Separation of Fungal Propagules by Partition in Aqueous Polymer Two-Phase Systems

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Conidia of Penicillium chrysogenum and Penicillium frequentans and sporangiospores of Rhizopus rhizopodiformis, Rhizomucor pusillus, and Mucor racemosus were subjected to partition in aqueous polymer two-phase systems. The partition behavior differed drastically between the conidia of the two Penicillium species and the sporangiospores of the three species of phycomycetes. This difference in partition behavior can be used for purification of fungi belonging to different taxonomical groups. P. frequentans was completely separated from M. racemosus by two extractions, whereas four extractions were needed to purify M. racemosus. This method was used on an air sample from a locality where wood fuel chips are handled. The conidia of the fungi Trichoderma viride and Rhizopus rhizopodiformis were removed completely by only two extractions.

Plate count techniques are often difficult to apply for enumeration of fungal propagules in liquid samples containing a mixture of unknown fungi. Some fungi, especially phycomycetes, often grow very fast over the agar plates and may thus prevent discovery or seriously inhibit growth of slower-growing fungal colonies on the plates. This may result in large underestimations of the CFU or decrease the precision of the estimates. Further, it makes isolation of all but the fast-growing fungi impossible.

One way to eliminate this problem is by adding various chemicals, e.g., rose bengal (7), Solacol (Aagrunol B.U., Groningen, The Netherlands) (4), or oxgall (6), to the agar substrate or by acidifying the substrate (5). All of these additions reduce the growth rate of fast-growing fungi. Another effect of such additions may be to increase the selective properties of the growth medium, thus preventing growth of other fungi. Another way of solving the problem of wide-spreading phycomycete colonies might be to eliminate the phycomycete sporangiospores before plating and to culture them separately. We here suggest the use of aqueous polymer two-phase systems for this purpose.

Aqueous two-phase systems consist of two polymers, usually dextran and polyethylene glycol, and water. Due to their high water content (about 90%), these systems are very mild toward biological substances. Macromolecules partition in these systems between the upper phase (polyethylene glycol rich) and the lower phase (dextran rich), whereas particles partition mainly between the upper phase, interphase, and lower phase.

The advantage of aqueous polymer two-phase systems as a separation method for macromolecules as well as cell particles and whole cells has been shown previously (1). Experiments in which bacterial cells have been used show that different species vary in their distribution patterns. Even different bacterial strains from the same species have been shown to differ in partition behavior (2). The distribution of a substance depends upon the size and surface properties of the particle. Particles showing great differences in partition behavior can easily be separated from each other by a simple extraction procedure, whereas particles with closely related partition behavior demand a multistep procedure, such as countercurrent distribution (2). The aim of this work was to study the partition of the conidia of two Penicillium species and of the sporangiospores of three species of phycomycetes and to develop a method for the separation and purification of conidia and sporangiospores through a simple extraction procedure in a dextran-polyethylene glycol two-phase system.

MATERIALS AND METHODS

Preparation of conidia and sporangiospores. Penicillium chrysogenum Thom, Penicillium frequentans Westling, Mucor racemosus Fres., Rhizopus rhizopodiformis (Cohn) Zopf, and Rhizomucor pusillus (Lind) Schipper were grown on 2% (wt/wt) malt extract (L 39; Oxoid Ltd., London, England)-1.5% (wt/wt) purified agar (L 28; Oxoid)-30 mg of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per liter for 10 days at 21°C, except for Rhizomucor pusillus, which was grown at 40°C. Small portions of sterile water containing 0.01% (wt/wt) Tween 80 were added to each of the agar plates covered by the fungi. The plates were gently shaken, and the washing fluid containing the conidia or sporangiospores was transferred to glass bottles. To avoid aggregates of conidia, the glass bottles were placed in an ultrasonication bath for 10 min. This treatment did not decrease the viability of the fungal spores.

