

Rapid and Effective DNA Extraction Method with Bead Grinding for a Large Amount of Fungal DNA

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ABSTRACT

To identify a rapid method for extracting a large amount of DNA from fungi associated with food hygiene, extraction methods were compared using fungal pellets formed rapidly in liquid media. Combinations of physical and chemical methods or commercial kits were evaluated with 3 species of yeast, 10 species of ascomycetous molds, and 4 species of zygomycetous molds. Bead grinding was the physical method, followed by chemical methods involving sodium dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), and benzyl chloride and two commercial kits. Quantity was calculated by UV absorbance at 260 nm, quality was determined by the ratio of UV absorbance at 260 and 280 nm, and gene amplifications and electrophoresis profiles of whole genomes were analyzed. Bead grinding with the SDS method was the most effective for DNA extraction for yeasts and ascomycetous molds, and bead grinding with the CTAB method was most effective with zygomycetous molds. For both groups of molds, bead grinding with the CTAB method was the best approach for DNA extraction. Because this combination also is relatively effective for yeasts, it can be used to extract a large amount of DNA from a wide range of fungi. The DNA extraction methods are useful for developing gene indexes to identify fungi with molecular techniques, such as DNA fingerprinting.

Fungi play an important role in food spoilage, although their use in food fermentation is well established. A large number of incidents of food spoilage by fungi (24), contamination of food by toxigenic fungi (1, 17, 18), and food poisoning associated with mycotoxins have been reported (1). Therefore, easy and rapid methods to identify fungal species are required. Although morphological observation is the dominant method of identifying fungi, various culture methods are used. The subjective judgment of specific characteristics also complicates identification. Recently, molecular biology techniques have attracted interest because of their ease, rapidity, and objectivity. PCR-based assays and analyses of DNA sequences are used extensively to identify fungi in food or to detect toxin genes in fungi. Some researchers have reported that molecular methods can be used to identify strains that are difficult to identify morphologically (16, 22).

Genetic experiments require nucleic acids, including genomic DNA extracted from cells. It is difficult to extract DNA from fungal cells, especially molds, because fungi contain many nucleases, polysaccharides, and pigments. Therefore, many methods of DNA extraction have been developed for fungi (3, 5, 9, 26, 27, 29, 33) and compared for quantity and quality (2, 6, 8, 11, 15, 20). These methods have been used with many species of yeasts but only a few species of molds (2, 5, 6, 8, 11, 20, 33) or have been used with several species of only medically important fungi (7, 21). To

develop new tools for identifying fungal species, it is also necessary to search for genes or nucleotide sequences specific to species or groups and to understand the structure of the genome by conducting experiments that include sequencing of the whole genome, DNA-DNA hybridization, and DNA fingerprinting. Because these experiments require a large amount of DNA, rapid and effective DNA extraction methods are required. Other researchers have extracted DNA from cell suspensions or excised mycelial pieces from colonies on agar media (2, 5, 6, 11, 20, 27). Under these conditions, significant time and effort with many agar plates are required to obtain a large amount of DNA because of the limited number of cells. Earlier and more efficient harvesting can be accomplished by inoculating a culture into liquid medium and collecting hyphae rather than spores as mold samples for DNA extraction. Guo et al. (10) compared two extraction methods and reported that those methods generated high DNA yields with freeze-dried mycelia of 25 species of molds; however, the DNA yields of only four species were greater than 1,000 µg/g. There is, therefore, a need for more rapid and effective extraction methods to obtain large amounts of DNA from a wide range of species, including various molds that commonly occur in foods.

The present study was conducted to determine whether the extraction methods could yield a high quantity of high-quality DNA from fungal cells by culturing in liquid medium. Species for DNA extraction were selected from a wide range of fungi based on their importance in the field of food hygiene.

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TABLE 1. *Fungal strains*

Fungus type	Division	Species	Strain ^a
Yeasts		<i>Cryptococcus neoformans</i>	NBRC 0699
		<i>Rhodotorula rubra</i>	NBRC 0909
		<i>Saccharomyces cerevisiae</i>	NBRC 0233
Molds	Ascomycota	<i>Alternaria alternata</i>	NBRC 31188
		<i>Aspergillus niger</i>	NBRC 6661
		<i>Botrytis cinerea</i>	NBRC 31831
		<i>Byssoschlamys fulva</i>	NBRC 31877
		<i>Emericella nidulans</i>	NBRC 33017
		<i>Fusarium subglutinans</i>	IFM 50097
		<i>Geotrichum candidum</i>	NBRC 9541
		<i>Penicillium expansum</i>	NBRC 7604
		<i>Talaromyces bacillisporus</i>	NBRC 31150
		<i>Trichoderma viride</i>	NBRC 100100
	Zygomycota	<i>Absidia corymbifera</i>	NBRC 4009
		<i>Mucor hiemalis</i>	NBRC 9405
		<i>Rhizopus stolonifer</i>	NBRC 4781
		<i>Syncephalastrum racemosum</i>	NBRC 4828

^a NBRC, National Institute of Technology and Evaluation Biological Resource Center; IFM, Institute of Food Microbiology (currently the Medical Mycology Research Center, Chiba University).

