A simple, efficient and universal method for the extraction of genomic DNA from bacteria, yeasts, molds and microalgae suitable for PCR-based applications

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Abstract

The extraction of genomic DNA from microbial cells plays a significant role in PCR-based applications such as molecular diagnosis, microbial taxonomy, screening of genetically engineered microorganisms, and other such PCR-based applications. Currently, many methods for extraction of genomic DNA from microorganisms have been developed. However, these methods either require hazardous chemicals or consist of time-consuming steps for effective execution. In this study, we have established a simple and universal genomic DNA extraction method for different microorganisms including bacteria, yeasts, molds, and microalgae. Our method does not require harmful reagents such as phenol and chloroform for the extraction process to minimize the generation of hazardous wastes. The obtained genomic DNA products displayed high concentrations and represented a good purity level with the average 260 nm/280 nm absorbance ratios ($A_{260/280}$) that range from 1.6 to 2.0. The DNA molecules further remained considerably intact when analyzed on agarose gels. More importantly, these DNA products were qualified through successful PCR amplifications of 16S rRNA gene, rDNA internal transcribed spacer (ITS), or 18S rRNA gene from genomes of bacteria, fungi, and microalgae respectively. Furthermore, with the extracted genomic DNA products, the processes of the identification of the haploid and diploid states of the *Saccharomyces* yeast strains or detection of putative strains of *Aspergillus oryzae* and *Aspergillus flavus* that have been isolated from infected food materials through PCR analyses are facilitated. The genomic DNA extraction method established in this study is easy to manage, time saving and cost-effective, and environmentally friendly.

Keywords: ***bacteria, microalgae, molds, PCR, simple genomic DNA extraction, yeasts.***

Classification number: ***3.5***

Introduction

Across natural processes, microorganisms play important roles in nutritional cycles that are involved in the maintenance of the balance in ecological systems. In the context of applied microbiology, numerous microbial species are utilized for the production of foods, beverages, drugs, biofertilizers, or for the applications of environmental pollution treatment [1, 2]. The accurate identification of these microorganisms for specific purposes is usually performed on the basis of barcode ribosomal DNA sequences that include bacterial 16S rRNA, fungal ITS (internal transcribed spacer) region, and microalgal 18S rRNA [3-6]. Furthermore, the selected useful microorganisms can be subjected to further genetic improvement to enhance beneficial traits [1, 2, 7]. Consequently, the development of efficient genomic DNA extraction methods with respect to different microbial species is always considered a central step in PCR-based molecular biology applications that include molecular taxonomy, molecular diagnosis, recombinant DNA cloning studies, etc. These DNA extraction methods were developed according to either chemical reagents or commercial kits [8-12]. However, commercial kits employed for microbial genomic DNA extraction are expensive for large-scale screening experiments in laboratories, while conventional genomic DNA extraction methods are usually developed for a specific microbial group or require hazardous reagents such as phenol and chloroform for the cleanup step [8]. In this study, we have successfully established a simple and universal method for the rapid extraction of genomic DNA from different microbial species including bacteria, yeasts, molds, and microalgae. The extracted genomic DNA samples displayed superior quality and were determined as suitable for specific PCR-based applications.

Materials and methods

Microbial strains and cultivation conditions

All of the microbial strains and PCR primers are listed in Table 1 and Table 2

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Table 1. Microbial strains used in this study.

Name	Description	Source
Escherichia coli DH5α	The laboratory Gram-negative bacterial strain	Our collection
Agrobacterium tumefaciens AGL1	The laboratory Gram-negative bacterial strain employed for genetic transformation of plants and fungi	Our collection
Burkholderia vietnamiensis LU4.4	A Gram-negative bacterial strain isolated from rice rhizosphere displaying antifungal activity	Our collection
Lactobacillus fermentum H7	A Gram-positive lactic acid bacterial strain isolated from a fermented pickle	Our collection
Bacillus subtilis PY79	The laboratory Gram-positive bacterial strain	Our collection
Saccharomyces cerevisiae BY4741	The laboratory haploid yeast strain (*MATa*)	Euroscarf
Saccharomyces cerevisiae BY4742	The laboratory haploid yeast strain (*MATα*)	Euroscarf
Saccharomyces cerevisiae BY4743	The laboratory diploid yeast strain (*MATa*/*MATα*)	Euroscarf
Saccharomyces boulardii NOM	A probiotic yeast strain isolated from the commercial product Normagut (Germany)	Our collection
Saccharomyces boulardii PE	A probiotic yeast strain isolated from the commercial product Perenterol (Germany)	Our collection
Saccharomyces boulardii BIO	A probiotic yeast strain isolated from the commercial product Bioflora (France)	Our collection
Candida albicans JCM2070	An opportunistic yeast-causing candidasis in human	JCM, Japan
Candida glabrata RN4	A yeast strain of *Candida glabrata* isolated from a fermented sticky rice product	Our collection
Pichia anomala BMH9	A yeast strain isolated from a traditional yeast cake	Our collection
Hanseniaspora thailandica Y39	A yeast strain isolated from the peel of a red apple fruit	Our collection
Aspergillus oryzae RIB40	The laboratory strain used for the research of food production	Our collection
Aspergillus flavus NRRL3357	The laboratory strain used for the research of mycotoxin biosynthesis	Our collection
Aspergillus niger N402	The laboratory strain used for the research of production of enzymes and organic acids	Our collection
Penicillium chrysogenum VTCC-F1172	A fungal strain used for the research of penicillin production	VTCC, Vietnam
Magnarporthe oryzae MN1	A fungal pathogen that causes the rice blast disease isolated in Southern Vietnam	Our collection
Aspergillus sp. A1	The fungal strains isolated from mold-infected rice seeds in Hanoi	Our collection
Aspergillus sp. A2		
Aspergillus sp. A3		
Aspergillus sp. A4	The fungal strains isolated from mold-infected peanut seeds in Hanoi	Our collection
Aspergillus sp. A5		
Chlorella sp. PT01	A freshwater microalgal strain	VTCC, Vietnam
Chlorella sp. PT02	A marine microalgal strain	VTCC, Vietnam

