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METHODS PAPER

A simplified quick microbial genomic DNA extraction via freeze–thawing cycles

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Abstract

Effective isolation of high-quality genomic DNA is one of the essential steps in molecular biology, biochemistry, and genetic studies. Here we describe a simplified procedure based on repeated freeze–thawing cycles to isolate genomic DNA from different organisms of microbes (*Burkholderia pyrrocinia* JK-SH007, *Bacillus pumilus* HRl0, *Botrytis cinerea*) and nematodes (*Bursaphelenchus xylophilus*). The DNA extraction buffer includes 10% of CTAB; 4% of NaCl (W/V); 20 mM of ethylenediamine tetraacetic acid; 100 mM of Tris-HCl, pH 8.0 and 1% of polyvinylpyrrolidone. The released DNA was purified from the mixture using a phenol/chloroform mixture and precipitated in 70% ethanol to remove proteins, carbohydrates, phenols, RNA, etc. Our method is a reproducible, simple, and rapid technique for routine DNA extractions from various microorganisms and nematodes. Furthermore, the low cost of this method could be an economic benefit to large-scale studies.

Keywords Extraction · Genomic DNA · Freeze–thawing cycles · Microorganism · Nematoda

Introduction

Extracting a sufficient amount and high quality of genomic DNA from various organisms is one of the significant techniques in various biological study fields, which usually including genomic library construction, genome sequencing, Southern blot hybridization, probe-based restriction fragment length polymorphisms (RFLPs) and restriction endonuclease analysis. Generally, an easy-to-operate method to extract genomic DNA with high quality and yield could be extraordinarily useful in advancing molecular biology studies across a diverse group of micro-organisms.

At present, there are few methods suitable for isolating DNA from various organisms. In general, cell disruptions have a significant impact on DNA isolation, which usually includes enzymatic lysis and grinding treatment [1–3]. The isolation of DNA from bacteria usually involves the use of proteinase K in combination with lysozyme to lyse the cells [4, 5]. In general, the DNA extractions of Gram-positive bacteria and Gram-negative bacteria require different procedures [2]. The lysis of Gram-negative bacteria depends on proteinase K, while lysis of Gram-positive bacteria requires both lysozyme and proteinase K due to cell wall differences. However, many bacteria, such as *Staphylococcus aureus*, are resistant to lysozyme [6]. Furthermore, the enzymatic process is strongly dependent on the incubation time. Moreover, Gram-positive bacteria require a longer reaction time than Gram-negative bacteria. This time includes 1 h of lysozyme and no less than 15 min of proteinase K digestion reactions, as described in the enzymatic lysis method [7].

Besides, the grinding treatment process is usually the primary step used for many DNA extraction methods [8, 9], especially in the DNA extraction of filamentous fungi and nematodes. However, there exist some foreseeable challenges in the grinding process. Not only is the preparation cumbersome, but the loss of the sample during grinding

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could decrease the yield of DNA. Furthermore, the purity of DNA is affected by proteins, phenols, polysaccharides, and RNA. The process is especially affected by the polysaccharides, which can strongly inhibit the activities of restriction endonucleases and ligases [4].

The freeze–thawing cycle is one of the promising strategies for cells lysis, which can break down the cells and release their DNA and cell contents. The recombinant proteins can be isolated from *Escherichia coli* cells through repeated cycles of freezing and thawing, as described by Johnson et al. [10]. Meanwhile, Silva et al. use repeated freeze–thawing method to extract the yeast DNA for polymerase chain reaction (PCR) [11]. Therefore, freeze–thawing cycles raise crucial hope for DNA extraction as they can overcome the challenges mentioned above.

As presently, extraction methods described for the isolation of DNA are not highly universal for multiple organisms and involve many extraction steps, which can cause contamination and lower both the quality and quantity of DNA. It is urgent to discover an easy-to-operate, more efficient way to extract the high-quality, purer genomic DNA from a broader range of species and smaller samples.

Therefore, here we introduce a method that is suitable for extracting the genomic DNA from a wide range of micro-organisms. While elevating the yield and purity of the extracted DNA, it also requires fewer steps and takes less time than other methods for the extraction. Moreover, the extracted DNA is almost free of proteins, carbohydrates, phenols, RNA and other contamination.

