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New Safety and Rapid Method for Extraction of Genomic DNA from Bacteria and Yeast Strains Suitable for PCR Amplifications

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Molecular biology studies based on PCR amplification require the extraction of good quality genomic DNA. In this study, freeze- sudden thawing as a safety, new, quick and low-cost genomic DNA extraction technique from gram-negative and gram-positive bacteria, and *Saccharomyces* and non- *Saccharomyces* yeasts for PCR applications has been developed. This technique does not require any risky chemicals, enzymes and/or extra purification steps. DNA extracted by this method is suitable for PCR applications using random and specific primers such as RAPD-PCR for fingerprinting and DNA sequencing of the 16S rDNA or the D1/D2 domain of the 26S rDNA for molecular identification of bacterial and yeast isolates respectively.

Key words: Freeze-sudden thawing, Safety and rapid DNA extraction, Bacteria, Yeasts, PCR applications.

Extraction of high quality genomic DNA is a key step for successful molecular genetic differentiation and identification studies on natural microbes. So far, several DNA extraction methods are widely used to isolate DNA from bacteria and yeast including phenol extraction but they often involve multiple and time consuming steps[1]. Most protocols previously described for DNA extraction from bacteria and yeast strains, require expensive or toxic reagents[2,3], glass beads[4] and enzymatic digestion[5] in one of the steps for the extraction or purification. The freeze-thawing process performed by Borman *et al*.,[5] submitted the samples to six cycles of freeze-thawing in liquid nitrogen. DNA extraction using heat procedure developed by Liguori *et al*.,[6] was depended on the use of phosphate-buffered saline solution.

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The present study aims to test a new and rapid DNA extraction method from pure microbial prokaryotes (Bacteria: gram- negative and positive) and eukaryotes (yeasts: *Saccharomyces* and non-*Saccharomyces*). This method does not require any expensive or toxic chemicals, enzymes and/or extra purification procedures. The efficiency of the method was investigated by the PCR amplification products, and sequences as well as the DNA fingerprinting (RAPD-PCR) profiles.

MATERIALS AND METHODS

Isolation of Microorganisms

Four microorganisms, two bacteria (gram-negative and gram-positive) and two yeasts (*Saccharomyces* and non- *Saccharomyces*) were isolated from soil and fruit samples using Nutrient Agar (NA) and Yeast Extract-Malt Extract Agar (YMA) media for bacteria and yeast respectively.

DNA extraction using new Freeze-Sudden thawing protocol

A safety and quick technique designated as freeze and sudden thawing using one cycle PCR

program was used for DNA extraction from bacteria and yeast cells. Briefly, a big suitable colony from a fresh growth of bacteria or yeast isolates was transferred to sterile PCR micro-tubes (200 µl size) with 100 µl sterile deionised water and homogenized by vortex for 15 s. PCR micro-tubes were incubated at -20° C for 20 min then transferred directly to PCR machine for thawing at one cycle of 99 °C for 12 min. Samples were then homogenized by vortex for 10 s and centrifuged at 10,000 x g for 5 min. The supernatants which used as a DNA template were collected in new tubes and examined by agarose gel electrophoresis, then stored at -20 °C. To increase the DNA concentration the tubes were incubated at 45°C for 30 min.

DNA quantification and purity measurement.

The concentration of the extracted DNA was quantified as ng/µl by the measuring UV absorbance at λ260 and λ280. The purity was estimated on the basis of absorption ratio at λ260/λ280.

Random amplified polymorphic DNA (RAPD)

RAPD profiles for bacteria and yeast isolates were generated using the extracted DNA and OPA-3, 5' TGCCGCGCTG 3' as a random primer[7]. The RAPD-PCR reactions were performed in a 30 µl volume and each tube contained GoTaq green master mix (Promega, Madison, WI, USA), 2.0 µl of 10 pmol primer, and 2.0 µl template DNA. The amplification reactions were carried out in a thermo cycler under the following conditions: initial denaturation at 94º C for 5 min, followed by 45 cycles of 92 ºC for 1 min, 36º C for 1 min, 72º C for 2 min, and a final extension at 72º C for 10 min and subsequently cooled to 4º C.

16S rRNA gene amplification

Amplification of 16S rRNA gene was performed with universal primers: 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-CGGCTACCTTGTTACGACTT-3)[8,9] in a final volume of 50 µl containing for each tube GoTaq green master mix (Promega, Madison, WI, USA), 1 µl of each primer at a concentration of 0.5 mM, and 2 µl template DNA. PCR was performed with the following program: 5 min denaturation at 95 °C, followed by 36 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 1.5 min extension at 72 °C, and a final extension step of 7 min at 72 °C, and holding at 4 °C.

