂O; 6 mg H₃BO₃; 190 mg CoCl₂ x 6 H₂O; 2 mg CuCl₂ x 2 H₂O; 24 mg NiCl₂ x H₂O; 36 mg Na₂MoO₄ x 2 H₂O; 3 mg Na₂SeO₃ x 5H₂O; 4 mg Na₂W₄O₁₇ x H₂O. All salts were dissolved in 100 mL of filter sterilized distilled water. One mL of the stock trace element solution was added to each litre of medium.

**Bacillus group:** Medium #1 (DSM, 1993) supplemented with the same salt composition as for the *Pseudomonas* group. Samples were pasteurized for 10 minutes by heating in a water bath (Madigan 1999) for quantification.

Coliforms were incubated at 37°C and *Pseudomonas* and *Bacillus* spp were incubated at 30°C for 24 h. Bacterial populations were determined by the spread plate method (Batton et al., 1995).

Biological efficiency (BE)

After harvesting, BE was calculated using the following formula:

\[ BE = \frac{\text{Biomass (fresh weight)}}{\text{Substrate weight (dry basis)}} \times 100 \]

SEM visualization of bacterial contaminants

For Scanning Electron Microscopy (SEM) a radial section of 3 mm wide was cut covering all different areas from the centre to the edge of the pileus. Sections were rinsed for 5 min with 0.1 M phosphate buffer, pH 7.2, (PBS) and placed in 3% glutaraldehyde in PBS, overnight (Dykstra, 1993). The sections were removed 24 h later and rinsed twice in PBS (5 min each) then dehydrated with serial ethanol solutions (30, 50, 70, 95, 100, 100%) for 45 minutes. Samples were critical-point dried with CO₂ and mounted over an aluminum stub with silver paint. Finally samples were gold-palladium covered using a ion-sputter Denton Vacuum Desk-II and were observed under high vacuum conditions at 5 kV.

### Table I

Percent substrate (dry weight) used for each of six P. ostreatus crops

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Grass</th>
<th>Corn straw</th>
<th>Corn cob</th>
<th>Coffee pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>6*</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>30</td>
</tr>
</tbody>
</table>

* - Control (Grass 70%-Coffee Pulp 30%, composted)

### Table II

<table>
<thead>
<tr>
<th>Soaking time (h)</th>
<th>Grass (S1)</th>
<th>Corn straw (S2)</th>
<th>Corn cob (S3)</th>
<th>Grass-Coffee pulp (S4)</th>
<th>Corn cob-Coffee pulp (S5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>56.0 ± 1.7</td>
<td>54.9 ± 1.2</td>
<td>48.0 ± 0.6</td>
<td>51.0 ± 1.0</td>
<td>48.0 ± 0.5</td>
</tr>
<tr>
<td>12</td>
<td>71.5 ± 1.4</td>
<td>74.0 ± 0.5</td>
<td>71.6 ± 0.6</td>
<td>69.2 ± 1.7</td>
<td>73.0 ± 0.0</td>
</tr>
<tr>
<td>24</td>
<td>71.6 ± 0.5</td>
<td>76.0 ± 0.0</td>
<td>72.5 ± 1.0</td>
<td>70.5 ± 0.5</td>
<td>71.6 ± 0.5</td>
</tr>
<tr>
<td>36</td>
<td>73.5 ± 2.4</td>
<td>76.0 ± 1.7</td>
<td>72.0 ± 1.2</td>
<td>70.0 ± 0.5</td>
<td>71.6 ± 0.5</td>
</tr>
<tr>
<td>48</td>
<td>72.9 ± 0.0</td>
<td>76.0 ± 1.5</td>
<td>73.7 ± 0.3</td>
<td>70.5 ± 0.5</td>
<td>70.7 ± 1.1</td>
</tr>
</tbody>
</table>

Mean ± SE of the mean.
Substrate treatment of *Pleurotus* culture

**RESULTS**

**Temperature, moisture, and pH**

The temperature of the liquid used for soaking remained at 28-29°C during the entire operation. Temperature at the centre of the logs during the incubation also varied very little (25-28°C) for all treatments, with no statistical differences.

