A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia

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Current approaches for obtaining homokaryons of asexually growing *N. crassa* rely on serial passages of macroconidia, which are usually multinuclear (Davis and de Serres 1970 Methods Enzymol. 17A:79- 143). This method is time consuming and labor intensive. We have devised a simple method for purifying viable uninucleate microconidia from conidiating strains of *N. crassa* grown on Westergaard and Mitchell synthetic crossing medium (SC) supplemented with iodoacetate (IAA). Rossier, Oulevey and Turian (1973 Arch. Mikrobiol. 91:345-353) showed that standing liquid cultures containing 1x SC and 1 mM IAA produced microconidia. We have modified their method to reliably obtain microconidia from 150 mm slants of solid agar medium (0.1 x SC/0.5% sucrose/2% agar/1 mM IAA). We have purified these microconidia free of macroconidia and mycelia using Millipore Durapore Millex 5 µm filters. We are using this technique for the one step purification of homokaryons following DNA-mediated transformation. These methods may be generally useful for studies with heterokaryons.

EXPERIMENTAL

Sterile 16 x 150 mm glass culture tubes containing 6 ml of a sterile, molten solu-tion consisting of 2% agar/0.5% sucrose/0.1 x SC and plugged with pre-autoclaved foam plugs, were adjusted to 50-60°C in a water bath. Then 60 µl of 0.1 M sodium IAA (Sigma catalog # I2512, freshly prepared, filter-sterilized in water) was mixed with the contents of each tube (final concentration 1.0 mM). Tubes were slanted at room temperature until solidified, and stored refrigerated for up to one week.

Slants were inoculated using an agar plug of mycelia (obtained by coring a colony with a sterile pasteur pipet) or using a drop of macroconidia suspended in water. Cultures were incubated at 25°C with a 12 hour light/dark cycle for 7-10 days. The production of microconidia was checked by lightly scraping an area of the sparse surface growth (away from tufts of macroconidia) with a sterile, wet inoculating loop. The loopful of culture was transferred to 20 µl of water and examined microscopically.

Microconidia were harvested from cultures by adding 2.5 ml of sterile water to tubes followed by rigorous vortex mixing for 30-60 sec (more microconidia were obtainable by repeating this harvesting step). The conidial suspensions were passed through 5 µm Millex Durapore filter units (Millipore catalog number SLSV025LS) using sterile conditions. Typically 0.1-1% of the microconidia were recovered. The yield as determined by counting with a hemacytometer varied from 10(3)-10(6) microconidia per slant. Filtrates with low numbers of microconidia were concentrated by pelleting the microconidia in a clinical centrifuge (Beckman RT6000, 2000 x g, 5 min). Most of each supernatant was removed by aspiration and the pellets resuspended in the remaining liquid.

Microconidia were germinated at 34°C after spreading on freshly prepared Vogel's/sorbose agar plates. Microconidial viability varied from 1-20%, as determined by comparison of the number

of colony forming centers/ml after seven days growth to the number of microconidia/ml as determined by direct counting. We typically plated 2000 microconidia/plate to obtain homokaryotic cultures, and picked colonies after 2-3 days. Vegetative homokaryotic stocks were obtained by transferring individual colonies to slants of Vogel's sucrose medium.

RESULTS AND DISCUSSION

We analyzed purified microconidia microscopically using the fluorescent dye Hoechst 33258 to stain nuclei (Springer and Yanofsky 1989 Genes. Dev. 3:559-571). Purified microconidia were almost all uninucleate, although microconidia containing two nuclei and enucleated microconidia were also seen. In contrast, macroconidia from Vogel's minimal slants mostly contained 2-5 nuclei, and macroconidia from the slants of 0.1 x SC/0.5% sucrose/2% agar/1 mM IAA mostly contained 1-3 nuclei.

Genetic analyses of microconidia were consistent with the nuclear counting data. Microconidia were obtained from the forced heterokaryon *cyh-1 ad-3B a*m1 + *al-1 lys-4 a* grown on minimal medium. Equal numbers of microconidia (2000/plate) gave the following numbers of colonies when assayed on the four media shown below:

Medium	Plates	Colonies	Colonies/Plate
ade + lys	5	81	16.2
ade	7	62	8.8
lys	10	48	4.8
minimal	10	1	0.1

These results demonstrate that this method effectively resolved this heterokaryon into its individual ade-requiring and lys-requiring components. The single colony obtained on the minimal plate was subsequently shown to be heterokaryotic in origin, based on analysis of it microconidia.

We have been routinely using microconidia to obtain homokaryons of strains transformed with exogenously applied DNA. Homokaryotic His+ transformants of His- strains were readily obtainable in a single step by selecting for the growth of His+ microconidia on unsupplemented media. Drug resistant transformants could be identified by subsequent screening of cultures grown nonselectively, or by plating microconidia directly on drug-supplemented plates. Microconidia derived from hygromycin B resistant strains transformed with several hph expression constructs readily formed colonies when germinated on plates containing hygromycin B. When plated on benomyl-containing medium (1 µg/ml), microconidia derived from a benomyl-resistant homokaryon germinated poorly (25% control value) and the colonies obtained grew slowly. Seventeen control colonies obtained by germination of the microconidia on drug-free plates were subsequently tested for benomyl resistance: all were benomyl resistant, as expected.

We find that IAA is essential for increased production of microconidia although the mechanism by which it acts is not known (Rossier et al. 1973). We have not examined whether IAA can be autoclaved in the media. When comparing SC concentrations we found 0.1 x SC to give more reproducible results than 0.5 x or 1.0 x SC. We have not tested production of microconidia on slants containing any additional supplements. Growth at 34°C appears to reduce the number of

microconidia produced. We have not tried lower growth temperatures, or tested whether the light/dark cycle is necessary for microconidial development.

Nylon 66 (Schleicher and Schuell) with 5 µm pore size can also be used for this procedure but we have more limited experience with it. We don't know precisely the features responsible for the differential filtration of conidia. The poor yield of microconidia suggests that the separation by these membranes may involve properties in addition to pore size. In general, the filtered microconidia germinate over a period of 1-3 days. However, it is prudent to wait for 3 days to be sure that when a colony is picked it is not contaminated with a late germinating microconidium. Microconidia germinate equally well at 34°C or 25°C.

CONCLUSION

We believe from our preliminary observations that this technique can be of immediate use to Neurospora workers. We have routinely used it successfully to isolate homokaryons from primary transformants and have verified that we have obtained homokaryons by Southern analyses. Using our procedure we can pick a colony from a transformation plate and in 10 days obtain a homokaryon using only one 150 mm slant, one filtration step, one plate, and very little of the investigator's time.

ACKNOWLEDGEMENTS

We thank Charles Yanofsky and David Perkins for advice and use of equipment. We wish to acknowledge the advice and technical assistance of Brian White. D. Ebbole and M. Sachs contributed equally to this work.