MATERIALS AND METHODS

Strains. The strains tested in this study are listed in Table 1: three yeasts (*Cryptococcus neoformans*, *Rhodotorula rubra*, and *Saccharomyces cerevisiae*), 10 ascomycetous molds (*Alternaria alternata*, *Aspergillus niger*, *Botrytis cinerea*, *Byssoschlamys fulva*, *Emericella nidulans*, *Fusarium subglutinans*, *Geotrichum candidum*, *Penicillium expansum*, *Talaromyces bacillisporus*, and *Trichoderma viride*), and four zygomycetous molds (*Absidia corymbifera*, *Mucor hiemalis*, *Rhizopus stolonifer*, and *Syncephalastrum racemosum*). Strains were provided by the National Institute of Technology and Evaluation Biological Resource Center (NBRC; Chiba, Japan) and the Medical Mycology Research Center (Chiba University, Chiba, Japan). These species occur frequently in the field of food hygiene (28) and cover a wide range of taxonomic groups based on both morphological and molecular data (12, 13).

Sample preparation. All strains were cultured on potato dextrose agar (Eiken, Tokyo, Japan) slants at 25°C for 7 days. Mycelia or conidia from the slant culture were inoculated into a 1-liter Erlenmeyer flask containing 400 ml of potato dextrose broth (Difco, Becton Dickinson, Sparks, MD). Fungi were grown at 25°C and shaken at 100 rpm. After 70 h, the cells in the planktonic cell suspension of *S. cerevisiae*, *C. neoformans*, *R. rubra*, or *G. candidum* were clumped by centrifugation at 6,000 × g for 10 min, and mycelial pellets of *A. alternata*, *A. niger*, *B. cinerea*, *E. nidulans*, *F. subglutinans*, *P. expansum*, *T. bacillisporus*, *T. viride*, *A. corymbifera*, *M. hiemalis*, *R. stolonifer*, or *S. racemosum* were wrapped in filter paper and squeezed to remove as much liquid as possible. Cells of both the planktonic cell suspension and mycelial pellets from *B. fulva* were pelleted using each method described above as samples. Each fungal pellet was divided into 100-mg samples, placed in 10 microtubes, and stored at -80°C until used.

DNA extraction. Combinations of physical and chemical DNA extraction methods or commercial kits were evaluated. Bead grinding was used as the physical method because it is frequently used in DNA extraction techniques for fungal spores (2, 5, 6, 11, 20). Bead grinding was performed using the Multi-Beads Shocker

(Yasui Kikai Co., Osaka, Japan) at 2,500 rpm for 1 min at 0°C with 0.5-mm zirconia beads. This procedure was followed by each of three chemical methods of cell lysis, the sodium dodecyl sulfate (SDS) method (29), the cetyl trimethyl ammonium bromide (CTAB) method (26), and the benzyl chloride method (33), or two widely used commercial kits (2, 14, 23, 25, 31): Dr. GenTLE (from Yeast) High Recovery (Takara Bio Inc., Otsu, Japan) and DNeasy Plant Kit (QIAGEN, Hilden, Germany).

The SDS method followed that described by Tapia-Tussell et al. (29) with minor modifications. SDS is an anionic surfactant. A 800-μl aliquot of lysing buffer was added to a tube containing 100 mg of the fungal pellet sample, and cells were resuspended by vortexing. After incubation at 65°C for 30 min, lysates were purified by mixing with phenol-chloroform. The suspensions were then centrifuged. The upper aqueous phase was transferred to a new tube, 15 μl of RNase A (10 mg/ml; Novagen, Darmstadt, Germany) was added, and tubes were incubated at 37°C. After 3 h, samples were purified by mixing with chloroform and then centrifuged. The upper aqueous phase contained the DNA, which was precipitated with chilled isopropanol, rinsed with 500 μl of 70% ethanol, dried under a vacuum, and resuspended in 100 μl of Tris-EDTA buffer.

The CTAB method followed that described by Saghai-Marooif et al. (26) with minor modifications. CTAB is a cationic surfactant. A 800-μl aliquot of CTAB buffer was added to a tube containing 100 mg of the fungal pellet sample and vortexed. Cell lysis, RNA digestion, and DNA purification were completed in the same way as for the SDS method.