Table II shows changes in substrate humidity for each treatment, after soaking in lime and air draining. Moisture generally varied from 48% (corn cobs; corn cobs + coffee pulp at 0 hours soaking) to 76% (corn straw at 24-48 hours soaking.) Statistical analysis showed significant differences ($P=0.0001$) for time and the interaction of time with substrates. With minimum immersions time (0), moisture varied between 48 and 56% depending on the substrate, and for substrates soaked for 12 h or more, moisture after rinsing and draining was higher, at 70-76%. The pH of the substrate after soaking and draining and just before spawning is indicated in Table III. Observed values varied between 8.4 and 12.1. A statistical analysis indicated significant differences ($P=0.001$) for time and the interaction of time with substrates. Substrates for “0” soaking (immersion for 5 min followed by rinsing) had the highest pH values (11.1-12.1). These values decreased for all substrates with soaking time and their value varied with the substrate, from 10 (corn cob) to 8.4 (corn cob + coffee pulp). The pH observed for the control (grass + composted pulp) was 8.7.

**Colonization and contamination levels**

The growth of *P. ostreatus* during incubation was homogeneous for all treatments. It also appeared similar to the control and to other reports regarding pasteurized substrates on which this mushroom has been cultivated. (Villa Cruz et al., 1999; Morales et al., 1995). The substrate was fully colonized in 14-15 d and no fungal contamination was observed (Figure 1).

At harvesting, although most carpophores appeared normal, soft, and dry to the touch, some of the harvested logs contained carpophores with a shiny and wet appearance, as if they had recently been watered. (Care had in fact been taken not to water at the final stages of development.) Their appearance was different from that of normal irrigated mushrooms; the liquid on the pileum was slightly viscous and sticky to the touch, similar to a bacterial film. This film was probably the result of *Pseudomonas* bacteria. (Gea, 2002) See Figures 2 to 5.

Analysis of bacterial population was therefore performed on the substrate at the time of spawning, as seen in Figures 6 a-e. The bacterial population of each substrate at spawning was noted. In the grass substrate, the corn straw, and the mixture of grass and pulp, the bacterial population remained at low levels from 0-36 h of soaking for all three bacteria under study (*Pseudomonas*<sub>bacillii</sub>, and coliforms). In these three substrates a high level of bacteria, principally *Pseudomonas*, ($3x10^6-10^7$ cell ml$^{-1}$) was observed after soaking in the alkaline solution for 48 h. In the treatments containing corn cobs, there was more contamination, mainly from *Pseudomonas*, although bacilli and coliforms were also observed.

**Biological efficiency**

Table IV presents the BE obtained for each substrate spawned with *P. ostreatus* after 48 h of soaking. The efficiencies obtained vary from 82.9% (corn straw) to 126% (grass). Statistical analysis resulted in the identification of three significantly different groups: Group A, the grass substrate, with the highest BE (126%); Group B, which included corn straw; grass + coffee pulp; composted substrate), (BEs from 82.9 to 90%), and Group C (corn cobs), with the lowest BE (60.9%).

Table V presents the BEs obtained when *P. ostreatus* was spawned in grass previously treated by soaking in alkaline water for different times. BEs obtained varied from 32% (immersion for 5 min and then rinsed) to 95%

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**Table III**

<table>
<thead>
<tr>
<th>Soaking Time (h)</th>
<th>Grass (S1)</th>
<th>Corn straw (S2)</th>
<th>Corn cob (S3)</th>
<th>Grass-Coffee pulp (S4)</th>
<th>Corn cob-Coffee pulp (S5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.1 ± 0.20</td>
<td>11.4 ± 0.05</td>
<td>11.2 ± 0.04</td>
<td>11.6 ± 0.06</td>
<td>11.1 ± 0.06</td>
</tr>
<tr>
<td>12</td>
<td>11.5 ± 0.11</td>
<td>11.0 ± 0.01</td>
<td>10.2 ± 0.06</td>
<td>11.0 ± 0.05</td>
<td>10.1 ± 0.04</td>
</tr>
<tr>
<td>24</td>
<td>11.2 ± 0.05</td>
<td>11.4 ± 0.07</td>
<td>11.0 ± 0.01</td>
<td>10.5 ± 0.06</td>
<td>10.5 ± 0.04</td>
</tr>
<tr>
<td>36</td>
<td>9.9 ± 0.07</td>
<td>10.1 ± 0.15</td>
<td>10.8 ± 0.01</td>
<td>9.5 ± 0.05</td>
<td>9.4 ± 0.00</td>
</tr>
<tr>
<td>48</td>
<td>9.6 ± 0.09</td>
<td>9.7 ± 0.25</td>
<td>10.0 ± 0.00</td>
<td>8.9 ± 0.05</td>
<td>8.4 ± 0.01</td>
</tr>
</tbody>
</table>

