Procedure for preparing permeabilized hyphae for enzyme assays

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Procedure for preparing permeabilized hyphae for enzyme assays

Abstract
Procedure for preparing permeabilized hyphae for enzyme analysis.

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A method for achieving prolonged nutrient limited growth of Neurospora mycelium

It is difficult to achieve sustained growth under supplement limited conditions of filamentous fungi such as Neurospora which cannot easily be grown in a chemostat. Reducing the initial concentration of a nutrient provided to batch cultures usually results in a rapidly declining supplement concentration and succeeds only in advancing the time at which growth ceases. This seldom produces experimentally amenable variation in growth rate. In order to study the effects of prolonged growth of amino acid auxotrophic mutants at low rates of provision of the required amino acid we have adapted the approach of continuously supplying fresh medium supplemented with the desired amino acid concentration to batch cultures. The method described should prove applicable to other nutrient limitation conditions such as nitrogen or carbon starvation.

Replicate 1 L round, flat bottomed flasks were used, having three equally spaced 2 cm indentations to improve agitation and aeration. These contained an initial volume of 200 ml of medium inoculated with around 10⁷ conidia. The flasks were fitted with silicone rubber stoppers carrying a foil capped vent and a glass tube in contact with the medium through which a combined stream of sterile air (0.42 ml/min) and fresh medium (0.16 ml/min) was pumped by means of a multichannel proportioning pump. Pump tubing was presterilized by passing a 2% solution of hypochlorite, followed by sterile distilled water. The flasks were shaken at 200 rpm on a New Brunswick gyratory shaker in 29°C constant temperature room for periods not exceeding 45 h, or a final culture volume of around 600 ml. In the experiments shown in Fig. 1 conidia from an arg-5 strain were inoculated into replicate flasks containing Vogel's medium supplemented with 2.25% (w/v) glucose and 0.12 mM arginine, and supplied at a constant rate with fresh Vogel's/glucose medium containing various concentrations of arginine. Once the initial supply of arginine had been exhausted, growth occurred at a linear rate proportional to the concentration of arginine in the feeding solution. Mycelium growing at a linear rate cannot represent a true metabolic steady state, as is established under conditions of exponential growth. Nevertheless such experiments can yield valuable information on changes in enzyme synthesis (in this case the derepression of amino acid synthetic enzymes - Flint et al., in preparation) and in macromolecular synthesis during prolonged periods of nutrient limited growth. A somewhat different 'fed batch' culture technique, suitable for growth and sampling of germing conidia, has been described by Limon-Lason et al. (1977) BBRC, 78: 1234-1241 for the study of nitrogen and carbon limited conditions.

Using the conditions described above the growth of a wild strain in minimal medium was shown to be exponential and the rate independent of culture volume over the maximum duration of each experiment. Thus the combination of vigorous agitation and forced air ensures adequate aeration throughout, despite the change in culture volume.

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Procedure for preparing permeabilized hyphae for enzyme assays.

Vegetative hyphae and aerial hyphae were obtained from standing cultures (Table I). The vegetative hyphae formed a pad on the surface of the liquid medium and, after a few days, aerial hyphae grew from the mycelial pad. The aerial hyphae and then the mycelial pad were removed from the test tube with a sterile spatula. Immediately the material was agitated on a vortex mixer for 2 min. with 5.5 ml of the permeabilization solution. The permeabilization solution contained 0.5 ml of toluene: methanol (1:4 v/v) and 5 ml of 20 mM potassium phospho-
**Table I.**

<table>
<thead>
<tr>
<th>Days' Specific Activity</th>
<th>Vegetative hyphae</th>
<th>Aerial hyphae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles CO₂ min⁻¹ (mg lyophilized weight)⁻¹</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.323 ± 0.005d</td>
<td>0.007</td>
</tr>
<tr>
<td>4</td>
<td>0.885 ± 0.007</td>
<td>0.735 ± 0.02</td>
</tr>
</tbody>
</table>

**GAD** was assayed by measuring the amount of ¹⁴CO₂ released from glucose (Christensen and Schmit, 1980 J. Bacteriol. 144: 983).

**b** Standing conidiating cultures of *nada* (FGSC #2688) were grown in test tubes (18 x 150) on 5 ml of liquid complete medium containing 1% casamino acids, 0.5% yeast extract, 2% sucrose, 1% glycerol and Vogel salts (Vogel 1964, Am. Nat. 98: 435) at 25°C.

**c** Days after inoculation with 5 x 10⁵ conidia/tube.

The assays were done in duplicate.

We have found that this procedure for measuring enzyme activities in hyphae has the following advantages, as compared to preparing cell free extracts.

1. The permeabilization of the hyphae assures that all the enzyme activity, intracellular as well as cytosolic, is accessible for measurement.
2. Small molecules are removed rapidly during permeabilization; thus, dialysis is not necessary.
3. The cell interior can be rapidly exposed to EDTA and protease inhibitors minimizing enzyme degradation.
4. Lyophilized cells can be weighed to calculate specific activities.

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Rapid sampling and collection of Neurospora mycelial suspensions.

**Neurospora** liquid shaking cultures (l-24 h, 25° in liquid minimal medium density of about 1 mg dry wt/ml) can be sampled using a variety of dispensers which fit into 24/40 ground joint erlenmeyer flasks (variously named "Repeater P pets", "Tipets", "Prep petters", "Tilt-a-Pets", etc.). One can typically take 10 ml samples varying by 15% with such medium density culture as described above.

Such samples can be collected on Whatman GF/A glass fiber filters. We typically collect such samples on 2.4 cm diameter filters using Millipore or similar filter holders having sintered glass bases. The samples can then be placed into TCA, PCA, ethanol or liquid nitrogen for extraction or rapid freezing. If it is not necessary to wash the samples, it is not difficult to take a sample, filter it and move it into TCA-containing tube in 3 to 5 seconds. We have shown that such samples may have ATP/ADP ratios of 7, showing that they retain a high energy charge throughout the collection procedure.

The GF/A filters are very fast in filtering mycelia of Neurospora and other fungi. Conidia, however, tend to clog the filters fairly rapidly. Consequently, it is important that conidia used to grow the mycelia are relatively fresh with good germination frequencies to avoid slowed filtration due to ungerminated conidia.

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