A Small-Scale Method for Screening of Lignin-Degrading Microorganisms

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A new method to facilitate rapid screening of lignin-degrading microorganisms was developed. Fungal strains are cultivated in tissue culture plates containing 14C-ring-labeled dehydrogenation polymerize (DHP) (synthetic lignin). Evolved 14CO2 is trapped in barium-saturated filter paper and is detected by exposing the paper to X-ray film. Analysis of the autoradiograms, carried out by density measurement with an image analysis program, allows for a semiquantitative estimation of the amount of 14CO2 evolved. The method is especially useful for screening for new, powerful lignin-degrading strains in both man-made and natural environments. It eliminates the need for special equipment for their cultivation and trapping of 14CO2 as well as laborious sample analysis. The method has in this study been used to test three novel fungal isolates and a laccaseless mutant of the basidiomycete Pycnoporus cinnabarinus. Their ligninolytic capacities were compared with those of the potent lignin degrader Ceriporiopsis subvermispora.

Higher fungi which cause white rot in wood are the most efficient lignin degraders in nature. They are the only microorganisms known to date capable of completely degrading lignin to carbon dioxide and water (5). White rot fungi that can selectively delignify wood and the enzymes involved in this process are important because of their potential application for the removal of lignin from ligninocellulosic materials, which would facilitate biopulping, biobleaching (1, 8), and detoxification of environmental pollutants (2, 7).

The lignin-degrading ability of a microorganism is commonly evaluated by measuring \(^{14}\text{C}\)CO\(_2\) evolution from \(^{14}\text{C}\)-labeled lignin preparations, such as \(^{14}\text{C}\)-ring-labeled dehydrogenation polymerize (DHP). The measurement of \(^{14}\text{C}\)CO\(_2\) evolution is the most sensitive and accurate method for testing ligninolytic activity (5). Standard experiments are carried out in culture flasks and require special equipment for trapping of \(^{14}\text{C}\)CO\(_2\) (6). Besides the fact that specifically radiolabeled lignins or lignin model compounds and the disposal of the radioactive waste produced during such experiments are expensive, screening a large number of microorganisms is very laborious and time-consuming. In our efforts to study the mechanisms of lignin degradation by white rot fungi, we developed a small-scale method for fast and easy evaluation of their ligninolytic capacity. The method has been modified after a protocol used by Tabor et al. (10) for the screening of bacterial mutants for their biosynthetic pathway for amines.

The ligninolytic capacities of three unidentified basidiomycetes isolated from decaying hardwood and a mutant strain of the white rot fungus Pycnoporus cinnabarinus (ATCC 200748) rendered laccaseless by UV radiation (3) were evaluated and compared to that of cultures of Ceriporiopsis subvermispora [L-6322-Sp (8501)]. The fungal strains were grown on 2\% (wt/vol) malt extract agar (15\% agar) plates for 10 days. Agar blocks (0.5 by 0.5 cm) from these precultures were transferred into the wells of sterile tissue culture plates (Falcon 3046) containing 3 ml of sterile liquid basal medium (4). The fungi were cultivated at 25°C for 3 days to establish mycelial growth prior to addition of radiolabeled DHP. \(^{14}\text{C}\) ring-labeled DHP of coniferyl alcohol was prepared by the ‘Zutropfverfahren’ (E. Odier and P. Heckman, Institut National de la Recherche Agronomique, Paris-Griognon, France) and previously characterized as described in reference 5. On day 4, \(^{14}\text{C}\)-DHP (5,000 dpm) dissolved in 5 \(\mu\)l of dimethylformamide was added to each well. After 24 h, sterile filter papers (Whatman 3 MM, cut exactly to the size of the tissue plate) were soaked in sterile saturated barium hydroxide solution and placed over the wells. Plastic lids were tightly fastened and clamped to the plates, which were then incubated at 30°C for an additional 5 days. Evolved \(^{14}\text{C}\)CO\(_2\) was trapped in the filter papers as insoluble BaCO\(_3\).