26S rRNA gene amplification.

Extracted genomic DNA from yeasts and universal primers NL1 (Forward 5'–GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (Reverse 5' GGTCCGTGTTTCAAGACGG – 3')[10, 11] were used to amplify the region of D1/D2 domain of 26S rRNA gene. PCR was performed in a final volume of 50 µl as mentioned above. The amplification was carried out by PCR under the following conditions: initial denaturation at 95 °C for 5 min, followed by 36 cycles at 94 °C for 2 min, 52 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 7 min, and holding at 4 °C.

Gel electrophoresis

Ten µl of the amplified mixture from RAPD-PCR or 5 µl from specific PCR products (16S or 26S rRNA gene) was analyzed using 1.5% 0.5×TBE agarose gel electrophoresis. 100-bp DNA Ladder were used as marker. The gel was stained with ethidium bromide, visualized under UV light, and photographed.

rRNA gene sequencing and phylogenetic analysis

DNA for sequencing was amplified with forward and reverse primers (27F and 1492R) for bacteria and (NL1 and NL4) for yeasts. PCR products of the correct size were purified and sequenced in both directions using an ABI automated sequencer. The bacteria and yeast rRNA genes sequences obtained were then aligned with known sequences of 16S and 26S rRNA genes in GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov/ BLAST/). Percent homology scores were generated to identify bacteria and yeast isolates. Phylogenetic trees for bacteria and yeasts were constructed with MEGA version 4.0 using a neighbor-joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1,000 replicates was performed[12].

GenBank accession number

The nucleotide sequences of 16S rRNA and 26S rRNA genes of isolated strains B1, B2, Y1, and Y2 reported in this study has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession Numbers KF761672 (*Bacillus amyloliquefaciens*), KF554299 (*Pseudomonas plecoglossicida*), KF761673

(*Saccharomyces cerevisiae*) and KF761674 (*Rhodotorula mucilaginous*) respectively.

RESULTS AND DISCUSSION

The studies on molecular identification and differentiation of microorganisms have been mainly based on the extraction of genomic DNA from microbial isolates. High molecular weight genomic DNA was obtained using Freeze-and-sudden thawing method with one cycle PCR program from gram-negative and positive bacteria, and *Saccharomyces* and non- *Saccharomyces* yeasts. The λ260/λ280 ratio was greater than 1.8

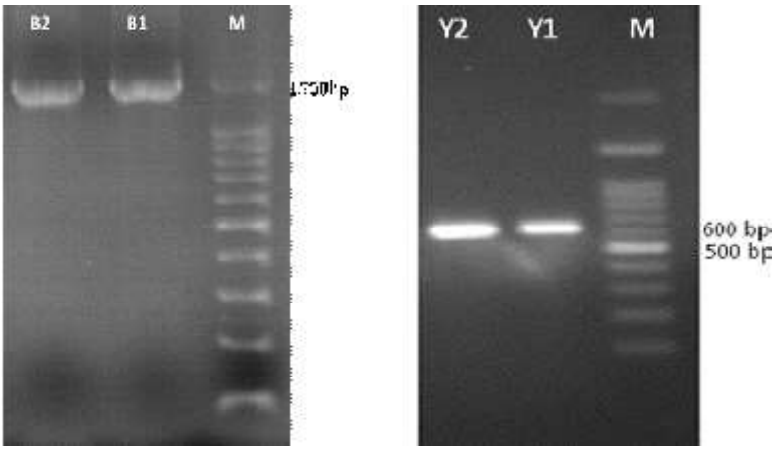

Fig. 1. Agarose gel electrophoretic profiles of PCR products of the 1500 bp and 600 bp fragments of the partial 16S and 26S ribosomal RNA genes of isolated bacteria B1 and B2 (left) and yeasts Y1 and Y2 (right) respectively. M- 100-bp DNA Ladder

