Fungal Genetics Reports

Volume 38 Article 7

Filtration-enrichment methods for selecting auxotrophs and other mutants in Ascobolus immersus and similar filamentous fungi.

B. C. LAMB
Imperial College of Science

S. HELMI
University of Alexandria

Recommended Citation

LAMB, B. C., and S. HELMI (1991) "Filtration-enrichment methods for selecting auxotrophs and other mutants in Ascobolus immersus and similar filamentous fungi.," *Fungal Genetics Reports*: Vol. 38, Article 7. https://doi.org/10.4148/1941-4765.1453

Filtration-enrichment methods for selecting auxotrophs and other mutants in Ascobolus immersus and similar filamentous fungi.

Abstract

Filtration-enrichment is a very successful method for obtaining auxotrophs, but traditional methods cannot be applied directly to many fungi lacking suitable asexual spores, mainly because of a heavy carry-over of parental nutrients into sexual spores. A procedure has been devised to apply filtration-enrichment to such fungi by using hyphal mutagenesis, crossing, germination of sexual spores, fragmentation of germination hyphae into small propagules, and then two cycles of filtration-enrichment in liquid minimal medium (LMM).

Filtration-enrichment methods for selecting auxotrophs and other mutants in *Ascobolus immersus* and similar filamentous fungi.

B.C. Lamb and S. Helmi(2)- Department of Biology, Imperial College of Science, Technology and Medicine, London SW7 2BB, United Kingdom. (2)Current address: Dept. of Environmental Studies, Institute of Graduate Studies and Research, University of Alexandria, Egypt. Filtration-enrichment is a very successful method for obtaining auxotrophs, but traditional methods cannot be applied directly to many fungi lacking suitable asexual spores, mainly because of a heavy carry-over of parental nutrients into sexual spores. A procedure has been devised to apply filtration-enrichment to such fungi by using hyphal mutagenesis, crossing, germination of sexual spores, fragmentation of germination hyphae into small propagules, and then two cycles of filtration-enrichment in liquid minimal medium (LMM). It has been optimized in *Ascobolus immersus*, Pasadena strains, for mutagenesis, hyphal fragmentation and recovery, filter pore size, duration and number of growth periods, and final isolation of colonies. Many variations on these factors were tried. The optimum conditions described here gave yields of about 30% of auxotrophs. The method is easily applicable to other fungi, such as Sordaria, whether homothallic of heterothallic. It could also be used in fungi where the asexual spores are large, with a heavy carry-over of parental nutrients, as in Cochliobolus, and in those Fusarium species which have sexual reproduction.

The ascospores of *A. immersus* are about 55 um x 33 um and so cannot be used for direct selection in LMM as the massive carry-over of parental nutrients allows a mutant (e.g. lysine-requiring) ascospore to produce hyphae growing in unsupplemented LMM almost as fast as wild-type ones, giving dense colonies up to 20 mm diameter. Lamb (1982 Micr. Gen. Bull. 52:2-4) suggested reducing this carry-over by germinating the ascospores, then fragmenting the hyphae in a blender into lengths of 1-3 coenocytic cells, when the terminal septa will usually seal in the contents, and the fragments can regenerate hyphae and colonies. The short healed fragments can then be used as if they were germinated conidia in selective methods, with a nutrient carry-over about two orders of magnitude less than in ascospores. Mutagenizing the hyphae used in a cross allows segregation-delay to occur before meiosis, so that each haploid sexual spore will either be wholly wild-type, or wholly mutant, even if not uninucleate. The present methods are based on those of Lamb (1982, loc. cit.), but with various practical improvements which more than tripled the yield of auxotrophs.