respectively.

Four bacterial species that include *Escherichia coli*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, and *Burkholderia vietnamiensis* were grown in the LB medium (1% peptone, 0.5% yeast extract, 0.5% NaCl). One lactic acid bacterium *Lactobacillus fermentum* was cultivated in the MRS medium (1% sucrose, 1% peptone, 1% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.005% $MnSO_4$, 0.5% CH_3COONa, 0.2% K_2HPO_4, 0.2% NaH_2PO_4, 0.5% $CaCO_3$, 0.1% Tween 80, pH 6.5).

Six yeast species that include *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Candida albicans*, *Candida glabrata*, *Pichia anomala*, and *Hanseniaspora thailandica* were cultivated in the YPG medium (1% yeast extract, 1% peptone, 2% glucose, 1.8% agar). A single colony of each microbial strain (Table 1) was grown in a conical flask that contained 10 ml of a suitable medium at 30°C, 200 rpm until the OD_{600} value reached 1.5-2.0 and the respective cell biomass was then harvested.

Five different mold species including *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Magnaporthe oryzae*, and five putative strains of *A. oryzae* and *A. flavus* that were isolated from mold-infected rice seeds and mold-infected peanut seeds (Table 1) were cultivated in the potato dextrose medium (Himedia, India) or Czapek-Dox medium (comprising 3% sucrose, 0.3% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4$) at 30°C for 3-7 days.

Two microalgal strains *Chlorella* sp. PT01 and PT02 were cultivated in 100 ml conical flasks that contained 50 ml BBM (Bold's Base medium) [13]. The flasks were incubated at room temperature under white light of 2,000 lux intensity, subjected to a lighting cycle of 12 h/12 h (light/dark).

Preparation of the extraction buffer

This genomic DNA extraction protocol requires only a unique extraction buffer referred to as the GX buffer (2.5% SDS, 200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.2% β-mercaptoethanol). The buffer composition was adapted from certain published reports [8, 10, 14-17]. It is observed that the stock solutions, including 1 M Tris-HCl (pH 8.0), 0.25 M EDTA (pH 8.0), 2.5 M NaCl, can be autoclaved and stored at room temperature for subsequent use. Further, SDS (sodium dodecyl sulfate) should be added to the buffer after the other components. This buffer provided better results for genomic DNA extraction when freshly prepared. Alternatively, the ready extraction buffer can also be stored in the dark at room temperature for 2-3 weeks, and it requires to be heated at 60°C for 10 min before its application.

Genomic DNA extraction

The genomic DNA extraction method was adapted from some previously published protocols for fungi [7, 10, 14] with suitable modifications for each microorganism employed in this study.

For bacterial cells, the following procedure was performed: 2 ml of each bacterial culture with the OD_{600} values of 1.5-2.0 was centrifuged at 12,000 rpm for 1 min to harvest the cells. The cell pellet was resuspended in 70 µl TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)] and the tube was strongly vortexed for 15 s. Subsequently, 30 µl of lysozyme (10 mg/ml) was added to the tube. The resultant mixture was incubated at room temperature for 10 min. In the subsequent step, 600 µl of GX buffer and 3 µl proteinase K (20 mg/ml) were added to the tube. The tube was gently vortexed for 15 s and incubated at 60ºC for 30 min. To achieve neutralization, 300 µl of a 3 M sodium acetate solution (pH 5.2) was added to the tube. The supernatant phase (600-700 µl) obtained from a

Table 2. Primers used in this study.

Name	Sequence (5'-3')	Target sequence	Reference
16SfD1 16SrP1	AGAGTTTGATCCTGGCTCAG ACGGTTACCTTGTTACGA	Bacterial 16S rRNA gene	Weisburg, et al. (1991) [4]
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Fungal rDNA ITS	White, et al. (1990) [6]
18S1 18S12	TACCTGGTTGATCCTGCCAG CCTTCCGCAGGTTCACCTAC	Microalgal 18S rRNA gene	Honda, et al. (1999) [6]
ScMAT ScMATa ScMATα	AGTCACATCAAGATCGTTTATGG ACTCCACTTCAAGTAAGAGTTTG GCACGGAATATGGGACTACTTCG	*Saccharomyces* mating-type genes *MATa*, *MATα*	Illuxley, et al. (1990) [18]
AO-ITS-uni-F	ATGGCCGCCGGGGGCTCT	Specific to the rDNA ITS1 of *A. oryzae* and *A. flavus*	Chiba, et al. (2013) [19]
AFB-F AFB-R	AAGCAAACCAAGACCAACAAG AACAAGTCTTTTCTGGGTTCTA	Specific to aflatoxin biosynthesis gene cluster in *A. flavus*	Chiba, et al. (2013) [19]