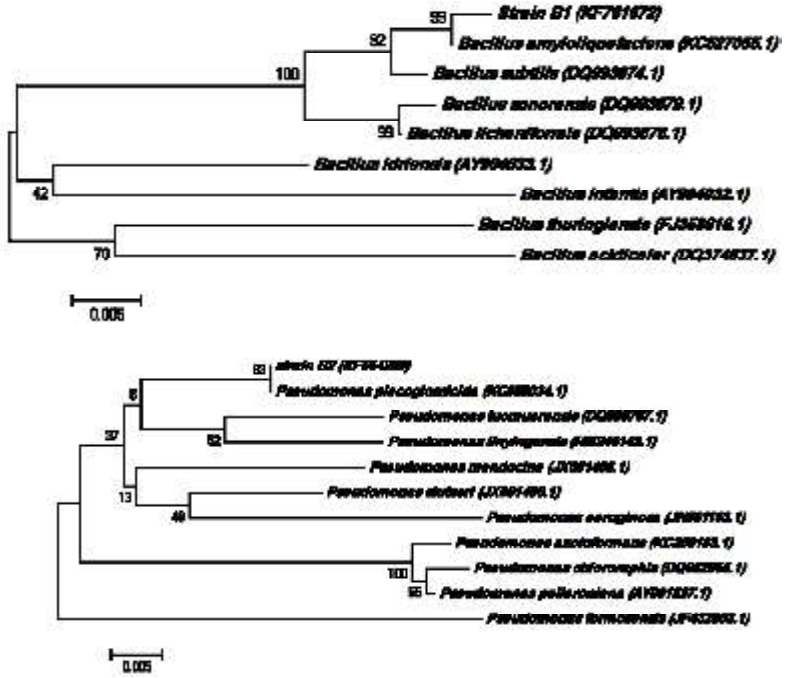

Fig. 2. Phylogenetic analysis of 16S rRNA gene of isolates B1 and B2 and other related species. By Neighbor-Joining method. Numbers at the nodes indicate bootstrap support (%) based on 100 replicates. The scale bar indicates 0.005 nucleotide substitutions per nucleotide position. GenBank accession numbers are given in parentheses

for all samples indicating the high purity of isolated genomic DNA. When the DNA samples were subjected to PCR amplification using specific primers for bacteria (27F and 1492R) and yeasts (NL1 and NL4) identifications, the 16S and 26S rRNA genes were successfully amplified with the expected sizes 1500 bp for bacteria (Fig. 1, left) and 600 bp for yeasts (Fig. 1, right). These results indicated that DNA were successfully amplified using specific primers for bacteria and yeasts identifications.

In order to confirm the correct affiliation of these microorganisms, PCR products were sequenced and compared with the sequences of 16S rRNA and 26S rRNA genes available in GenBank for each by means of BLAST search of the National Center for Biotechnology Information (NCBI) databases. Alignment results of the obtained sequences from the isolates, B1, B2, Y1, and Y2 show that high similarities ranged from 99 to 100% were found with the rDNA sequences of the bacterial strains *Bacillus amyloliquefaciens* and *Pseudomonas plecoglossicida* and yeast strains *Saccharomyces cerevisiae* and *Rhodotorula mucilaginos* respectively.

To confirm the taxonomic position of each strain in phylogeny, a number of sequences were selected from GenBank database for the construction of phylogenetic trees using MEGA4 program. As shown in (Fig. 2), the phylogenetic trees of 16S rRNA gene sequences indicated that the strains B1 and B2 shared one clade cluster with *Bacillus amyloliquefaciens* and *Pseudomonas plecoglossicida* respectively. For yeast strains, Phylogenetic analysis based on 26S rRNA gene sequences showed that isolates Y1 and Y2 and *Saccharomyces cerevisiae* and *Rhodotorula mucilaginos* were in the same clade cluster respectively (Fig. 3).

RAPD-PCR for the four strains B1, B2, Y1 and Y2 were performed by using OPA-3 as a random primer, the results of DNA fingerprints gave distinct band profiles which indicated a clear differentiation between the two bacteria (Fig. 4,