Materials and methods

Strains

Burkholderia pyrrocinia JK-SH007 [12] (Gram-negative bacteria), *Bacillus pumilus* HRl0 [13] (Gram-positive bacteria), *Botrytis cinerea* [14] (filamentous fungi) and *Bursaphelenchus xylophilus* (nematodes) [15].

Culture methods

Burkholderia pyrrocinia JK-SH007 and *B. pumilus* HRl0 were cultivated in Nutrient Agar medium at 28 °C and 200 rpm. *B. cinerea* was cultivated in Potato Dextrose Agar (PDA) medium at 25 °C. *B. xylophilus* was cultivated on *B. cinerea* growing on PDA at 25 °C.

Solutions

1. A modified DNA extraction buffer: 10% cetyltrimethylammonium bromide (CTAB); 4% NaCl (W/V); 20 mM ethylenediamine tetraacetic acid; 100 mM Tris-HCl, pH 8.0; 1% polyvinylpyrrolidone (W/V)
2. 10 mg/mL RNase
3. Phenol–chloroform–isoamyl alcohol (25:24:1)
4. Chloroform–isoamyl alcohol (24:1)
5. 3 M sodium acetate (pH 5.2)
6. Ethanol (70% and absolute)

DNA isolation protocol

The method of genomic DNA extraction presented in this report is an improvement based on the enzymatic lysis method [16], the CTAB-grinding method [17], the CTAB–SDS method [18], and the repeated freeze–thawing cycles. In the following, we describe the methodology of extracting the DNA from different microbes via freeze–thawing cycles (Fig. 1). All the experiments were conducted at least triplicate.

1. The bacteria (0.1 g, wet weight), fungi (0.1 g, wet weight), and nematodes (0.1 g, wet weight) were collected by centrifuging at 10,000×*g* for 1 min and sus-

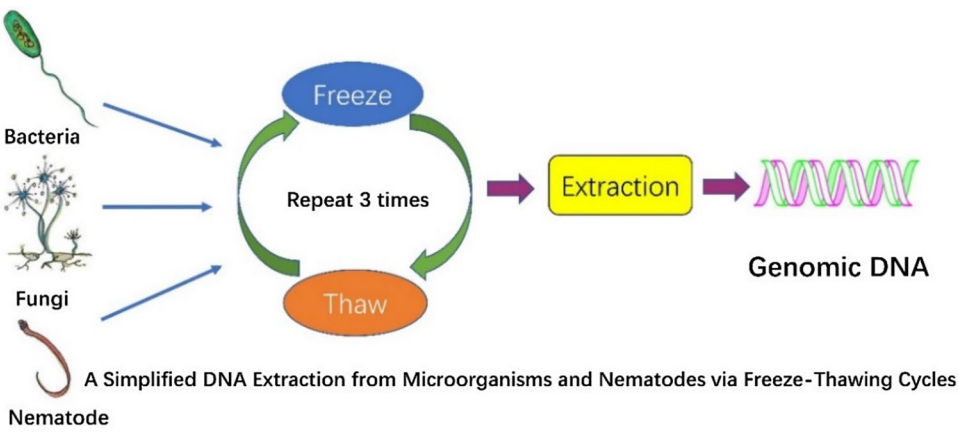

Fig. 1 The DNA extraction from microorganisms and nematodes via freeze–thawing cycles