centrifugation at 12,000 rpm, 4ºC for 20 min was transferred to a new 1.5 ml microcentrifuge tube. The genomic DNA was precipitated with 700 µl of cold isopropanol before it was subjected to centrifugation at 12,000 rpm, 4ºC for 20 min. The obtained pellet was washed with 500 µl of 70% ethanol and recollected by centrifugation. The DNA pellet was subsequently dried in a SpeedVac machine (Thermo Scientific, USA) and dissolved in 50 µl of TE buffer. This genomic DNA product was treated with 3 µl of RNase A (10 mg/ml) at 60ºC for 30 min for the removal of RNA and stored at -20ºC for ensuing applications.

For yeasts and microalgae, the following processes were performed: Yeast cells were collected from 2 ml of each culture obtained through a centrifugation at 4,000 rpm for 5 min, while microalgal cells were harvested at 8,000 rpm for 15 min. To break the cells, 600 µl of GX buffer and 150 mg of 0.1 mm diameter glass beads (Carl Roth, Germany) were added to the tube. The tube was strongly vortexed for 30 s and subsequently added with 3 µl of proteinase K (20 mg/ml). Subsequently, the tube was incubated at a temperature of 60°C for 30 min. The remaining steps of the extraction procedure were performed as those described above for the extraction of genomic DNA from bacteria.

For molds: 1 ml of each fungal spore suspension (10^6 spores/ml) was added to a 250 ml conical flask containing 100 ml of potato dextrose broth or Czapek-Dox liquid. The flask was subjected to a shaking incubator at 200 rpm, at a temperature of 30°C for 3 days. Fungal mycelium was collected by filtration through Miracloth (Calbiochem, Germany), and 200 mg of the obtained biomass was distributed to a 2 ml microcentrifuge tube. The fungal biomass was crushed directly in the tube for 1 min using a clean glass rod. Subsequently, 600 µl of GX buffer and 3 µl of proteinase K (20 mg/ml) were added to the tube. The tube was vortexed for 15 s and incubated at 60ºC for 30 min. The next steps of the extraction procedure were performed as described above for the extraction of genomic DNA from bacteria.

Analysis of the extracted genomic DNA products

The genomic DNA products were analyzed on 0.7% agarose gels through electrophoresis and the DNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific, USA) for the 260/280 nm absorbance ratios ($A_{260/280}$).

Verification of genomic DNA quality by PCR

All genomic DNA products were diluted to the concentration of 100 ng/µl as DNA template for PCR. Taq DNA polymerase as GoTaq® Green MasterMix (Promega, USA) was utilized for all PCR amplifications in accordance to the manufacturer's instruction. The universal primer pairs including 16SfD1/16SrP1 [4], ITS1/ITS4 [6], and 18S1/18S12 [5] (Table 2) were employed for specific amplifications of bacterial 16S rRNA gene, fungal rDNA ITS, and microalgal 18S rRNA gene, respectively. The thermal cycling parameters were determined as follows: 94°C (6 min); 30 cycles of 94°C (30 s), 58°C (30 s), 72°C (40 s to 1.5 min); 72°C (10 min); 4°C (∞). The obtained PCR products were analyzed on 0.7% agarose gels and visualized under UV light of the Gel Doc XR System (Bio-Rad, USA).

Determination of haploid and diploid states in Saccharomyces yeast strains: Three strains of the baker's yeast *S. cerevisiae*, including BY4741 (haploid, *MATa*), BY4742 (haploid, *MATα*), BY4743 (diploid, *MATa*/*MATα*), and three strains of the commercial probiotic *S. boulardii*, including NOM, PE, BIO (Table 1) were cultivated in the YPG liquid medium for genomic DNA extraction. The yeast ploidy states were determined through the PCR by employing the primer pairs ScMAT/ScMATa and ScMAT/ScMATα (Table 2) that are known to specifically amplify the mating-type genes *MATa* and *MATα* respectively [18]. The thermal cycling parameters are as follows: 94°C (6 min); 30 cycles of 94°C (30 s), 58°C (30 s), 72°C (30 s); 72°C (10 min); 4°C (∞). Each yeast strain was examined separately for the genes *MATa* and *MATα*. Thereafter, the obtained PCR products were mixed together for a comparative analysis on a 0.7% agarose gel.

Detection of A. oryzae and A. flavus strains by singleplex and multiplex PCR: The genomic DNA samples extracted from the fungal isolates including *A. oryzae* RIB40, *A. flavus* NRRL3357, and *Aspergillus* sp. (A1, A2, A3, A4, A5) were employed for singleplex PCR using five different primers in pairs that include ITS1/ITS4, AO-ITS-uni-F/ITS4, and AFB-F/AFB-R (Table 2). The universal primer pair ITS1/ITS4 is widely employed the amplification of the ITS region of rDNA in fungi [6], whereas the primer pair AO-ITS-uni-F/ITS4 was designed for specific amplification of the rDNA ITS in *A. flavus* and *A. oryzae* [19]. The primer pair AFB-F/AFB-R was designed to amplify the specific sequence located between *aflR* and *aflJ* of the aflatoxin biosynthesis gene cluster in *A. flavus* [19]. For multiplex PCR, five primers were applied simultaneously in a single reaction with the thermal cycling parameters as follows: 94°C (6 min); 30 cycles of 94°C (30 s), 60°C (30 s), 72°C (1.5 min); 72°C (10 min); 4°C (∞). Two standard strains *A. oryzae* RIB40 and *A. flavus* NRRL3357 were employed as the reference controls. The obtained PCR products were analyzed on 1.2% agarose gels.