The benzyl chloride method followed that described by Zhu et al. (33). Benzyl chloride can destroy the cell walls of fungi by reacting with polysaccharides and (similar to phenol) can extract proteins and other cell debris from the aqueous phase. A 500-μl aliquot of extraction buffer, 100 μl of 10% SDS, and 300 μl of benzyl chloride were added to a tube containing 100 mg of the fungal pellet sample and vortexed. The tube was incubated at 50°C for 30 min with shaking to mix thoroughly, 300 μl of 3 M NaOAc (pH 5.0) was added, and the tube was kept on ice for 15 min. After centrifugation, the upper aqueous phase was transferred to a new tube, 15 μl of RNase A was added, and the tube was incubated at 37°C for 3 h. DNA purification with chloroform and precipitation

with isopropanol were completed in the same way as for the SDS method.

The GenTLE kit was used according to the manufacturer's instructions with minor modifications. After salt precipitation of DNA, additional steps were performed. The precipitations were dissolved in 800 μ l of distilled water, 15 μ l of RNase A was added, and mixtures were incubated at 37°C for 3 h. DNA purification with chloroform and further precipitation with isopropanol were completed in the same way as for the SDS method.

The DNeasy Plant kit was used according to the manufacturer's instructions. All DNA extractions were performed in triplicate.

Quantity and quality of extracted DNA. The extracted DNA was evaluated spectrophotometrically to check quantity and quality using the NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Wilmington, DE). Quantity was calculated based on the absorbance at 260 nm (A_{260}). Quality was assessed by the ratio of UV absorbance at 260 and 280 nm (A_{260}/A_{280}). A ratio of A_{260}/A_{280} greater than 1.8 is generally considered an acceptable indicator of DNA purity. To test amplification ability and the absence of PCR inhibition in all DNA extracts in this study, a PCR assay was carried out with TaKaRa Ex Taq (Takara Bio Inc.). For DNA from yeast cells and ascomycetous molds, forward primer FF1 (5'-GTT AAA AAG CTC GTA GTT GAA C-3') and reverse primer FR1 (5'-CTC TCA ATC TGT CAA TCC TTA TT-3') were used (32). For DNA from zygomycetous molds, forward primer P1 (5'-ATC TGG TTG ATC CTG CCA-3') and reverse primer Fun-R1 (5'-TTG TTA CGA CTT TTA CTT CCT CT-3') were used (30). These primer pairs were designed to amplify specific fragments (of 630 and 1,730 bp, respectively) in two regions of the 18S rRNA gene, which is one of the most widely used genes in systematics studies based on molecular biological techniques. Amplification reactions were performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). PCR products were electrophoresed with PCR markers (Novagen, Madison, WI) as the molecular maker using a 1.5% (wt/vol) agarose gel (Agarose L03 Takara, Takara Bio Inc.) in Tris-borate-EDTA buffer. To check the amount of intact and degraded DNA in each extract, all extracts were electrophoresed with lambda DNA digested with HindIII marker (COSMO BIO Co., Ltd., Tokyo, Japan) as the molecular maker using a 1.0% (wt/vol) agarose gel. DNA fragments and PCR products were visualized by staining with ethidium bromide and UV transillumination.

Statistics. Significance tests among proportions of the number of species were performed by multiple comparisons with Fisher's exact test. These comparisons were considered significant with P values controlled at an error rate ($\alpha = 0.05$) with Bonferroni correction. Pairwise comparisons between mean quantities of DNA were performed with the Mann-Whitney U test.

RESULTS

Quantity of DNA extracted from fungi. The physical extraction method was combined with each of the three chemical extraction methods and the two commercial kits for a total of 10 combinations. DNA extracted from pellets of fungal cells using each of the 10 combinations were converted to the quantity of DNA per 1 g of fungal cells. The mean quantity of DNA was determined (with the standard deviation) for triplicate samples (Table 2). The mean amount of DNA extracted from ascomycetous and

zygomycetous molds ranged from 11.5 to 5,430.7 μ g/g of pellet. This finding supported the possibility of extracting DNA from all mycelial pellets cultured in liquid medium in this study. The mean DNA yield for all species in this study with the GenTLE kit or DNeasy Plant kit tended to be lower than that obtained using the three chemical methods: SDS, CTAB, and benzyl chloride.

