Note
A Simple Method for Enrichment of Uninucleate Conidia of Aspergillus oryzae

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Aspergillus oryzae produces multinucleate conidia, which makes the obtaining of homokaryons labor-intensive. Analysis of conidia by flow cytometry clarified the relationship that conidia of lower nuclear number were smaller in size. Based on this, we have developed a simple way to enrich uninucleate conidia with a membrane filter. Our results also suggest that the method is useful for elimination of heterokaryons.

Key words: Aspergillus oryzae; uninucleate conidia; homokaryon; flow cytometry; membrane filtration

Aspergillus oryzae is an important filamentous fungus for fermentation industries. It has been improved by induction of mutation\(^1,2\) and manipulated by transformation.\(^3\),\(^4\) A. oryzae produces multinucleate conidia,\(^5,6\) which makes purification of homokaryotic strains time-consuming and labor-intensive. For example, the strain reproduced just after transformation is heterokaryotic, and untransformed nuclei can coexist with transformed ones even in a conidium. To obtain homokaryotic transformants, which are stable, we therefore have to repeat the isolation of conidia across the generations until all reproduced colonies become a uniformly transformed type. On the other hand, only a single isolation of conidia is sufficient to obtain homokaryons of Aspergillus nidulans because this fungus produces uninucleate conidia exclusively.\(^7\) If we can enrich uninucleate conidia of A. oryzae, the probability of obtaining homokaryons will increase.

First of all, the nuclear number in conidia of A. oryzae was analyzed by flow cytometry as follows. A. oryzae RIB40 (from the National Research Institute of Brewing) was grown on agar plates of malt extract (ME) medium, which consists of 2% malt extract (Difco), 0.1% Bacto-peptone (Difco), 2% glucose, and 2% agar. After 5 days of cultivation at 30°C, conidia were collected with 0.85% NaCl containing 0.08% Tween 80 (Tweem saline). DNA in conidia was stained with fluorescent dye by the method described by De Lucas et al.\(^9\). Briefly, conidia were fixed with 70% ethanol, washed with Tween saline, incubated with 1 mg/ml RNase A, and stained with 25 μg/ml propidium iodide (PI). The treated conidia were put into a flow cytometer (FACScan, Becton Dickinson). The intensity of fluorescence (FL), which reflects DNA content, was measured on each of 10,000 or 20,000 conidium. A typical FL histogram is shown in Fig. 1(A). There were four major peaks at an interval of intensity. Assuming that the ratio of peak heights reflects the distribution of nuclear number in conidia, the distribution obtained by flow cytometry was similar to that obtained by fluorescence microscopy (Fig. 1(C)). These nuclear number distributions were also similar to those obtained previously by others with microscopic observation of a derivative of RIB40 with different methods for nuclear staining.\(^9\) The similarity in our results of flow cytometry and microscopy was also confirmed with other strains of A. oryzae though nuclear number distributions of some of these strains were very different from that of RIB40 (data not shown), suggesting the assumption is proper.

Conidial size was then related to nuclear number on a computer since we had measured the intensity of forward scattering (FSC), which reflects conidial size, besides FL on each conidium. Namely, regions were set in the FL histogram so that each would contain one of the major peaks (Fig. 1(A)) and FSC histogram was drawn of conidia within each region (Fig. 1(B)). It was found that conidia in a region of less FL intensity tended to have less FSC intensity, suggesting that conidia of lower nuclear number are small in size. Conidial size was furthermore estimated by flow cytometry with latex beads of different sizes as calibrators. Most of the uninucleate conidia were distributed from 4 to 7 μm with maximum frequency about 5 μm, while whole conidia were from 4 to 9 μm with a maximum at about 7 μm. Such a relationship between nuclear number and conidial size was also observed in conidia of other strains (data not shown). These results suggested the possibility that uninucleate conidia of A. oryzae were enriched by size fractionation.

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Abbreviations: FL, fluorescence; FSC, forward scattering; PI, propidium iodide
The analysis of conidia by flow cytometry showed that the conidia of A. oryzae RIB40 obtained from culture on ME plates were stained with PI as described in the text and put into a flow cytometer. (A) FL histogram. (B) FSC histograms of conidia in region 1 (thick line), region 2 (dotted line), and region 3 (solid line) in panel A. Region 4 histogram is removed to avoid complexity. (C) Nuclear number distribution of the same conidial sample observed with an Olympus BH-2 fluorescence microscope.

To investigate the possibility, we filtered conidia of A. oryzae RIB40 with a membrane filter as a size fractionation method. Based on the size of conidia described above, an Isopore membrane filter (Millipore) with 5-μm pore size was chosen. As shown in Fig. 2(A), the membrane filtration increased the frequency of uninucleate conidia in the pass-through fraction while it decreased that of multinucleate conidia. The enrichment of uninucleate conidia was also confirmed by fluorescence microscopy (data not shown). Uninucleate conidia of other strains were also enriched by the membrane filtration method; a result on A. oryzae ATCC22788, which produces a smaller percentage of uninucleate conidia than RIB40, is shown in Fig. 2(B). These results suggest that uninucleate conidia of A. oryzae are enriched by the membrane filtration method. The recovery of RIB40 conidia after the membrane filtration was about 0.1%, but this depended on the strain.