Results and discussions

Establishment of a universal genomic DNA extraction method for different microorganisms

In this study, a unique procedure has been established for the extraction of genomic DNA from several microbial species including bacteria, yeasts, molds, and microalgae. Only the first step of the microbial biomass treatment is specific for each cell type. For lysis of bacterial cells, lysozyme was utilized to break down the peptidoglycan layer of the bacterial cell wall. Since this enzyme works more effectively in the presence of EDTA [20, 21], the bacterial cells in our procedure were treated with lysozyme in the TE (Tris-EDTA) buffer. The cells of yeasts and microalgae were broken mechanically in the extraction buffer (GX buffer) with glass beads, while the mycelia of the molds were crushed by hand with a glass rod. The overview of the genomic DNA extraction procedure is illustrated in Fig. 1.

The results revealed that the established method worked effectively for both Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Agrobacterium tumefaciens*, *Burkholderia vietnamiensis*, *Lactobacillus fermentum*, and *Bacillus subtilis* (Fig. 2A). To test the efficacy of this method for yeasts, five different yeast species including *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Pichia anomala* and *Hanseniaspora thailandica* were employed. Since the yeast cell wall is easily disrupted with glass beads through the process of vortexing [22], we added glass beads with a diameter of 0.1 mm and GX buffer to a 2 ml microcentrifuge tube containing the yeast biomass, and subsequently, the tube was vortexed strongly to break the cells. Following the subsequent steps for the genomic DNA extraction (Fig. 1), the results indicated that the genomic DNA products extracted from the yeasts as well as from

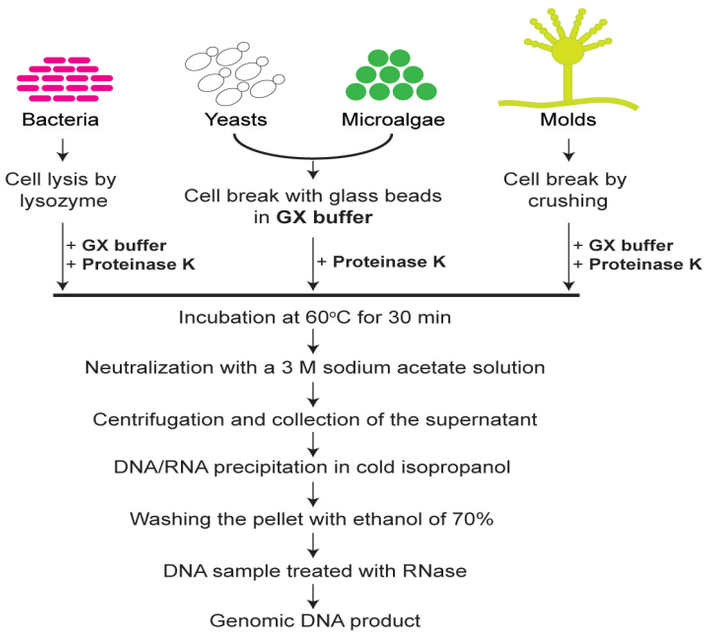

Fig. 1. The universal procedure of genomic DNA extraction for different microorganisms.

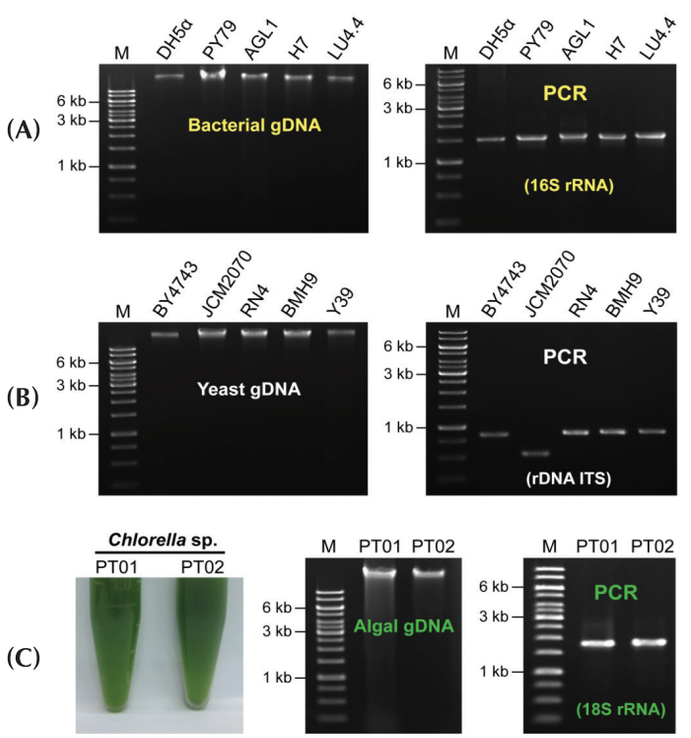

Fig. 2. Extraction of genomic DNA from bacteria, yeasts and microalgae. **(A)** The genomic DNA (gDNA) products extracted from five bacteria and the PCR products of the 16S rRNA genes on agarose gels. **(B)** The genomic DNA samples extracted from five yeasts and the PCR products of the rDNA ITS on agarose gels. **(C)** The analysis of the genomic DNA products extracted from two microalgae and the respective PCR products of the 18S rRNA genes on agarose gels.

