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Note

Rapid preparation of PCR-amplifiable fungal DNA by benzyl bromide extraction

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For isolation of fungal DNA for PCR amplification, we compared three DNA isolation methods: enzymatic cleavage and the use of benzyl chloride or benzyl bromide. Since benzyl bromide is more reactive, its use enabled us to readily isolate the total nucleic acids as a DNA template source from various fungi, including dematiaceous hyphomycetes, for RAPD analysis.

Key Words——benzyl bromide; DNA preparation; fungal DNA; PCR; RAPD.

In the past, genetic studies on fungi have required a rapid method for isolating DNA from many samples in sufficient amounts to permit the analysis of restriction fragment length polymorphisms (RFLP). However, with the development of the polymerase chain reaction (PCR) method, such large amounts of DNA have now become unnecessary. At present, random amplified polymorphic DNA (RAPD) (Goodwin and Annis, 1991) and arbitrarily primed PCR (AP-PCR) (Williams et al., 1990) analyses are widely being used for genome mapping, strain identification, and population studies (Welsh et al., 1991; Fujimori and Okuda, 1994; Lehmann et al., 1992; Okuda et al., 1995). However, the difficult task of preparing DNA from fungi possessing firm cell walls, especially the dematiaceous fungi, must be accomplished first before any PCR method can be applied. Although a number of methods have been published for preparing fungal DNA, such as those requiring freezing with liquid nitrogen (Biel and Parrish, 1986; Omori et al., 1990), extraction with phenol-chloroform, or gradient sedimentation with cesium chloride (Hudspeth et al., 1980; Yoon et al., 1991), all require a lot of time and large samples, and are inappropriate for some fungal taxa. Recently, a method involving extraction with benzyl chloride was reported as a simple, fast procedure for the isolation of genomic DNA (Zhu et al., 1993). Even though benzyl chloride readily destroys the cell walls of several fungi, it was not applicable to some dematiaceous fungi. To solve this problem, we decided to use benzyl bromide for the extraction since it reacts with hydroxyl residues to a greater extent than benzyl chloride does. We expected to achieve a simple separation of impurities, such as polysaccharides, proteins, and cellulose, from the aqueous phase containing the fungal DNA.

Total nucleic acids extraction: A liquid medium, PYG (2% polypeptone, 1% yeast extract and 2% glucose) was used to cultivate fungi. A loop full of conidia was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of this medium, which was then incubated on a rotary shaker at 27°C at 220 rpm for 3 days. This base culture was then subjected to one of the following three methods for the preparation of total nucleic acids (including both DNA and RNA).

Method 1 Two hundred mg of wet mycelium grown in the PYG medium was washed three times in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), then the mycelium was suspended in 350 μl of the same TE buffer containing 0.5 mg of chitinase and 300 to 400 mg of glass beads (0.4 to 0.5 mm in diam). The suspension was agitated for 1 min and incubated at 37°C for 1 h. Then 100 μl of proteinase K solution (1 mg/ml) was added, and the suspension was heated at 55°C for 1 h, treated with phenol-chloroform, precipitated with ethanol and the pellet (total nucleic acids) was dissolved in TE buffer (Fujimori and Okuda, 1994).

Method 2 The washed mycelium as described above (method 1) was suspended in 500 μl of extraction buffer consisting of 100 mM Tris-HCl, pH 9.0, and 40 mM EDTA with the addition of 100 μl of 10% SDS and 600 μl of benzyl chloride, as slightly modified from the method of Zhu et al. (1993). The suspension was heated at 50°C for 30 min, prior to the addition of 300 μl of 3 M sodium acetate, then cooled at 0°C for 15 min. It was then centrifuged at 12,000 rpm for 10 min, precipitated with isopropanol and the pellet was dissolved in TE buffer.

Method 3 The benzyl chloride in method 2 was replaced with benzyl bromide. The pellet (total nucleic acids) was dissolved in TE buffer.