Two-phase system. The two-phase system used for studying the partition behavior of the different species of fungi consisted of 5% (wt/wt) dextran 500 (molecular weight, 500,000; Pharmacia Fine Chemicals, Uppsala, Sweden), 4% (wt/wt) polyethylene glycol Carbowax 6000 (Union Carbide Corp., New York, N.Y.), 10 mM potassium phosphate buffer (pH 6.9), sodium thiocyanate (0, 20, 40, 80, or 160 mM), and water to a total of 3.0 g. The water used was filtered through a Millipore R/Q water purifier (Millipore Corp., Bedford, Mass.) and sterilized. All chemicals were of analytical grade.

Partition of conidia and sporangiospores. To study the partition behavior, 1 ml of fungal conidia or sporangiospores suspended in sterile water containing 0.01% (wt/wt) Tween 80 was transferred to test tubes containing the phase systems.

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FIG. 1. Principles of the procedure for extraction of a suspension containing conidia from *P. frequentans* and sporangiospores from *M. racemosus*. The upper phase of system 1 is extracted four times with fresh lower phase (systems 3, 5, 7, and 9). In the same way, the lower phase of system 1 is extracted four times with fresh upper phase (systems 2, 4, 6, and 8). with various amounts of electrolytes. The contents of the test tubes were mixed by inversion 30 times and then allowed to stand for 25 min at room temperature (21°C) for phase separation. After phase separation, 1 ml of the upper phase was withdrawn and diluted with 2 ml of 10 mM potassium phosphate buffer (pH 6.9). The same amount of buffer was added to the remaining part of the system for phase dissolving. The absorbance of the upper phase and the remaining part of the system was measured at 560 nm, and, with a knowledge of the phase volumes, the amount of conidia or sporangiospores in the upper phase relative to that in the interphase and lower phase could be estimated.

**Extraction procedure.** For the extraction procedure, 1 ml of a suspension containing $8.5 \times 10^7$ conidia of *P. frequentans* and $3.0 \times 10^7$ sporangiospores of *M. racemosus* was added to the phase system described above. After mixing and phase separation, the two phases were extracted as shown in Fig. 1. The upper phase of system 1 was extracted four times with fresh lower phase, and the lower phase of system 1 was extracted four times with fresh upper phase. Two 10-μl samples were taken from the upper phase of systems 1, 3, 5, 7, and 9 and from the lower phase of systems 1, 2, 4, 6, and 8 (Fig. 1). The withdrawn volume was corrected by adding the same amount of fresh upper and lower phase, respectively. One of the samples was diluted twice and applied to a Bürker counter chamber for microscopic counting, while the other samples were diluted and spread on agar plates. The cultivation medium was the same as described above except for addition of malic acid to 0.5% to prevent the fast-growing *M. racemosus* from overgrowing the more slowly growing *P. frequentans*. The agar plates were incubated for 5 days at room temperature (21°C) before the number of CFU from the two species was determined.

**RESULTS AND DISCUSSION**

The partition of sporangiospores and conidia from five species of fungi is shown in Fig. 2, where the relative concentration in the upper phase, the interphase, and the lower phase is plotted as a function of the amount of sodium thiocyanate present in the system. The results show that an increase in ionic strength did not influence the partition of the conidia of the *Penicillium* species. However, a small increase in ionic strength markedly decreased the affinity of sporangiospores of *M. racemosus* and *Rhizomucor* *pucillus* for the upper phase, transferring them to the interphase and the lower phase. *Rhizopus rhizopodiformis* had the same partition pattern as the other phycomycetes but was affected to a smaller extent by the increase in salt concentration. The difference in partition behavior between the *Penicillium* species and the phycomycetes is probably due to differences in surface properties rather than conidal size since, in the absence of electrolytes, sporangiospores of *M. racemosus*, with an average size of 5.5 to 8.5 by 4 to 7 μm, partition in