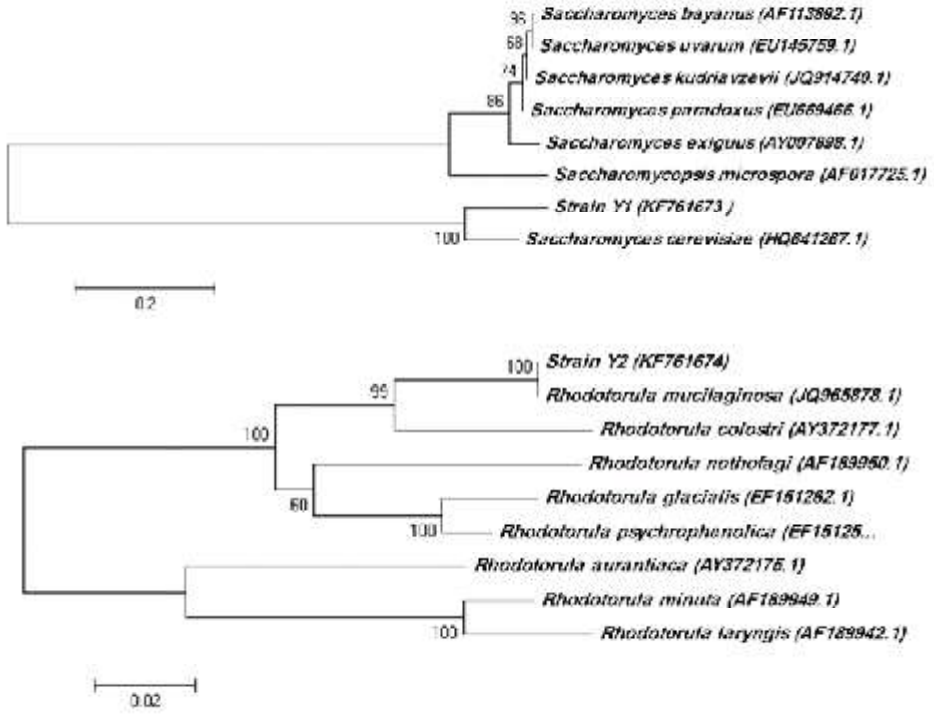

Fig. 3. Phylogenetic analysis of 16S rRNA gene of isolates Y1 and Y2 and other related species. By Neighbor-Joining method. Numbers at the nodes indicate bootstrap support (%) based on 100 replicates. The scale bar indicates 0.2 and 0.02 nucleotide substitutions per nucleotide position for Y1 and Y2 respectively . GenBank accession numbers are given in parentheses

left) and between the yeasts (Fig. 4, right). Identification results and DNA fingerprints profiles indicated that the amount of the extracted DNA by the present protocol were yielded with high quality and good enough for a variety of PCR-based applications.

Many current protocols for DNA extraction from microorganisms are too laborious, time-consuming and or generate poor yields compared to the methods of DNA extraction from the animal cells or viruses[13]. Most protocols previously described for DNA extraction from bacteria and yeast strains, used toxic reagents[2,3], glass beads[4] and enzymatic digestion[5] in one of the steps for the extraction or purification. The freeze-thawing process performed by Borman *et al*.[5] submitted the samples to six cycles of freeze-thawing in liquid nitrogen. The freeze-sudden thawing protocol reported in this study for DNA

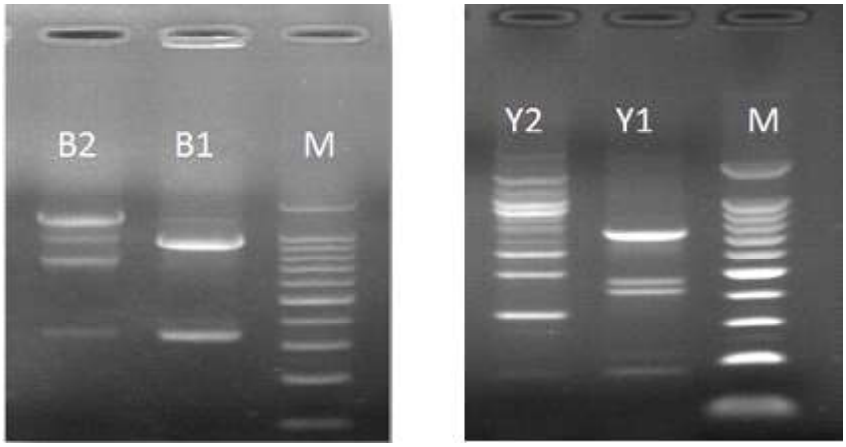

Fig. 4. Agarose gel electrophoresis of RAPD products for bacterial isolates B1 and B2 (left) and yeast isolates Y1 and Y2 (right) obtained by OPA-3 primer. M- 100-bp DNA Ladder

extraction using one cycle PCR program is, considerably faster and low-cost than conventional DNA extraction methods, whereas does not require any hazardous chemicals, enzymes and purification procedures. This method is suitable for genomic DNA extraction from gram-negative and positive bacteria, and *Saccharomyces* and non- *Saccharomyces* yeasts for PCR applications including identification and differentiation works.

CONCLUSION

Protocol described in this study is considerably useful for a quick and successful extraction of genomic DNA from different bacterial and yeast species with unlike cell wall composition. DNA extracted by this method is suitable for a variety of PCR-based applications including genetics differentiation and molecular identification works on bacterial and yeast species. The operation of this protocol is very low cost and safety where does not require any hazardous chemicals, enzymes and/or extra purification procedures.

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