PCR amplification: The procedure for PCR was as

Table 1. Total nucleic acids yields obtained from different fungi.

Fungus/strain	1[a]	2	3
Absidia cylindrospora CBS 100.37	2.9[b],[c]	20.1[c]	34.1
Acremonium fusioides NR 6849	13.3	10.4	11.8
Alternaria alternata IFO 4024	6.3[c]	31.3	36.9
Aspergillus niger IAM 3001	4.4[c]	5.9	1.4
Aspergillus versicolor NR 6603	4.3[c]	68.0	46.5
Aureobasidium pullulans IFO 5060	57.0	25.9	29.6
Beauveria bassiana NR 5756	19.7[c]	45.7[c]	53.2
Candida albicans NR 6236	44.6	71.0[c]	83.4[c]
Chaetomium sp. NR 6877	20.9	44.1	33.0
Chrysosporium asperatum IFO 7582	6.5	2.3	1.8
Cladosporium cladosporioides NR 6305	7.3[c]	48.9	49.2
Curvularia lunata NR 5681	4.4	1.2[c]	0.5
Eupenicillium javanicum NR 6495	3.4	53.0	47.9
Fusarium oxysporum NR 7145	12.1	4.0	3.0
Gliomastix murorum NR 5635	19.7	29.6	23.1
Kluyveromyces fragilis ATCC 8635	118.3	62.4	61.9
Mucor hiemalis IFO 8448	14.3	7.6	6.7
Myrothecium verrucaria NR 5611	19.7	21.9	18.1
Neosartorya fischeri NRRL 4161	13.9	53.2	26.6
Paecilomyces inflatus IFO 3196	19.7	27.7	30.6
Penicillium funiculosum NR 6620	2.8	1.8	2.1
Penicillium sclerotiorum NR 6440	7.3	47.2	32.5
Phialomyces sp. NR 6649	9.9	19.3	17.1
Scopulariopsis brevicaulis IFO 5841	4.2[c]	3.8	2.9
Talaromyces flavus FRR 1265	17.5	18.5	9.4
Trichoderma harzianum ATCC 18647	20.9	73.5	72.2
Verticillium albo-atrum IFO 5922	20.9	57.9	58.3

a) These numbers indicated the methods in this paper.
b) Isolated total nucleic acids (μg) from each 200 mg mycelia.
c) RAPD bands were not observed.
Strains with a prefix of NR were isolated in our laboratory. Some reference strains were purchased from American Type Culture Collection, Rockville, U.S.A. (ATCC); Centraalbureau voor Schimmelcultures, Baarn, the Netherlands (CBS); Division of Food Research, CSIRO, North Ryde, N.S.W., Australia (FRR); Institute for Fermentation, Osaka, Japan (IFO); Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan (IAM); Culture Collection Unit, Fermentation Section, Northern Utilization Research Branch, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. (NRRL).

reported by Fujimori and Okuda (1994). For the template source, the absorption of each sample at A_{260} was measured and the concentration was adjusted to 3.0. The primer used was R28 (5′-ATG GAT CCG C), reported by Goodwin and Annis (1991).

The three methods all facilitated the rapid isolation of total nucleic acids from several fungi as easily amplifiable DNA (Table 1). At first we tried to use RNase in each purification method, but when those samples were used as template DNA, some strains gave no RAPD bands. Thus, we used total nucleic acids comprising DNA and RNA as the template. The yields were estimated by absorption ($A_{260}=1$ represent 50 μg total nucleic acids). The amount of the total nucleic acids extracted with either benzyl chloride or benzyl bromide was generally higher than that obtained by the enzyme treatment method: of the 27 strains tested, this was seen in 16. For a few strains, methods 1 and 2 did not generate total nucleic acids of a high enough quality to be used as a DNA template source (Table 1). For example, in dematiaceous fungi such as *Cladosporium cladosporioides* NR 6305 and *Curvularia lunata* NR 5681, no RAPD band pattern could be obtained from samples prepared by methods 1 or 2. Also no RAPD bands of *Beauveria bassiana* NR 5756 could be amplified from samples obtained by methods 1 or 2 (Fig. 1, A). However, a number of RAPD bands from these three genera were successfully amplified from the total nucleic acids prepared as DNA template by method 3. Preparation of DNA template from *Aspergillus niger* IAM 3001, *Aspergillus versicolor* NR 6603, and *Eupenicillium javanicum* NR 6495 was not successful by enzymatic cleavage (Fig.