FIG. 2. Partition behavior of *P. chrysogenum* (■), *P. frequentans* (▲), *Rhizomucor pucillus* (○), *M. racemosus* (●), and *Rhizopus rhizopodiformis* (□) in a two-phase system composed of 5% (wt/wt) dextran 500, 4% (wt/wt) polyethylene glycol 6000, 10 mM potassium phosphate buffer (pH 6.9), and various amounts of sodium thiocyanate.
the same way as conidia of *P. frequentans*, which are 3.0 to 3.5 μm in diameter. An increase in ionic strength affects the sporangiospores of *M. racemosus*, transferring them from the upper, polyethylene glycol-rich phase to the interphase and the lower, dextran-rich phase, whereas the conidia of *P. frequentans* still prefer the upper phase. These differences in distribution pattern thus enable the conidia and sporangiospores to be separated by means of a few extractions.

The results of the extraction procedure are presented in Table 1. The upper phase of phase system 1 was almost free of *M. racemosus* sporangiospores. After two extractions, no sporangiospores were found with either of the two determination techniques. However, the percentage of *Penicillium* conidia in the upper phase relative to the interphase and the lower phase steadily increased throughout the extractions. After four extractions, no *Penicillium* conidia could be detected in the lower phase or at the interphase.

This extraction procedure was tested on a spore sample collected from the air when wooden fuel chips were handled. The chips consisted of 50% spruce and pine wood and 50% birch wood and had a diameter of 3 to 10 cm. About 10⁹ fungal propagules per m² were present in the air when the chips were handled. The sample subjected to partition in the two-phase system contained 10² propagules. The fungi present in the sample were, to 96%, *Penicillium* sp. and *Aspergillus fumigatus* Fres. About 3% of the fungi were *Trichoderma viride* Pers. ex Gray and *Rhizopus* sp., which grow rapidly on malt extract and cover an agar plate (diameter, 9 cm) within a few days. The phase system was 5% (wt/wt) dextran 500–4% (wt/wt) polyethylene glycol 6000–10 mM potassium phosphate buffer (pH 6.9)–160 mM sodium thiocyanate. Ninety-seven percent of the slowly growing conidia are extracted into the upper phase of phase system 0. No fast-growing fungi could be detected at the dilution used when the propagules were cultivated. After extraction 1, 99% of the slowly growing conidia were in the upper phase. When two extractions were performed, 4% of all fungi were left at the interphase and in the lower phase. About 75% of these originated from the fast-growing fungal species.

The advantages of this method are the simplicity and rapidity with which the experiments can be performed. Only ordinary test tubes are used for the partition, and the time needed for the separation described is only 20 to 25 min. The growth of the fungi is not affected by the polymers used in the phase systems.

The method might be particularly attractive for the selective isolation of specific fungi, such as plant-pathogenic ones, from diverse habitats. By use of other polymers and salts in the phase system, it may be possible to selectively extract spores or conidia of almost any fungus. Further experiments are in progress.

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**LITERATURE CITED**


**TABLE 1. Distribution of *P. frequentans* and *M. racemosus* after four extractions in a liquid two-phase system**

<table>
<thead>
<tr>
<th>Extraction no.</th>
<th>Microscopic counts</th>
<th>Viable counts</th>
<th>% in upper phase</th>
<th>% in lower phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. frequentans</em></td>
<td><em>M. racemosus</em></td>
<td><em>P. frequentans</em></td>
<td><em>M. racemosus</em></td>
</tr>
<tr>
<td>0</td>
<td>88</td>
<td>0.8</td>
<td>91</td>
<td>1.4</td>
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<tr>
<td>1</td>
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<td>0.5</td>
<td>97</td>
<td>0.0</td>
</tr>
<tr>
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<td>0.0</td>
<td>98</td>
<td>0.0</td>
</tr>
<tr>
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<td>98</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0.0</td>
<td>100</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* The phase system used was composed of 5% dextran 500, 4% polyethylene glycol 6000, 10 mM potassium phosphate (pH 6.9), and 160 mM NaSCN.

*b* As compared with the interphase and lower phase.