The mean amount of DNA recovered using each combination in each taxonomic group of fungi was calculated and compared. For yeasts (Table 2), DNA yields from *S. cerevisiae* with combinations that included the SDS or CTAB method with or without bead grinding were 2,078.1 to 2,883.9 μ g/g, although DNA yields for *R. rubra* and *C. neoformans* with the same combinations were 98.4 to 860.4 μ g/g. DNA yields for three yeasts with the combination of bead grinding and benzyl chloride were 831.7 to 1,481.0 μ g/g, whereas DNA yields with benzyl chloride method were 195.2 to 453.2 μ g/g. For ascomycetous molds (Table 2), DNA yields for all species obtained with the combination of bead grinding and the SDS method were 1,660.8 to 5,430.7 μ g/g, higher than DNA yields obtained with the other nine combinations (12.5 to 3,695.8 μ g/g). For zygomycetous molds (Table 2), DNA yields for all species, excluding *R. stolonifer*, with combinations of the three chemical extraction methods with or without bead grinding were 1,174.3 to 4,934.0 μ g/g. DNA yields for *R. stolonifer* with these combinations were only 138.3 to 589.3 μ g/g.

Five extraction methods were compared without bead grinding to clarify the best method of DNA extraction applicable to a wide range of species in each taxonomic group. With 1,000 μ g/g defined as a large amount of DNA, the number of species from which more than 1,000 μ g/g was extracted was calculated for each method. Significance tests on the results were not performed for yeasts and zygomycetous molds separately because numbers for individual species were not sufficient to give appropriate test results. The five methods were thus compared based on their ability to extract a large amount of DNA from a wide range of mold species, including Ascomycota and Zygomycota. Of the 14 mold species tested, 10 (71.4%) yielded more than 1,000 μ g/g with the SDS method, 9 (64.3%) yielded this amount with the CTAB method, 8 (57.1%) yielded this amount with the benzyl chloride method, 1 (7.1%) yielded this amount with the GenTLE kit, and none yielded this amount with the DNeasy Plant kit. Multiple comparisons of these results with the Bonferroni correction provided the following result: SDS, CTAB, and benzyl chloride methods were significantly better than both the GenTLE and DNeasy Plant kits ($P < 0.01$). Results were not significantly different for the SDS, CTAB, and benzyl chloride methods.

The physical extraction method of bead grinding also was evaluated to determine whether it improved DNA extraction. Three chemical extraction methods (SDS, CTAB, and benzyl chloride methods) were used because they recovered more than 1,000 μ g/g from significantly more species. Pairwise comparisons between mean quantities of DNA obtained with each of the three chemical