Strains, media and general methods were as described by Yu-Sun (1966 Genetica 37:569-580) and Helmi and Lamb (1983 Genetics 104:23-40). Crosses were made in the light at 17.5 C and growth was at about 24-26 C. Nylon filters of defined pore size were monofilament bolting cloth (Henry Simon Ltd., Stockport, UK). For mutagenesis and crossing, three squares of cellophane, 2.5 cm square, were spread on crossing medium in 9 cm diameter petri dishes. A small inoculum of (-) mating type was placed centrally on each square and allowed to grow for two days, then the original agar inoculum block was removed, leaving a thin layer of fully exposed hyphae. For chemical mutagenesis, the cellophane (plus hyphae) was removed and immersed in mutagen

solution, then washed several times in LMM. For UV mutagenesis, the hyphae were kept in the dark for four hours after irradiation. At the same time as (-) inoculation, a (+) mating type strain was inoculated at one edge of petri dishes of crossing medium. After mutagenesis, the cellophane squares with treated (-) hyphae were placed, hyphae downwards, on the opposite side of the dishes to the non-mutagenized (+) inoculum. The cellophane was removed about 4 hours later, leaving the (-) hyphae on the agar. These crosses were left at 17.5 C until ready. The efficiency of mutation was scored from the frequency of 4w+(red ascospores):4w (white ascospores) segregations in dehisced octads of ascospores in w+ x w+ crosses. The optimum mutagenic treatments to use on one strain in a cross were: UV, 240 secs at 800 ergs/sec/c2; ICR-170, 10 ug/ml for 2700 secs, NMG, 50 ug/ml for 600 secs. These gave, respectively, 4.9, 4.9 and 2.2% of 4w+:4w octads from samples of about 12,000 asci, compared with spontaneous frequencies of about 0.1%.

For filtration-enrichment, dehisced ascospores were separated from each other with 5 ml of 0.1% w/v pronase solution per collecting lid for 30 min. Red ascospores from w+(+) x w+(-) crosses were transferred to 75 ml liquid medium in a 500 ml flask. They were heat-shocked for 2 hours at 50 C, then left at 37 C overnight. White ascospores from w1-(+) x w1-(-) crosses just incubated overnight at 37 C in 75 ml distilled water. At least 2000 spores were used per flask. 125 ml water were added to the flasks of germinated spores to cover the macerator probe blades fully, before maceration at full speed in a blender for 60 secs. Samples were examined with a microscope to check whether most fragments were 1-3 cells long, with further maceration if necessary.

The suspension was filtered through a 305 um pore filter to remove most large fragments; some smaller fragments were also lost by trapping. The fragments in the filtrate were collected on a 0.8 um pore membrane filter, then were washed thoroughly with LMM to remove unwanted nutrients, before resuspending the fragments in 150 ml LMM in 500 ml flasks. The flasks were put on shakers and incubated for about 7 hours before filtering through a 305 æm filter to remove prototrophs, followed by a further cycle of 7 hours incubation and a final 305 um filtration. Microscopic examination was used at each stage, to monitor fragment lengths and concentrations, and reconstruction experiments using a known lysine-requiring mutation provided an additional check on the methods. 305 um was the best pore size for removing fragments larger than 1-3 cells, though a few larger fragments sometimes penetrated, end-on. The losses of different sized fragments varied with conditions, especially whether suction was applied during filtering. Typical figures for losses (as percentages of fragments of stated cell number trapped by the filter) at one 305 æm filtration were: 1-3 celled fragments, 71% loss; >3 cells, unbranched, 98%; >3 cells, branched, 100%. Monitoring of fragments showed gains in numbers of small fragments from mechanical fragmentation of hyphae during shaking to resuspend fragments after collection, and considerable losses of fragments due to fusion (or firm intertwining) of fragments, in spite of shaking during the two growth periods.

In Neurospora, Strauss (1958 J. Gen. Microbiol. 18:658-669) found that many auxotrophs died after 2 days in LMM, but our reconstruction experiments with a mixture of wild-type and lysine-requiring fragments showed almost 100% survival of both types of fragment if left unfiltered in LMM for 6 days. Those results are given in Table 1. They also show the expected rise in percentage of auxotrophs after the first filtration. The absolute number of (non-growing)

auxotrophs per flask only declined slightly after the first filtration in the 2000 um pores experiment (the 5 day result is odd) but declined much more on filtering through 305 um pores, consistent with some loss of auxotrophs by trapping in the smaller pores. The persistence of and increase in numbers of prototrophs, even after several rounds of filtration, shows fragmentation of grown prototrophic colonies during filtering, some fragments being small enough to pass through. This increase in number of prototrophic colonies did not occur in the unfiltered control flasks. The 24 hour period between filtrations in the reconstruction experiment was poorer for auxotroph isolation than the shorter periods used in other experiments.

