Fluorescent staining of Neurospora nuclei with DAPI

E. Martegani
Universita di Milano

F. Trezzi
Universita di Milano

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Abstract
Fluorescent staining of Neurospora nuclei with DAPI
walls as a carbon source. We have adapted this method for the isolation of a preparative scale of Trichoderma enzymes that can be used for the formation of protoplasts from hyphae of Neurospora crassa.

The growth medium for Trichoderma viride contained per liter: 2 g KH$_2$PO$_4$, 1 g (NH$_4$)$_2$SO$_4$, 0.39 urea, 0.3 g MgSO$_4$·7H$_2$O, 0.3 g CaCl$_2$, 1 g bactopeptone and 1 ml of a trace element solution. The composition of the trace element solution was per 100 ml: 50 mg FeSO$_4$ 7H$_2$O, 15.6 mg MnSO$_4$·H$_2$O, 16.7 mg ZnCl$_2$, 20 mg CoCl$_2$ and 0.1 ml 19% HCl. When glucose was used as the only carbon source, 5 g per liter was added to the growth medium. With cell walls as carbon source, 50 g of Neurospora cell walls (wet weight) plus 0.59 glucose were added per liter medium. The cell wall preparation was obtained as the 2000xg pellet of Neurospora hyphoe disrupted in a grindmill (H. Wiess et al., 1970 Eur. J. Biochem. 14: 75). This pellet was suspended in distilled water and rehomogenized in the grindmill. The washed cell walls were collected by centrifugation. The washing procedure was repeated four times. For the production of cell wall digesting enzymes 100 ml of medium containing glucose as the only carbon source was inoculated with 10$^5$ Trichoderma conidia (per ml) and grown at 30° on a rotary shaker. Conidia were obtained from solid grown cultures as described for Neurospora. After 24 hours the 100 ml culture was added to a 10 liter bottle containing 7 liters of medium supplied with Neurospora cell walls and glucose. The culture was aerated vigorously and growth was evident after two days as foam production (excessive foaming could be suppressed by adding antifoam). After 5 to 7 days of growth, cells and cell walls were removed by filtration through a Büchner funnel. The turbid filtrate was clarified by centrifugation for 10 min of 2000xg. The enzyme was precipitated from the supernatant with ammonium sulphate at 75% saturation. The precipitate was dissolved in 10 to 20 ml distilled water and dialyzed overnight at 4° against 2 x 5 liter distilled water. Insoluble material was removed by centrifugation and the enzyme preparation (100 to 300 mg) was stored at -20° or lyophilized.

Protoplasts from Neurospora crassa were prepared from cultures in the early log phase. Hyphae were collected on a Büchner funnel and washed twice with ice cold distilled water. 10 g hyphoe (wet weight) were suspended in 50 ml of 500 mM sorbitol, 200 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 50 mM maleic acid, adjusted to pH 5.8 and 20 mg of the Trichoderma enzyme preparation. Incubation was carried out at 30° in a 250 ml erlenmeyer with gentle shaking. Protoplast formation was complete in 60-90 minutes (see Figure 1). - - - Lob. Physiol. Chem., State University Groningen, Groningen, The Netherlands.

<table>
<thead>
<tr>
<th>Growth (mg dry weight) of 74A in different minimal media</th>
<th>Ascorbic acid 100 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td>2% sucrose with NH$_4$NO$_3$</td>
<td>327</td>
</tr>
<tr>
<td>2% glycerol with NH$_4$NO$_3$</td>
<td>30</td>
</tr>
<tr>
<td>2% glycerol with L-asparagine</td>
<td>108</td>
</tr>
</tbody>
</table>

An improved glycerol minimal medium.

Charlang, G. W.

Anyone who has grown Neurospora in a liquid glycerol medium knows the frustrations of low yield and difficulties of harvesting such cultures. We have found a way to significantly improve yield by using an organic nitrogen source and/or ascorbic acid.

The medium consists of Vogel's salts (without NH$_4$NO$_3$), plus glycerol (2%) and L-asparagine (0.5%). Tween 80 (3 drops or 42 mg per flask) is added before autoclaving. We inoculate wild type 74A at a concentration of 10$^4$ conidia per ml in 50 ml of this medium (125 ml flasks). The flask cultures are incubated at 30° C with shaking for 48 hours; some typical results (dry weight in mg per flask) are given in the table.

<table>
<thead>
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<th>Ascorbic acid 100 µg/ml</th>
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<tr>
<td>2% sucrose with NH$_4$NO$_3$</td>
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<tr>
<td>2% glycerol with NH$_4$NO$_3$</td>
</tr>
<tr>
<td>2% glycerol with L-asparagine</td>
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</tbody>
</table>

Ascorbic acid improves yield with or without asparagine. The ascorbic acid solution is freshly prepared in sterile distilled water and filter sterilized before adding it to the autoclaved medium. (Supported by Grant NGR 05-002-121 from the National Aeronautics and Space Administration.) - - - Division of Biology, California Institute of Technology, Pasadena, CA 91125.

Mortegani, E. and F. Trezzi.

Fluorescent staining of Neurospora nuclei with DAPI.

A simple procedure is described for fluorescent staining of Neurospora crassa nuclei with the bisbenzimida DAPI (4', 6' diamidino-2-phenylindole·2 HCl). DAPI binds selectively to DNA, and the intensity of the blue fluorescence obtained is proportional to the DNA content of each nucleus (Schneider et al., 1977 Cytobiologie 15: 357). A technique was developed to determine microfluorimetrically the relative DNA content of nuclei in exponentially growing hyphae.