TABLE 2. Yields of fungal DNA extracted by various methods

Fungus type	Division	Species	Mean ± SD DNA extracted (µg/g of fungal cells)															
			SDS method				CTAB method				Benzyl chloride method				Dr. GentLE (for Yeasts) High Recovery kit			
			Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding		
Yeasts		<i>Cryptococcus neoformans</i>	860.4 ± 358.9	412.0 ± 213.5	641.4 ± 298.3	175.2 ± 30.7	837.1 ± 247.6	195.9 ± 47.2	422.2 ± 324.7	1,34.5 ± 147.6	108.0 ± 11.5	38.0 ± 26.1						
		<i>Rhodotorula rubra</i>	285.9 ± 89.3	98.4 ± 72.3	423.8 ± 32.8	206.7 ± 58.7	1,481.0 ± 809.0	453.2 ± 318.0	366.9 ± 360.1	406.0 ± 209.5	299.7 ± 41.3	22.1 ± 11.8						
		<i>Saccharomyces cerevisiae</i>	2,602.7 ± 263.6	2,078.1 ± 286.0	2,883.9 ± 686.6	2,743.0 ± 381.2	1,220.7 ± 462.3	195.2 ± 41.7	810.2 ± 413.3	1,803.5 ± 366.2	318.8 ± 84.1	4.6 ± 1.4						
		Molds	Ascomycota	<i>Alternaria alternata</i>	2,780.7 ± 156.2	1,467.4 ± 313.0	2,554.4 ± 1,248.6	1,798.4 ± 279.5	2,100.6 ± 131.8	1,678.1 ± 595.4	809.6 ± 763.0	349.1 ± 116.4	199.8 ± 56.7	86.6 ± 18.0				
				<i>Aspergillus niger</i>	2,572.4 ± 1,494.8	769.4 ± 459.7	1,843.1 ± 1,221.5	266.6 ± 139.1	1,927.8 ± 468.2	381.4 ± 89.0	1,664.0 ± 508.5	196.9 ± 124.4	164.0 ± 118.0	30.8 ± 21.7				
Zygomycota		<i>Botrytis cinerea</i>	3,944.8 ± 1,210.9	2,742.0 ± 441.0	2,203.1 ± 606.6	1,347.4 ± 596.4	3,695.8 ± 1,198.0	2,340.2 ± 853.5	304.6 ± 34.0	280.0 ± 89.3	266.9 ± 168.5	192.2 ± 85.9						
		<i>Byssosclamyces fulva</i>	2,847.3 ± 621.4	2,119.2 ± 431.9	2,083.9 ± 586.4	1,730.1 ± 668.3	1,839.7 ± 347.4	990.4 ± 402.8	749.4 ± 35.2	438.1 ± 38.2	288.0 ± 68.7	133.4 ± 49.7						
		<i>Emeticella nidulans</i>	1,660.8 ± 203.2	2,340.4 ± 108.9	1,559.2 ± 298.8	1,935.4 ± 721.7	677.3 ± 26.3	2,355.0 ± 192.0	627.7 ± 77.4	135.2 ± 79.1	65.7 ± 22.1	105.4 ± 40.7						
		<i>Fusarium subglutinans</i>	2,226.8 ± 100.6	773.5 ± 121.0	1,193.4 ± 460.7	711.1 ± 371.9	1,171.5 ± 401.4	267.2 ± 163.3	82.0 ± 20.7	531.5 ± 40.2	162.3 ± 38.3	106.6 ± 47.2						
		<i>Geotrichum candidum</i>	1,660.8 ± 110.9	482.7 ± 134.6	752.5 ± 23.3	424.9 ± 117.9	745.1 ± 289.1	188.5 ± 16.3	593.8 ± 278.9	930.0 ± 60.9	37.9 ± 7.8	12.5 ± 6.3						
		<i>Penicillium expansum</i>	2,909.0 ± 621.6	1,356.6 ± 331.8	850.9 ± 431.2	502.7 ± 169.9	1,664.0 ± 932.7	903.2 ± 386.1	472.3 ± 224.9	543.7 ± 210.2	206.0 ± 123.3	140.0 ± 93.3						
		<i>Talaromyces bacillisporus</i>	3,426.1 ± 221.3	2,406.1 ± 485.1	3,273.9 ± 663.1	1,539.1 ± 68.1	3,451.6 ± 315.9	1,745.2 ± 586.0	730.2 ± 64.3	240.0 ± 40.5	367.4 ± 80.5	23.1 ± 9.7						
		<i>Trichoderma viride</i>	5,430.7 ± 202.9	3,022.3 ± 541.2	2,637.6 ± 1,227.4	1,050.7 ± 496.6	3,432.9 ± 1,039.8	2,230.4 ± 650.7	1,851.1 ± 191.9	1,116.5 ± 316.1	1,768.7 ± 702.2	562.4 ± 328.7						
		<i>Absidia corymbifera</i>	4,065.9 ± 1,437.4	2,569.6 ± 1,141.4	3,252.2 ± 310.9	3,556.0 ± 596.9	2,048.2 ± 420.6	1,292.0 ± 601.2	1,461.6 ± 262.0	951.8 ± 61.6	128.4 ± 21.6	44.1 ± 9.5						
		<i>Mucor hiemalis</i>	2,832.9 ± 360.1	1,562.7 ± 102.5	3,379.6 ± 256.0	2,757.6 ± 211.2	2,883.2 ± 231.7	1,174.3 ± 184.0	170.0 ± 44.1	158.4 ± 23.4	274.1 ± 63.3	42.4 ± 10.1						
<i>Rhizopus stolonifer</i>	248.9 ± 13.6	433.3 ± 260.5	589.3 ± 107.6	445.8 ± 103.6	281.2 ± 39.1	138.3 ± 54.8	73.3 ± 24.9	119.9 ± 18.1	26.3 ± 16.2	11.5 ± 5.5								
<i>Syncephalastrum racemosum</i>	4,943.0 ± 272.4	4,397.6 ± 373.7	4,493.6 ± 325.0	3,560.8 ± 498.5	3,690.5 ± 311.4	1,819.3 ± 770.8	1,190.0 ± 496.4	598.0 ± 256.8	101.3 ± 4.7	29.5 ± 13.0								

TABLE 3. Statistical comparison of DNA yields with or without bead grinding for three chemical extraction methods

Fungus type	Division	Species	DNA yield comparison ^a		
			SDS method	CTAB method	Benzyl chloride method
Yeasts		<i>Cryptococcus neoformans</i>	—	>	>
		<i>Rhodotorula rubra</i>	—	>	>
		<i>Saccharomyces cerevisiae</i>	—	—	>
Molds	Ascomycota	<i>Alternaria alternata</i>	>	—	—
		<i>Aspergillus niger</i>	—	>	>
		<i>Botrytis cinerea</i>	—	—	—
		<i>Byssoschlamys fulva</i>	—	—	>
		<i>Emericella nidulans</i>	<	—	<
		<i>Fusarium subglutinans</i>	>	—	>
		<i>Geotrichum candidum</i>	>	>	>
		<i>Penicillium expansum</i>	>	—	—
		<i>Talaromyces bacillisporus</i>	>	>	—
		<i>Trichoderma viride</i>	>	>	>
	Zygomycota	<i>Absidia corymbifera</i>	—	—	—
		<i>Mucor hiemalis</i>	>	>	>
		<i>Rhizopus stolonifer</i>	—	>	>
		<i>Syncephalastrum racemosum</i>	—	>	>
No. of species yielding significantly more DNA					
With bead grinding			7	9	11
Without bead grinding			1	0	1