The filters were then removed and exposed to X-ray film (Kodak XAR-5) for 5 days. Dark circles on the autoradiogram corresponding to the positions of individual wells were formed by evolved and trapped \(^{14}\text{C}\)CO\(_2\). The intensity of the dark circles disclosed the ligninolytic capacity of the respective fungal culture. Analysis of the autoradiograms was performed with a Macintosh Power PC 9500 computer by using a scanner (UMAX Powerlock II) and the public domain NIH image analysis program (developed at the National Institutes of Health and made available for the public via http://rsb.info.nih.gov/nih-image/).

All cultivations were performed in triplicate with independent culture plates. Uninoculated samples containing 3 ml of liquid basal medium (pH 4.6) and radiolabeled DHP (5,000 dpm) were used as a control on each culture plate.

Each of the fungal strains studied developed a mycelial layer on the surface of the culture medium during the 10-day cultivation. One of the new fungal isolates (Fig. 1C) gave the strongest signal on autoradiograms of all strains, indicating an even higher ligninolytic activity than that of the efficient lignin degrader C. subvermispora (9) (Fig. 1F). Weak autoradiographic signals were obtained with one of the new strains (Fig. 1B) and the laccaseless mutant of P. cinnabarinus (Fig. 1E). The inability of the laccaseless mutant to release significant...
amounts of $^{14}$CO$_2$ is in good agreement with our previous result, which was obtained by the conventional method for measuring $^{14}$CO$_2$ evolution (3). The third new isolate (Fig. 1A) used in this study gave a signal almost as strong as those obtained for C. subvermispora (Fig. 1F). The background was low on all X-ray films, and the control well did not show any $^{14}$CO$_2$ evolution (Fig. 1D). All samples, run in triplicate on separate plates under identical conditions, gave identical results. Data obtained from density reading analysis of the autoradiograms are presented in Table 1.

The method described here allows easy screening of ligninolytic activity in a large number of microorganisms. It has clear advantages over conventional culture flask experiments since the fungal organisms can be simultaneously cultivated in small volumes of media containing minimal amounts of $^{14}$C-

TABLE 1. Density reading of the autoradiograms of the fungal strains

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Mean density$^a$</th>
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<tbody>
<tr>
<td>Basidiomycete A</td>
<td>30 ± 0.2</td>
</tr>
<tr>
<td>Basidiomycete B</td>
<td>17 ± 0.2</td>
</tr>
<tr>
<td>Basidiomycete C</td>
<td>67 ± 0.5</td>
</tr>
<tr>
<td>P. cinnabarinus laccaseless mutant</td>
<td>7 ± 0.2</td>
</tr>
<tr>
<td>C. subvermispora</td>
<td>42 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ The values, in uncalibrated units, are the means ± the standard deviations from three independent density measurements of each of three independent autoradiograms obtained.

This allows the testing of large numbers of microorganisms and their lignin-degrading abilities in a short period of time.

Both man-made environments, such as rivers and ponds, which for a long time have received black liquor from pulping or wastewaters from pulp bleaching, as well as natural environments can rapidly be screened for useful lignin-degrading microorganisms. The method can also be used for the screening of mutants deprived of their ability to produce one or several lignin-degrading enzymes as well as for optimization of culture conditions for efficient delignification. Various substrates, such as pulp in which the lignin has been $^{14}$C labeled, can also be used. Mineralization experiments can be downsized even further by using 24- or 96-well microtiter plates. The technique, as described above, allows a semiquantitative analysis of evolved $^{14}$CO$_2$ by using density readings of the autoradiograms. Alternatively, quantitative analysis can be carried out by scintillation counting of the filter papers.

In conclusion, the method is a useful tool for rapid screening of large numbers of microorganisms for their ligninolytic capacity to obtain more potent and specific lignin-degrading white rot fungi than those already known.

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REFERENCES