Of six methods tried (see Table 2, legend) for isolating the auxotrophs after the second filtration in our main experiments, the two giving best yields were those involving a further selection stage on solid minimal medium (SMM). The best method was to spread 1 ml filtrate on a plate of SMM, then after 24 hours all small colonies, but not big ones, were isolated under a dissecting microscope into individual 75 mm tubes of solid complete medium. This gave the highest proportion of isolates with slow growth on SMM (40%) and the highest proportion of auxotrophs (about 30% of total fragments isolated). Auxotrophs included requirements for: uracil, riboflavin, inositol, aspartic acid, lysine, phenylalanine, methionine, cysteine, tryptophan, leucine, threonine, and a number of double, complex or unidentified requirements. These types are typical of previous ones obtained non-selectively in Ascobolus by Yu-Sun (1964 Genetics 50:987-997). The method also yields morphological mutants, especially ones with restricted growth. They included: dense, compact colonies, profusely branched mycelium, clock, wave, slow growth, sparse growth.

Table 1. Reconstruction experiments using a white-mycelium auxotroph, *lys-31*, and a yellow-mycelium prototroph, *yel-7*, in liquid minimal medium in multi-replicated flasks, plating out the whole flask contents at termination, to study survivability of auxotrophs, with one filtration a day.

Incubation time until termination (days)	No. of filtrations per flask	Viable colonies per flask	Auxotrophs per flask	White auxotrophs *%
	(i) Using 305 um pore filters			
0	0	105	21	20
1	1	48	15	31
2	2	67	11	16
3	3	93	16	17
4	4	115	10	9
5	5	142	5	4
6	6	218	2	1
Unfiltered control:				
6	0	91	17	19
	(ii) Using 2000 um pore filters			
0	0	231	99	43
1	1	156	111	71
2	2	143	98	69
3	3	105	45	43
4	4	97	50	52
5	5	12	3	25
6	6	259	43	17
Unfiltered control:				

6	0	225	104	46

* All auxotrophs were white; all prototrophs were yellow

Table 2. Comparison of methods for final isolation of auxotrophs after two cycles of filtration enrichment, from the cross 1w78,2+ x ECw78,7- (both strains have white ascospores)

Method number*	Total fragments isolated	Isolates with slow growth on minimal medium,%	Auxotrophs obtained %
1	125	22.4	9.6
2	20	40.0	30.0
3	60	13.3	5.0
4	49	22.4	14.3
5	77	40.3	31.0

* 1: 1 ml of filtrate plated on SCM. After 5 hours, fragments were checked microscopically for growth, with growing hyphae being isolated into individual tubes of SCM.
2: 1 ml of filtrate plated on SMM. After 24 hours, all small colonies, but not large ones, were individually isolated.
3: 2 ml of filtrate were mixed in petri dishes with 23 ml molten SCM (with 20 g/l agar) at 48 C. After 12 or more hours, growing colonies were isolated into separate small tubes of SCM.
4: Fragments were collected on a 0.8 um pore filter and resuspended in 250 ml liquid complete medium. After incubation with shaking for 36 hours, individual colonies were isolated with fine forceps into separate tubes of SCM.
5: As for 4, but with isolation of growing colonies onto SMM. Only colonies with little or no growth in these SMM tubes were then transferred to tubes of SCM.
6: From the filtrate, individual fragments were isolated with a fine pipette under a microscope. They were placed in separate tubes of SMM and any colonies with good growth after 48 hours were discarded. 1 ml of molten SCM, at 48 C, was added to each of the remaining tubes to stimulate growth of auxotrophs. Too few results were obtained to give meaningful data, but the auxotroph yield was low.

Acknowledgements:
We are grateful to previous students in this lab who contributed to this work, including Dr. A. Ghikas, M. Sladdin, Miss B. Davies, Miss F. Williams, Dr. W.M. Howell, Miss S. Rutherford, Miss B. Gardiner, Dr. S.A. Zwolinski and Miss J.A. Goodeve.