^a —, yield of DNA with bead grinding was not significantly different from that obtained without grinding; >, yield of DNA with bead grinding was significantly higher than that obtained without grinding ($P < 0.05$); <, yield of DNA with bead grinding was significantly lower than that obtained without grinding ($P < 0.05$).

extraction methods with or without bead grinding indicated that mean quantities of DNA with bead grinding were higher overall than those obtained without bead grinding (Table 3). The mean quantity of DNA was significantly higher with bead grinding than without bead grinding for 7 (41.2%) of the 17 species with the SDS method, 9 species (52.9%) with the CTAB method, and 11 species (64.7%) with the benzyl chloride method. In contrast, the mean quantity of DNA was significantly lower with bead grinding than without bead grinding for 1 (5.9%) of the 17 species, *E. nidulans*, with both the SDS method and the benzyl chloride method. This result suggests that bead grinding has an interactive effect with chemical extraction methods for most fungal species.

Quality of extracted fungal DNA. Of the three chemical extraction methods and two commercial kits, only the SDS, CTAB, and benzyl chloride methods (not the GenTLE and DNeasy Plant kits) were evaluated for DNA quality because these methods recovered more than 1,000 $\mu\text{g/g}$ from significantly more species. The quality of DNA extracted was determined in six combinations of bead grinding and the three chemical extraction methods. The A_{260}/A_{280} ratios and the mean A_{260}/A_{280} ratio of three trials for each species and each of the six combinations were calculated. A mean A_{260}/A_{280} ratio lower than 1.8 was obtained for 1 (5.6%) of 18 combinations in yeasts (*R.*

rubra with the SDS method), 5 (8.3%) of 60 combinations in ascomycetous molds (*A. niger*, *B. fulva*, *F. subglutinans*, and *G. candidum* with the benzyl chloride method and *B. fulva* with the benzyl chloride method with bead grinding), and 1 (4.2%) of 24 combinations in zygomycetous molds (*S. racemosum* with the SDS method). Multiple comparisons among these percentages indicated no significant differences; thus, there was no difference among the three taxonomic groups in terms of the extraction of low-purity DNA.

DNA extracted with the SDS, CTAB, benzyl chloride methods with or without bead grinding was compared by electrophoresis in agarose gel. *S. cerevisiae*, *A. alternata*, and *M. hiemalis* were selected as representatives of yeast, ascomycetous molds, and zygomycetous molds, respectively (Fig. 1A). The DNA extracted by the SDS, CTAB, or benzyl chloride method from all species in this study contained a large amount of intact DNA, especially when these methods were used in combination with bead grinding. The electrophoresis profiles of the DNA extracted with the DNeasy Plant kit were similar to those for the DNA obtained with the SDS, CTAB, and benzyl chloride methods. In contrast, electrophoresis profiles of DNA extracted with the GenTLE kit revealed degraded DNA (data not shown). The DNA extracted in this study also was evaluated to determine its appropriateness for use as a PCR template, because PCR assays are used extensively for