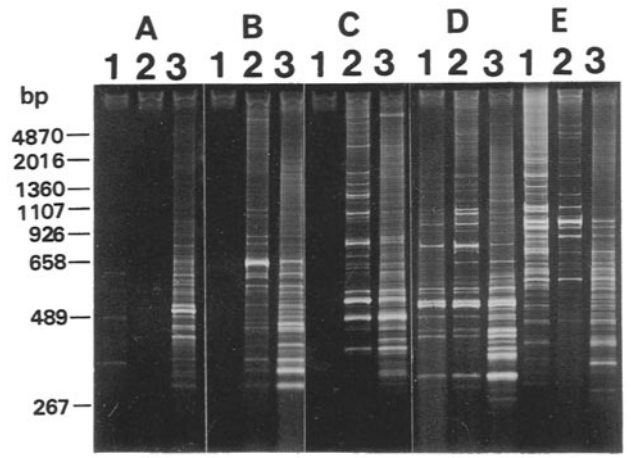

Fig. 1. Comparison of three types of total nucleic acids preparation methods for obtaining RAPD patterns.
PCR program: 30 cycles of denaturing at 92°C for 45 sec, annealing at 34°C for 60 sec, and extension at 72°C for 90 sec. A, *Beauveria bassiana* NR 5756; B, *Aspergillus niger* IAM 3001; C, *Eupenicillium javanicum* NR 6495; D, *Trichoderma harzianum* ATCC 18647; E, *Fusarium oxysporum* NR 7145; 1, method 1 (enzyme treatment); 2, method 2 (benzyl chloride); and 3, method 3 (benzyl bromide).

1, B, C), whereas extraction of total nucleic acids with benzyl chloride or benzyl bromide yielded RAPD bands. We succeeded in isolating PCR amplifiable fungal DNA by benzyl bromide extraction from all strains except *Candida albicans.* Although a high yield of total nucleic acids was obtained from *C. albicans* NR 6236 (Table 1), the benzyl chloride and benzyl bromide extractions did not yield RAPD bands. Moreover, many more polymorphic DNA bands were visualized by benzyl bromide than by benzyl chloride extractions (Fig. 1).

We also compared the amplification patterns derived from these three methods. Although most band patterns were identical or at least similar, we did obtain some different amplification patterns (Fig. 1, D, E). By each method, however, reproducible fingerprint patterns were apparently achieved. Although the reasons for the differences in the amplification band patterns of the differently isolated DNA template sources are unknown, it may be argued that different purification methods resulted in different proportions of genomic DNA in the total nucleic acids solution (e.g., mt DNA, rDNA, RNA, etc.).

Our results unambiguously demonstrated that the outcome of RAPD band patterns may depend on how the parameters are controlled. We have previously established a standard method for RAPD by controlling the parameters (template concentration, reaction cycles, and different thermostable DNA polymerases) (Fujimori and Okuda, 1994). If the same set of conditions, including those for the DNA preparation procedure, is applied, the same patterns of PCR products will be generated.

We conclude that the extraction of total nucleic acids as fungal template DNA for the RAPD with benzyl bromide is rapid, reliable, and useful. This method is especially useful for isolating total nucleic acids from the dematiaceous hyphomycetes. When such extracts were used for PCR amplification, it was possible to visualize more RAPD band patterns than could be seen by other extraction methods. Thus, very minor differences between similar strains could be distinguished.

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