the bacteria displayed sharp bands with lesser amounts of smearing of DNA on agarose gels (Figs. 2A, 2B). Particularly, these DNA products exhibited high concentrations that ranged from 753 to 6,059 ng/µl and superior purity with the $A_{260/280}$ values ranging from 1.81 to 2.02 (Table 3). When the same procedure as that for yeasts was applied to the microalgal strains including *Chlorella* sp. PT01 and PT02, the results revealed that this method also worked suitably for these green microalgae (Fig. 2C). In comparison to the bacteria and yeasts, the genomic DNA products extracted from the microalgae exhibited lower concentrations (99-177 ng/µl) with the $A_{260/280}$ values ranging from 1.57 to 1.87 (Table 3). More importantly, all the extracted genomic DNA products could be employed productively as the DNA template for PCR amplifications of the bacterial 16S rRNA gene, the yeast rDNA ITS sequence or microalgal 18S rRNA gene using the respective primer pair (Fig. 2, Table 2).

For genomic DNA extraction from molds, we crushed fungal biomass directly in a 2 ml microcentrifuge tube with a glass rod (Fig. 1). Five mold species including *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum* and *Magnaporthe oryzae* (Fig. 3A, Table 1) were utilized to test this procedure. The obtained genomic DNA products were superior in quality with the $A_{260/280}$ values ranging from 1.86 to 1.96 and high DNA concentrations of 1,466-6,528 ng/µl (Fig. 3B, Table 3). It is worth mentioning that the crushing of fungal cells in the tubes with a clean glass rod facilitates the prevention of cross-contamination among fungal samples and reduces the cost when compared to the grinding of the fungal biomass in liquid nitrogen using a mortar and a pestle. The obtained fungal genomic DNA products were evaluated for quality by PCR. The universal primer pair ITS1/ITS4 (Table 2) was utilized for amplification of the ITS region of fungal rDNA. The results indicated that the ITS region was successfully amplified from the genomes of all five fungal species (Fig. 3C).

Table 3. The concentration and purity of the extracted genomic DNA products.

Microbial species	DNA concentration (ng/µl)	$A_{260/280}$
Bacteria		
Escherichia coli DH5α	1,183 ± 203	1.81
Bacillus subtilis PY79	2,288 ± 139	1.92
Agrobacterium tumefaciens AGL1	859 ± 170	1.85
Lactobacillus fermentum H7	753 ± 208	1.90
Burkholderia vietnamiensis LU4.4	1,135 ± 52	1.92
Yeasts		
Saccharomyces cerevisiae BY4743	858 ± 61	1.94
Candida albicans JCM2070	6,056 ± 55	1.98
Candida glabrata RN4	2,701 ± 239	1.84
Pichia anomala BMH9	1,762 ± 276	2.02
Hanseniaspora thailandica Y39	2,341 ± 38	1.95
Molds		
Aspergillus oryzae RIB40	4,766 ± 91	1.87
Aspergillus flavus NRRL3357	2,669 ± 291	1.96
Aspergillus niger N402	6,177 ± 543	1.89
Penicillium chrysogenum VTCC-F1172	6,528 ± 711	1.86
Magnaporthe oryzae MN1	1,466 ± 104	1.90
Microalgae		
Chlorella sp. PT01	177 ± 12	1.87
Chlorella sp. PT02	99 ± 23	1.57

Although the genomic DNA extraction method established in this study works well for numerous microbial species, it does not always work suitably for all microorganisms. In fact, we tested this method for the Gram-positive pathogenic bacterium *Staphylococcus aureus*, but no DNA bands appeared on the agarose gel (data not shown). The reason behind this is that the cell wall of *S. aureus* is highly resistant to the digestion of lysozyme [23]. Additionally, we tested this method for some other fungal species. It also worked rather well for the citrus postharvest pathogen *Penicillium digitatum*, the antagonistic fungus *Trichoderma asperellum*, and the opportunistic human pathogenic fungus *Aspergillus fumigatus*. However, this method did not prove to work efficiently for the extraction of genomic DNA from the model filamentous fungus *Aspergillus nidulans*, the plant pathogen *Curvularia lunata*, and