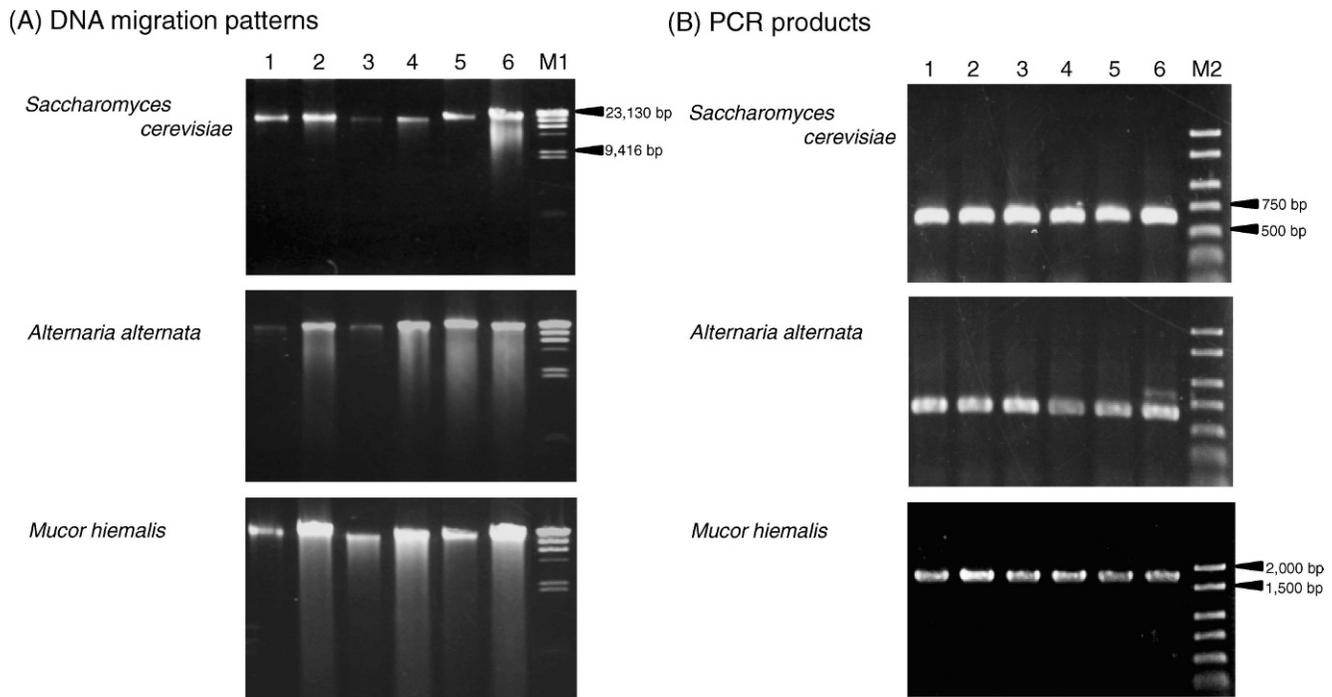


FIGURE 1. DNA migration patterns of extracted DNA from fungi (A) and the PCR products of the 18S rRNA gene (B). Lanes 1 and 2, SDS method without or with bead grinding, respectively; lanes 3 and 4, CTAB method without or with bead grinding, respectively; lanes 5 and 6, benzyl chloride method without or with bead grinding, respectively.

molecular biological experiments. PCR products whose template DNA was extracted by the SDS, CTAB, and benzyl chloride methods with or without bead grinding from *S. cerevisiae*, *A. alternata*, and *M. hiemalis* as representatives of yeast, ascomycetous molds, and zygomycetous molds, respectively, are shown in Figure 1B. The number of trials that successfully yielded PCR products from the 18S rRNA gene in triplicate were counted. In yeasts, gene amplifications were successful for all species and all trials for all combinations of DNA extraction methods. In all ascomycetous molds except *E. nidulans*, gene amplifications were successful for all species and all trials for all combinations of DNA extraction methods. The PCR for *E. nidulans* failed to amplify the gene in two extracts in triplicate obtained with the CTAB method with and without bead grinding. In zygomycetous molds, gene amplifications were successful for *M. hiemalis* and *R. stolonifer* in all trials for all combinations of DNA extraction methods. For *A. corymbifera*, the PCR failed to amplify the gene in one or two of three extracts obtained with the SDS method with or without bead grinding, in one of three extracts obtained with the CTAB method with bead grinding, and in one or all of three extracts obtained with the benzyl chloride method with or without bead grinding. In *R. stolonifer*, the PCR failed to amplify the gene in all of three extracts obtained with the SDS method with bead grinding and in all or one of three extracts obtained with the benzyl chloride method with or without bead grinding.

DISCUSSION

In developing DNA extraction methods for fungi, researchers have frequently considered rapid and effective

extraction methods for a large amount of DNA targeting yeasts and molds with conidia (2, 5, 11, 20). The DNA extraction method for mold mycelia is still not considered sufficient. Culture in liquid medium is better for harvesting many fungal cells more rapidly than culture on agar medium. Because most molds grow mycelia but not conidia in liquid medium, it is necessary to consider rapid methods for extracting a large amount of DNA from mycelial cells. Although Fredricks et al. (8) and Zhu et al. (33) independently reported comparisons of DNA extraction methods for mycelia of molds, only single species, *Aspergillus fumigates* and *Magnaporthe grisea*, respectively, were tested. Although Tapia-Tussell et al. (29) developed a DNA extraction method with mycelia of five mold species, the recovery rate per weight of mycelium was not evaluated, and the developed method was not compared to other methods. Although Guo et al. (10) compared two extraction methods with mycelia of 25 mold species, DNA yields were not high enough for experiments that require a large amount of DNA; therefore, in the present study, DNA extraction methods were compared based on the quantity and quality of extracted DNA from mycelial pellets of a wide range of mold species after culture in liquid medium. Methods also were evaluated to determine which were effective for use in a wide range of fungi, including yeasts.