We subsequently investigated the possibility that heterokaryotic conidia, which are multinucleate, were eliminated by the membrane filtration method. For this purpose, a prototrophic fusion strain was made by the protoplast fusion method from two auxotrophs derived from RIB40: a niaD11 defective strain and a pyrG123 disrupted strain. Protoplast was prepared with Yatalase (Takara Shuzo), a cell-wall digesting enzyme. The fused prototrophic strain was maintained on agar plates of minimal medium (MM), which consists of 2% glucose, 0.3% NaNO3, 0.2% KCl, 0.1% KH2PO4, 0.05% MgSO4·7H2O, 0.002% FeSO4·7H2O, and 2% agar, pH 5.5. The strain produces multinucleate conidia of both heterokaryons and homokaryons, and uninucleate conidia. Among them, only heterokaryotic mul-
The heterokaryons and membrane colonies were examined.

We plated uninucleate conidia to assess the effect of the membrane filtration. To evaluate the percentage of the prototrophic conidia before and after the membrane filtration of conidia of the fusion strain by spreading equal numbers of conidia on plates of MM and ME (as a complete medium). As a result, 11% (61 colonies on MM plates to 552 colonies on ME plates) were prototrophs before the membrane filtration while 0.5% (4 to 771) were prototrophs after the filtration. Three of the four prototrophic colonies after the membrane filtration were, however, found to be heterozygous diploids by flow cytometry; their conidia gave only two FL peaks corresponding to nuclear numbers two and four. Such a change in FL has been observed with diploids of A. fumigatus and A. nidulans. The one prototrophic colony remaining was a heterokaryon. Thus our result suggests that heterokaryotic conidia are eliminated by the membrane filtration method.

We furthermore investigated the possibility that heterokaryotic conidia of a still unpurified transformant of A. oryzae were decreased by the membrane filtration method. For this purpose, a niaD defective derivative of A. oryzae IFO4206 was cotransformed with the niaD gene and a laccase cDNA of Schizopyllum commune. The ratio of uninucleate conidia of the host strain was about 2% estimated by flow cytometry, which ratio was much lower than that of uninucleate conidia of RIB40. We chose one transformant that consisted of three types of nuclei with regard to the copy number of the laccase cDNA integrated into chromosome. The copy numbers of the laccase cDNA were zero, one, and two estimated by Southern blot analysis and the laccase plate assay (data not shown). In the following experiment, we examined only the laccase activity for the estimation of copy number of the laccase cDNA because the level of laccase activity depended on the copy number of the laccase cDNA. The heterogeneity in the copy number of the niaD gene integrated into chromosome was not examined. The chosen transformant was grown on ME plates and its conidia were collected and put through the membrane filtration. The filtration step was omitted in the control experiment.

We then estimated the heterogeneity of each of 30 conidia to assess the effect of the membrane filtration. That is, 50 to 100 colonies obtained from each conidium were grown and their laccase activity was examined. In this assay, a conidium was regarded as a heterokaryon if at least one out of the 50 to 100 colonies showed a different level of laccase activity. As results of the experiments with and without the membrane filtration step, 43% (13 conidia out of 30) and 70% (21 conidia out of 30) were estimated to be heterokaryons, respectively. The results suggest that heterokaryotic conidia of still unpurified transformants are decreased by the membrane filtration method.

In this paper we described a simple method for enrichment of uninucleate conidia of A. oryzae. Though the method does not completely remove multinucleate conidia, it increases the probability of obtaining homokaryons. Similarly, uninucleate conidia should be enriched with a flow cytometer equipped with a cell sorter by adjusting the range of FSC intensity (but not FL intensity, since fluorescent dye staining of nuclei kills conidia) for collection. Maruyama et al. sorted uninucleate conidia of a recombinant A. oryzae with a flow cytometer by monitoring their FL intensity, with the nuclei fluorescently labeled by the expression of a histone-GFP fusion protein. Though the threshold of collecting size is fixed in our membrane filtration method, it is simple and requires no high-cost equipment or maintenance. For the selection of a membrane, uniformity of pore size and pore shape seems to be important since we could not enrich uninucleate conidia at all with a membrane filter of varied pore size and pore shape (data not shown).

Our membrane filtration method should also be useful for obtaining mutants of A. oryzae efficiently. When mutation is induced against conidia, the multinucleate nature of A. oryzae conidia causes the following problem. A recessive mutation occurring in a nucleus of a multinucleate conidium is phenotypically masked by some intact coexisting nuclei, and is not detected until the mutant nuclei are purified to homokaryons. Use of the membrane filtration method before the induction of mutations should increase the probability of obtaining mutants without isolation steps of homokaryotic colonies.

By the way, it is notable that the ratio of uninucleate conidia was changed by the medium. For example, 30% of conidia were uninucleate after cultivation of RIB40 on MM plates but 11% of conidia were uninucleate after cultivation of the same strain on agar plates of Sabouraud dextrose (Difco) estimated by flow cytometry (data not shown), suggesting another approach to enrich uninucleate conidia.

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