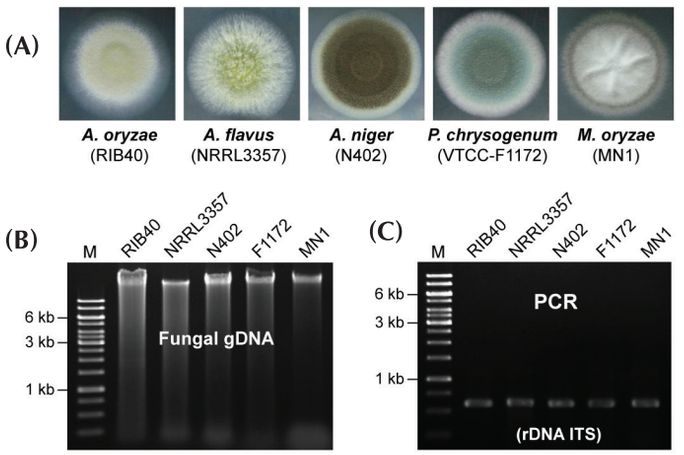

Fig. 3. Extraction of genomic DNA from different molds. (A) The morphology of the tested molds on the PDA medium at 30°C for 3-7 days. **(B)** The extracted genomic DNA (gDNA) products on a 0.7% agarose gel. **(C)** Analysis of the PCR products of the ITS on a 0.7% agarose gel.

the medicinal mushroom *Cordyceps militaris*, although the obtained DNA products were still functional for successful PCR amplifications (data not shown). Therefore, this method requires to be improved for certain specific microorganisms.

Simple identification of haploid and diploid states in Saccharomyces yeast strains by PCR

The baker's yeast *S. cerevisiae* can exist as diploid strains that possess *MATa* and *MATα* mating-type genes or haploid strains that carry only *MATa* or *MATα* gene [24]. The probiotic yeast *S. boulardii* is employed commonly for the treatment of antibiotic-associated diarrhea caused by *Clostridium difficile* infection in human. This probiotic yeast and *S. cerevisiae* share almost identical genomes [25]. In this study, we demonstrated that the ploidy states of three *S. boulardii* strains that were isolated from the commercial probiotic yeast products (Table 1) could be rapidly identified through PCR amplifications. Three standard *S. cerevisiae* strains, including BY4741 (haploid, *MATa*), BY4742 (haploid, *MATα*) and BY4743 (diploid, *MATa*/*MATα*), were adopted as controls and three *S. boulardii* isolates named NOM, PE, BIO were cultivated in the YPG liquid medium for genomic DNA extraction adhering to the above established method. The genomic DNA products extracted from all six yeast strains displayed high quality as indicated on an agarose gel (Fig. 4A). The extracted DNA products were utilized as the template for PCR with the specific primer pairs (Table 2); and further, the obtained data indicated that the haploid strains BY4741 and BY4742 possess either *MATa* (544 bp) or *MATα* (404 bp) gene respectively. Conversely, the diploid strain BY4743 carries both *MATa* and *MATα* genes (Fig. 4B). These results are consistent with the results previously reported [26]. Interestingly, all three probiotic strains (NOM, PE, BIO) of *S. boulardii* exist as diploids that carry both the mating-type genes *MATa* (544 bp) or *MATα* (404 bp) like the diploid strain BY4743 of *S. cerevisiae* (Fig. 4B).

Quick detection of Aspergillus oryzae and Aspergillus flavus strains by PCR

A. oryzae and *A. flavus* play significant roles in the food industry and food safety. *A. oryzae* has been commonly employed for the industrial production of soy sauce, miso, sake, soybean sauce paste in Asian countries, while *A. flavus* produces the carcinogenic aflatoxins. Since these fungal species are extremely closely related to each other and share similar morphology and genome homology amounting to 99.5%, their recognition is easily confused [2, 27].

In this study, we cultured five isolates *Aspergillus* sp. (A1, A2, A3, A4, A5) that share similar phenotypes of *A. oryzae* and *A. flavus* for genomic DNA extraction. The extracted genomic DNA products were good in quality displaying sharp bands on the agarose gel (Fig. 4C). With the utilization of singleplex PCR with the universal primer pair ITS1/ITS4, we amplified successfully the ITS region of rDNA with the same size of 595 bp from the genomes of all five *Aspergillus* sp. isolates, as well as from the genomes of the standard strains *A. oryzae* RIB40 and *A. flavus* NRRL3357. For the specific amplifications of the ITS region from *A. oryzae* and *A. flavus*, the primer pair AO-ITS-uni-F/ITS4 was utilized. The primer AO-ITS-uni-F was designed to bind only to the ITS1 sequence of *A. oryzae* and *A. flavus* [19]. The PCR with this primer pair resulted in a DNA band of 486 bp for all tested strains that include the reference strains *A. oryzae* RIB40 and *A. flavus* NRRL3357. To discriminate between *A. oryzae* and *A. flavus*, the primer pair AFB-F/AFB-R that specifically binds to the aflatoxin biosynthesis gene cluster of *A. flavus* was utilized [19]. With this PCR, only a DNA band of 116 bp appeared for the *A. flavus* strains (Fig. 4D). From the obtained results, we suggested that the strains A1, A2, A4, A5 belong to *A. flavus* and A3 is *A. oryzae*. Furthermore, these results were additionally confirmed through the performance of multiplex PCR in which all five primers (ITS1, AO-ITS-uni-F, ITS4, AFB-F, AFB-R) were combined in a single reaction. The multiplex PCR resulted in three bands (116 bp, 486 bp, 595 bp) for the *A. flavus* strains (NRRL3357, A1, A2, A4, A5) and only two bands (486 bp, 595 bp) for the *A. oryzae* strains (RIB40, A3) on an agarose gel (Fig. 4D).