DNA extraction methods were compared for each taxonomic group: yeasts, ascomycetous molds, and zygomycetous molds. For yeasts, the combination of bead grinding and SDS extraction yielded the largest amount of DNA for *C. neoformans* and *S. cerevisiae*. For *R. rubra*, the DNA yield with the combination of bead grinding and benzyl chloride method recovered a high quantity of DNA. Gene amplification by PCR was successful for all species

and in all trials with all combinations of DNA extraction methods. These results suggest that the combination of bead grinding and the SDS method is the best DNA extraction method for yeasts, and when sufficient DNA is not obtained, the combination of bead grinding and benzyl chloride as a secondary extraction method should increase the yield. For ascomycetous molds, the combination of bead grinding and SDS extraction recovered the largest amount of DNA for all species. Gene amplification by PCR was successful for all species and all trials with the combination of bead grinding and the SDS method. These results suggest that the combination of bead grinding and the SDS method is the best DNA extraction method for ascomycetous molds. For zygomycetous molds except *R. stolonifer*, the combination of each of three chemical extraction methods with or without bead grinding recovered large amounts of DNA for all species. Gene amplification by PCR was successful for all species and almost all trials with only the combination of the CTAB method with or without bead grinding. These results suggest that the CTAB method with or without bead grinding is the best DNA extraction method for zygomycetous molds, and the use of bead grinding should increase the yield. Thus, the combination of bead grinding and the SDS method is most effective DNA extraction method for yeasts and ascomycetous molds, and the combination of bead grinding and the CTAB method is most effective DNA extraction method for zygomycetous molds. However, bead grinding tends to promote DNA degradation (Fig. 1). The bead grinding process used in this study did not produce highly degraded DNA; however, care should be taken when using bead grinding in DNA extractions.

For DNA extracted by some method combinations from *E. nidulans*, *A. corymbifera*, and *S. racemosum*, gene amplification was not successful in some trials, although DNA yields were high. This finding indicates that species with the target gene in the extracted DNA not amplified by PCR were limited. Some researchers have previously reported that DNA extracts of *Candida* spp. and *Stachybotrys* spp. contained PCR inhibitors (4, 19). Therefore, some components derived from fungal cells could cause PCR inhibition.

In this study, the best method for DNA extraction differed according to the fungal taxonomic group. However, when identifying mold isolates, it may not be possible to distinguish between ascomycetous and zygomycetous molds because isolates sometimes do not exhibit important diagnostic characteristics such as sporulation and hyphal elongation. Therefore, the efficacy of DNA extraction methods was evaluated for a wide range of fungi across taxonomic groups. Five DNA extraction methods were compared by the percentage of 14 mold species that yielded more than 1,000 µg/g. A significantly higher percentage of species yielded high amount of DNA with the SDS, CTAB, and benzyl chloride methods than with both the GenTLE and DNeasy Plant kits ($P < 0.01$). This result suggests that the SDS, CTAB, and benzyl chloride methods are more likely to recover large amounts of DNA from molds. DNA yields with bead grinding were better than those without bead grinding in all 14 species of molds except *E. nidulans*

(Table 3), suggesting that bead grinding has an interactive effect with DNA extraction by chemical methods in many fungal species. The three combinations of bead grinding plus the SDS, CTAB, or benzyl chloride method were considered most likely to recover a large amount of DNA from molds; therefore, these three combinations were compared based on the DNA yield. PCR of *S. racemosum* failed to amplify the target gene in all trials with DNA extracted by both combinations of SDS or benzyl chloride method plus bead grinding. In contrast, gene amplification was successful for all mold species in at least one of three trials with the combination of bead grinding and the CTAB method. Overall, the combination of bead grinding and the CTAB method should be the first choice method for effectively extracting DNA from a wide range of molds. The combination of bead grinding and the CTAB method also yielded a relatively large amount of DNA from all species of yeasts used in this study (Table 2). Gene amplification by PCR using DNA extracted with this combination was successful in all trials for all yeasts, suggesting that bead grinding plus the CTAB method is relatively effective for DNA extraction in a wide range of fungi, including yeasts.

Our study is the first to clarify which extraction methods provide a high yield of high-quality DNA from mycelial pellets from a wide range of fungi growing rapidly in liquid medium. These methods should be very useful in genetic studies focused on identifying fungi important in the area of food hygiene, including many species of molds (28).

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