Mycelia were washed with cold 0.1 M phosphate buffer pH 7 (PB) and fixed by resuspending in sufficient fixative buffer (0.1 M phosphate buffer pH 7 + 0.3% Formolin) to obtain a suspension with an absorbance of 450 nm (A$450$) of 0.250.
After two hours of room temperature, 10 ml of the fixed hyphoe were centrifuged at 3000rpm for 10 min, the hyphoe were resuspended in 10 ml of PB and recovered again by centrifugation. The washed, fixed hyphoe were then resuspended in 10 ml PB containing 0.2 pg/ml of DAPI and left for 15-16 hours at 25°C. Although the dilute DAPI solution is unstable, a stock solution of 1 mg/ml in distilled water can be stored for weeks at -20°C. After staining, the hyphoe were recovered by centrifugation, washed twice with PB, resuspended in a small volume of buffer and mounted. Observations were made with an optical fluorescence microscope (Leitz Ortholux equipped with 1 mm UG-1 excitor filter, 5 mm BG-38 red absorbing filter and a barrier filter K.430 or K.460). Nuclei appear as bright, light-blue spherical bodies, while the cytoplasm is almost completely dark, except for small foci of fluorescence which are probably due to mitochondrial DNA. Septa are also visible as weak dark-blue linier.

With a suitable microfluorimeter (we employed a Leitz-MPV microphotofluorimeter with a KNOTT-MFLK photoelectric unit), the intensity of the fluorescence of individual nuclei can be measured, and the relative DNA content of each nucleus can therefore be determined. The staining with DAPI appears very stable under UV light, with no appreciable fading. -- Centro del C.N.R. per la Biologia Cellulare e Molecolare delle piante, Istituto di Scienze Botaniche, Università di Milano, 20133 Milano, Italy.

Perlman, J. and J.F. Feldman.

A new culture method for biochemical study of the circadian clock.

The band strain of N. crassa exhibits a well-characterized circadian rhythm of conidiation under conditions of constant temperature and darkness (Sargent et al., 1966 Plant Physiol. 41: 1343-1349). Biochemical and physiological studies of the circadian clock have been very difficult because most cultures used in such experiments have been grown on solid medium to observe the bonding pattern. We have now been able to show that homogeneous, non-bonding cultures also exhibit normal circadian rhythmicity. Included in our studies were lawn-inoculated cultures on solid medium, shaking liquid cultures, and standing liquid cultures. Methods and results are presented only for the latter because that system has been characterized the most extensively and potentially may be the most useful.

Cultures were maintained on Horowitz complete slants. All other medium, both liquid and solid, was Vogel's salts containing 1.2% sodium acetate and 0.05% casamino acids (solid medium contained 1.5% agar). A conidium from 6-8 day old slants were suspended in distilled water or (in later experiments) liquid medium, and filtered through glass wool. The concentration of conidia in the filtrate was measured (Klett-Summerson colorimeter, blue filter), and an aliquot immediately added to a large volume of stirring liquid medium to give a final concentration of 2 x 10^5 spores/ml. Using an automatic pipetter, 25 ml of the stirring suspension were added to each of several dozen 100 x 15 mm plastic disposable Petri dishes. Six growth tubes with solid medium were inoculated at one end with about 50 microliters of undiluted filtrate. All plates and growth tubes were put in constant light at 25°C. After about a day, they were transferred to constant darkness in an environmental growth chamber, also at 25°C. The Petri dish cultures had visible growth by 24 hours, and subsequently formed a mycelial mat which covered the surface. At several different times after the cultures were placed in the dark, six pieces of mycelium from each of three plates were cut with a cork borer (11 mm diam.) and transferred to fresh growth tubes. (The size of the transferred pieces has been varied greatly with identical results.) The growing fronts of control and experimental growth tubes were marked in red safelight (G.E. BCJ, 60 watt) at the same time each subsequent day. After about 7 days in the dark, the timer of occurrence, or phases, of the first conidial bands of the experimental growth tube were determined by linear regression analysis and compared to the corresponding band of the control tube.

The phases observed in the growth tubes inoculated with pieces of the standing liquid cultures were very close to those of the controls at all timer sampled. (In some experiments small and consistent phase advances were seen in the experimental tubes.) The sampling manipulations therefore da not affect the phase of the clock, and it may be concluded that the liquid cultures have a normal circadian clock whose phase is set, like that of the controls, by the light-to-dark transition. The phases of experimental growth tubes start to differ from that of the controls at approximately the time when the liquid cultures reach stationary phase (about 55-60 hours of age). It seems likely that either the clock of older cultures "runs down," or that older cultures are susceptible to phase resetting when cut and transferred. This appears to be largely independent of the length of time they spend in constant light. Preliminary experiments suggest that a 3-hour period in constant light (possibly even less) suffices to set the phase of these cultures.

Experiments are underway to relate the age of the cultures and nuclear division timer to the functioning of the clock. Other types of experiments, particularly the addition of various agents to determine their effects on the clock, are now possible.

Rigby, D.J., T.J. Balls and A. Radford.

Semi-quantitative analysis of protease activity.

A technique has been developed in this laboratory by which Neurospora proteases can be quickly and simply assayed semi-quantitatively. The method is particularly useful when many sampler must be assayed. The assay is bored on the digestion of the gelatin matrix of the emulsion of photographic film, digestion progressively