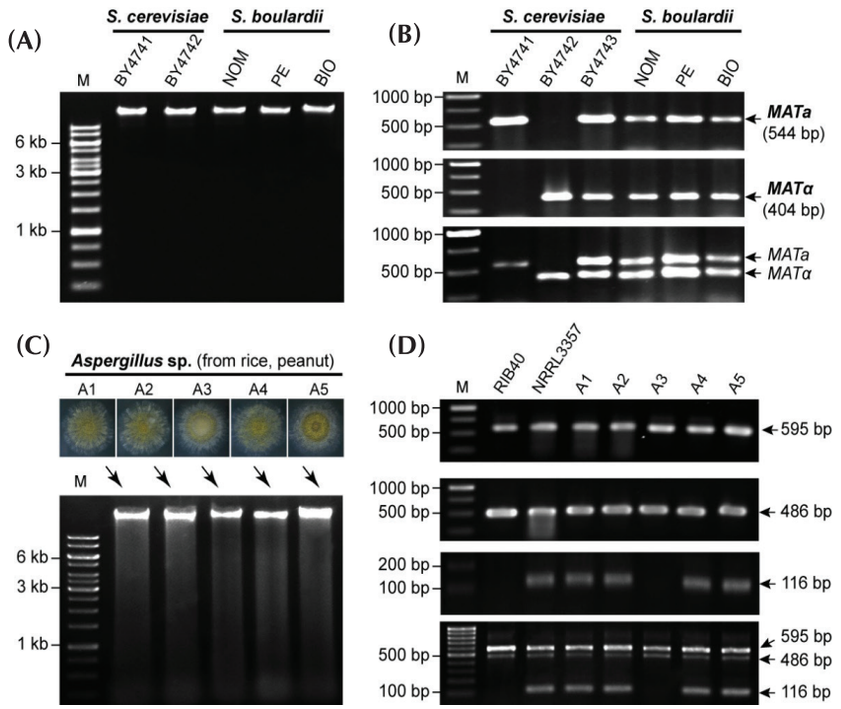

Fig. 4. Genetic identification of the closely related fungal species by PCR. (A) Genomic DNA products extracted from three standard strains of the baker's yeast *S. cerevisiae* and three newly isolated strains of the probiotic yeast *S. boulardii*. **(B)** Determination of haploid and diploid states in the *Saccharomyces* yeasts with the utilization of singleplex PCR amplifications with the primer pairs ScMAT/ScMATa and ScMAT/ScMATα specific to the yeast mating-type genes *MATa* and *MATα* respectively. **(C)** The genomic DNA products extracted from the molds *A. oryzae* RIB40, *A. flavus* NRRL3357, and *Aspergillus* sp. (A1-A5). **(D)** Quick detection of putative strains of *A. oryzae* and *A. flavus* isolated from infected food materials through singleplex and multiplex PCR amplifications.

In summary, the genomic DNA products obtained with our genomic DNA extraction method are completely suitable for PCR-based applications. The method described in this study is significantly uncomplicated in terms of execution and considerably more secure, since it does not employ toxic chemicals such as phenol and chloroform like other DNA extraction methods [17, 28-30]. Our method further provides higher DNA concentrations as compared to the simple method reported by Cenis (1992) [10], and even the DNA concentrations are 10 times higher in comparison to genomic DNA concentrations obtained from the phenol-chloroform method employed by Umesha (2016) [30].

Conclusion

The genomic DNA extraction method established in this study is universal, simple to handle, safe and cost-effective for the extraction of high-quality genomic DNA from various microbial cell types. The obtained genomic DNA products can be utilized for different research purposes, especially for PCR-based applications.

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REFERENCES

[1] B.K. Singh (2010), "Exploring microbial diversity for biotechnology: The way forward", *Trends Biotechnol.*, **28(3)**, pp.111-116.

[2] M. Machida, O. Yamada, K. Gomi (2008), "Genomics of *Aspergillus oryzae:* Learning from the history of Koji mold and exploration of its future", *DNA Res.*, **15(4)**, pp.173-183.

[3] C.L. Schoch, K.A. Seifert, S. Huhndorf, V. Robert, J.L. Spouge, C.A. Levesque, W. Chen, Fungal Barcoding Consortium (2012), "Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*", *Proc. Natl. Acad. Sci.*, **109(16)**, pp.6241-6246.

[4] W.G. Weisburg, S.M. Barns, D.A. Pelletier, D.J. Lane (1991), "16S ribosomal DNA amplification for phylogenetic study", *J. Bacteriol.*, **173(2)**, pp.697-703.

[5] D. Honda, T. Yokochi, T. Nakahara, S. Raghukumar, A. Nakagiri, K. Schaumann, T. Higashihara (1999), "Molecular phylogeny of Labyrinthulids and Thraustochytrids based on the sequencing of 18S ribosomal RNA gene", *J. Eukaryot. Microbiol.*, **46(6)**, pp.637-647.

[6] T.J. White, T. Bruns, S. Lee, J. Taylor (1990), "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics", *PCR protocols: A Guide to Methods and Applications*, **18(1)**, pp.315-322.

[7] K.T. Nguyen, Q.N. Ho, T.H. Pham, T.N. Phan, V.T. Tran (2016), "The construction and use of versatile binary vectors carrying pyrG auxotrophic marker and fluorescent reporter genes for *Agrobacterium*-mediated transformation of *Aspergillus oryzae*", *World J. Microbiol. Biotechnol.*, **32(12)**, p.204.