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**Table IV**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substrate</th>
<th>Biological Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Grass</td>
<td>126.0 a</td>
</tr>
<tr>
<td>2</td>
<td>Corn straw</td>
<td>82.9 b</td>
</tr>
<tr>
<td>4</td>
<td>Grass coffee pulp</td>
<td>84.0 b</td>
</tr>
<tr>
<td>5</td>
<td>Corn cob coffee pulp</td>
<td>87.2 b</td>
</tr>
<tr>
<td>6</td>
<td>Composted</td>
<td>90.0 b</td>
</tr>
<tr>
<td>3</td>
<td>Corn cob</td>
<td>60.9 c</td>
</tr>
</tbody>
</table>

*The same letter in the same column means no statistical difference at the level $P=0.05$. C.V. = 16.8%
TABLE V
Biological efficiency of Pleurotus ostreatus ECS-0152 cultivated on Digitaria decumbens grass, previously soaked in water with lime for times indicated

<table>
<thead>
<tr>
<th>Immersion time (h)</th>
<th>Biological efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37.3 b</td>
</tr>
<tr>
<td>12</td>
<td>93.7 a</td>
</tr>
<tr>
<td>24</td>
<td>95.1 a</td>
</tr>
<tr>
<td>36</td>
<td>93.2 a</td>
</tr>
<tr>
<td>48</td>
<td>92.5 a</td>
</tr>
</tbody>
</table>

* The same letter in the same column means no statistical difference at the α=0.5 level. C.V.=16.1%
Substrate treatment of pleurotus culture

(24 h soaking). Analysis resulted in the identification of two statistically different groups at the 0.5% level: Group A (control, and all soaking treatments from 12 to 48 h) had higher BEs, and Group B (immersion for 15 min) had a lower BE.

DISCUSSION

The technical feasibility of preparing substrate for the cultivation of *P. ostreatus* by soaking in a 0.5% alkaline solution without heat treatment was investigated. BEs varying from 37 to 126% were obtained for various substrates and mixtures of substrates tested in three harvests. The results, with BEs ranging from 37% to 126% BE, include data obtained with a control prepared by composting, and were comparable with results reported with other substrates prepared by various pasteurization methods. (Bermudez et al., 1994; Block et al., 1958; Gonzalez and Arzeta, 1994).

From this fact it may be concluded that adjusting pH to alkaline levels is a good means of inhibiting the growth of competitor fungi and bacteria without seriously affecting the growth of *P. ostreatus*. This confirms reports by Stolzer and Grabbe (1999) and Hernandez et al. (2002), who suggested the use of alkaline pHs to cultivate this mushroom. In our experiment, the common deuteromycete contaminants *Trichoderma, Monilia, Penicillium*, and *Aspergillus*, were not observed. The pileus of some of the carpophores displayed some abnormal moist and slimy shininess, presumably from *Pseudomonas* bacteria, which cause yellow blotch on *Pleurotus* spp. (Stamets and Chiltons, 1983; Fermor, 1987; Gea, 2002), but the pronounced deformation of
the carpophores reported by Bessette et al. (1985) was not observed. Since Pseudomonas bacteria proliferate in moist areas, and are disseminated primarily from water and secondarily through spawn and other materials used for mushroom cultivation (Stamets, 1983), high moisture along with a high initial bacterial population on the substrate explain the appearance of the symptoms described on the affected mushrooms.

The substrate which produced the best BE and least contamination was the Digitaria decumbens grass. This substrate has already been reported as suitable for the cultivation of P. ostreatus; however in that report it was pasteurized and composted (Hernandez et al., 2003). Other substrates tested were less efficient; however, it is probable that with better moisture control, higher BEs could be obtained. Methods employing water soaking are known to present difficulties in controlling the initial moisture of the substrate, since humidity varies between lots and even within a single lot. This causes differences in fruiting and a higher risk of contamination (Lopez et al., 1996).

The effect of soaking in alkaline solution on the bacterial population at the end of the soaking was monitored for each substrate. The grass, straw, and straw + coffee pulp in 0.5% lime did not display bacterial growth in the first 36 h of soaking, although after 48 h, bacterial growth did occur. The corn cobs and coffee pulp + corn cobs, however, displayed bacterial contamination from 12 h of soaking onwards. The size of the corn cob particles was probably a factor in this, since it is a dense material and even when broken into 3 cm pieces was not well penetrated by the lime, resulting in greater bacterial growth. Taking the BE and contamination results into consideration, the recommended soaking time for straw in 0.5% lime is 12-36 h.

The preparation of substrates by soaking in alkaline solution is a technique which this study indicates has potential. However good control of the moisture of the substrate must be maintained to limit proliferation of substrate bacteria.

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REFERENCES


