Protoplasts from Neurospora crassa

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Recommended Citation
Agsteribbe, E. (1979) "Protoplasts from Neurospora crassa," Fungal Genetics Reports: Vol. 26, Article 15. https://doi.org/10.4148/1941-4765.1703

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Abstract
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Protoplasts from yeast and molds are usually prepared by incubation of cells or hyphae with commercially available snail gut enzyme. However, in the case of Neurospora crassa, protoplast formation proceeds slowly and is incomplete. De Vries and Wessels (1973 J. Gen. Microbiol., 73; 13) have shown that the cell walls of a number of molds contain constituents that cannot be hydrolyzed by the enzymes present in snail gut preparations. For complete digestion of cell walls these authors used exoenzymes produced by the mold Trichoderma viride when grown in a liquid medium with cell

Figure 1. -- Phase contrast micrographs (magnification 500x) of Neurospora crassa hyphae and protoplasts before (A) and after incubation for 30 (B), 60 (C) and 90 (D) minutes with cell wall-digesting enzymes from Trichoderma viride.
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 0.1 ml 50 g FeSO$_4$•7H$_2$O, 15.6 g MnSO$_4$•H$_2$O, 16.7 mg ZnCl$_2$, 20 mg CaCl$_2$ 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 by suspending the mycelia in a grindmill. The washed cell walls were collected by centrifugation. 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$^\circ$C 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 at 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$^\circ$ 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$^\circ$ 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 hyphae (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$^\circ$C in a 250 ml erlenmeyer with gentle shaking. Protoplast formation was complete in 60-90 minutes (see Figure 1). 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 50ml of this medium (125 ml flasks). The flask cultures are incubated at 30$^\circ$C with shaking for 48 hours; some typical results (dry weight in mg per flask) are given in the 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.)

| Growth (mg dry weight) of 74A in different minimal media | Ascorbic acid 100 $\mu$g/ml |
|---------------------------------------------------------|--------------------------|
| Medium                                                  | Ascorbic acid 100 $\mu$g/ml |
|---------------------------------------------------------|--------------------------|
| 2% sucrose with NH$_4$NO$_3$                            | 327                      |
| 2% glycerol with NH$_4$NO$_3$                           | 30                       |
| 2% glycerol with L-asparagine                           | 108                      |

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Martegoni, 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 bisbromotriphenylmethane dye 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.