[8] G.S. Mahuku (2004), "A simple extraction method suitable for PCR-based analysis of plant, fungal, and bacterial DNA", *Plant Mol. Biol. Rep.*, **22(1)**, pp.71-81.

[9] M. Greco, C.A. Saez, M.T. Brown, M.B.

Bitonti (2014), "A simple and effective method for high quality co-extraction of genomic DNA and total RNA from low biomass *Ectocarpus siliculosus*, the model brown alga", *PLoS One,* **9(5)**, pp.e96470.

[10] J.L. Cenis (1992), "Rapid extraction of fungal DNA for PCR amplification", *Nucleic Acids Res.,* **20(9)**, p.2380.

[11] N. Mahmoudi, G.F. Slater, R.R. Fulthorpe (2011), "Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soils", *Can. J. Microbiol.*, **57(8)**, pp.623-628.

[12] M.K. Lee, H.S. Park, K.H. Han, S.B. Hong, J.H. Yu (2017), "High molecular weight genomic DNA mini-prep for filamentous fungi", *Fungal Genet. Biol.*, **104**, pp.1-5.

[13] J.R. Stein (1973), *Handbook of Phycological Methods: Culture Methods and Growth Measurements*, Cambridge University press, p.448.

[14] C. Illuxley, E.D. Green, I. Dunham (1990), "Rapid assessment of *S. cerevisiae* mating type by PCR", *Trends Genet.,* **6(8)**, p.236.

[15] T. Chiba, Y. Takahashi, K. Sadamasu, A. Nakama, A. Kai (2013), "Discrimination of *Aspergillus flavus* group fungi using phylogenetic tree analysis and multiplex PCR", *Shokuhin eiseigaku zasshi. Journal of the Food Hygienic Society of Japan,* **55(3)**, pp.135-141.

[16] S. Aljanabi, I. Martinez (1997), "Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques", *Nucleic Acids Res.,* **25(22)**, pp.4692-4693.

[17] A. Kalia, A. Rattan, P. Chopra (1999), "A method for extraction of high-quality and high-quantity genomic DNA generally applicable to pathogenic bacteria", *Anal. Biochem.,* **275(1)**, pp.1-5.

[18] A. Velegraki, M. Kambouris, A. Kostourou, G. Chalevelakis, N. Legakis (1999), "Rapid extraction of fungal DNA from clinical samples for PCR amplification", *Med. Mycol.,* **37(1)**, pp.69-73.

[19] K. Wilson (1997), "Preparation of genomic DNA from bacteria", *Current Protocols in Molecular Biology*, chapter 2.4.1-2.4.5

[20] S.T. Harrison (1991), "Bacterial cell disruption: a key unit operation in the recovery of intracellular products", *Biotechnol. Adv.,* **9(2)**, pp.217-240.

[21] L. Chitarra, P. Breeuwer, R. Van Den Bulk, T. Abee (2000), "Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*", *J. Appl. Microbiol.,* **88(5)**, pp.809-816.

[22] C.C. Stowers, E.M. Boczko (2007), "Reliable cell disruption in yeast", *Yeast,* **24(6)**, pp.533-541.

[23] A. Bera, S. Herbert, A. Jakob, W. Vollmer, F. Götz (2004), "Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*", *Mol. Microbiol.,* **55(3)**, pp.778-787.

[24] J.E. Haber (2012), "Mating-type genes and MAT switching in *Saccharomyces cerevisiae*", *Genetics,* **191(1)**, pp.33-64.

[25] L. Edwards-Ingram, P. Gitsham, N. Burton, G. Warhurst, I. Clarke, D. Hoyle, S.G. Oliver, L. Stateva (2007), "Genotypic and physiological characterization of *Saccharomyces boulardii*, the probiotic strain of *Saccharomyces cerevisiae*", *Appl. Environ. Microbiol,* **73(8)**, pp.2458-2467.

[26] T. Schmidlin, M. Kaeberlein, B.A. Kudlow, V. MacKay, D. Lockshon, B.K. Kennedy (2008), "Single-gene deletions that restore mating competence to diploid yeast", *FEMS Yeast Res.,* **8(2)**, pp.276-286.

[27] C. Rank, M.L. Klejnstrup, L.M. Petersen, S. Kildgaard, J.C. Frisvad, C. Held Gotfredsen, T. Ostenfeld Larsen (2012), "Comparative Chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357)", *Metabolites,* **2(1)**, pp.39-56.

[28] S. De, G. Kaur, A. Roy, G. Dogra, R. Kaushik, P. Yadav, R. Singh, T.K. Datta, S.L. Goswami (2010), "A simple method for the efficient isolation of genomic DNA from Lactobacilli isolated from traditional Indian fermented milk (dahi)", *Indian J. Med. Microbiol.,* **50(4)**, pp.412-418.

[29] J.A. Van Burik, R. Schreckhise, T.C. White, R. Bowden, D. Myerson (1998), "Comparison of six extraction techniques for isolation of DNA from filamentous fungi", *Med. Mycol.,* **36(5)**, pp.299-303.

[30] S. Umesha, H. Manukumar, S. Raghava (2016), "A rapid method for isolation of genomic DNA from food-borne fungal pathogens", *3 Biotech.,* **6(2)**, pp.1-9.