pended in 50 μL ddH_2O, and then 400 μL of extraction buffer was added and mixed thoroughly.
2. The tube in step 1 was instantly frozen with liquid nitrogen for 10 s and melted at 65 °C for 1 min (freeze–thawing cycle) twice.
3. 10 μL of 10 mg/mL RNase was added to the tube and mixed thoroughly, and then the tube was incubated at 37 °C for 10 min (if a significant amount of RNA still exists, an additional 10 μL RNase can be added and the incubation time can be extended to 1 h).
4. 50 μL of 5 M NaCl and 100 μL of 10% SDS were transferred to the mixture above, mixed through vigorous manual shaking, and then the freeze–thawing cycle was repeated again.
5. An equal volume of a mixture of phenol–chloroform–isoamyl alcohol solution (25:24:1) was added into the solution in step 4, mixed through vigorous manual shaking, followed by centrifugation at 10,000×*g* for 10 min, and the supernatant was collected for further processing.
6. An equal volume of chloroform-isoamyl alcohol (24:1) was added into the supernatant, mixed through vigorous manual shaking, followed by centrifugation at 10,000×*g* for 10 min (repeated twice, centrifuged for 5 min in the second time), and then the supernatant was collected.
7. Two times volume of the absolute cold ethanol and 10% volume of 3 M sodium acetate were added to the supernatant obtained from step 6 for precipitating DNA, mixed through light manual shaking, and then centrifuged at 10,000×*g* for 2 min to collect the DNA pellet and discard the supernatant.
8. Finally, 100 μL of 70% ethanol was added to the DNA pellet for washing away impurities by pipetting up and down and centrifuged again at 10,000×*g* for 2 min to collect the DNA pellet by discarding the supernatant, then the pellet was dried in air at room temperature, followed by adding 50 μL ddH_2O or TE buffer to dissolve the DNA.

Quality and yield of DNA

The yield of DNA isolated from different organisms was measured using NanoDrop 2000C (ThermoFisher Scientific, USA). The purity of DNA was determined by electrophoresis on a 1% agarose gel, and calculating the ratios of absorbance at 260 nm to 280 nm and 260 nm to 230 nm.

Validation of restriction digestion

The quality of isolated DNA was validated by restriction digestion and PCR amplification. It was found that the DNA could be digested by the restriction enzyme EcoRI (1 μg genomic DNA/unit, incubated at 37 °C for 3 h).

The digested DNA was purified using the ethanol precipitation method and electrophoresed in a 1% agarose gel.

PCR amplification of 16S rDNA and ITS-rDNA

PCR amplification and product detection of 16S rDNA was performed on the extracted genomic DNA of *B. pyrrocinia* JK-SH007. The universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′/1492R:5′-GGTTACCTTGTTACGACTT-3′) of 16S rDNA of the bacteria were selected for genomic DNA amplification. The amplification system was 20 μL: 2 × *Easy*Taq® PCR SuperMix (Transgen) at 10 μL and 1 μL of 10 μM forward and reverse primers respectively, 1 μL of the template, and 7 μL of ddH_2O to fill the reaction volume up to 20 μL. The PCR reaction conditions were: denaturing at 94 °C for 5 min and the cycle starts at 94 °C for 30 s, then annealing at 55 °C for 30 s, extending at 72 °C for 1.5 min for 33 cycles in total and then a final extension at 72 °C for 10 min.

PCR amplification and product detection of ITS-rDNA was performed on extracted genomic DNA from *B. cinerea* and *B. xylophilus*, and the ITS-rDNA was amplified by a general primer (ITS7: 5′-GTGARTCATCGARTCTTTG-3′/ITS4: 5′-TTCCTSCGCTTATTGATATGC-3′). The amplification system was 20 μL: 2 × *Easy*Taq® PCR SuperMix at 10 μL and 1 μL of 10 μM primers for each one, 1 μL of the template and 7 μL of ddH_2O to fill the volume up to 20 μL. The PCR reaction conditions were: denaturing at 94 °C for 5 min and again at 94 °C for 30 s, then annealing at 55 °C for 30 s, extending at 72 °C for 1 min for 33 cycles in total and then extending at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel, and photos were taken under ultraviolet light stained by GelRed (Biotium, USA).

Results

DNA yield and purity

The universality and efficacy of the method presented were evaluated by isolating genomic DNA from various organisms, including Gram-negative bacteria (*B. pyrrocinia* JK-SH007), Gram-positive bacteria (*B. pumilus* HRl0), filamentous fungi (*B. cinerea*), and nematodes (*B. xylophilus*). Traditionally, the extractions of genomic DNA from Gram-positive and Gram-negative bacteria require different protocols [2]. Thus, it is a challenge to extract genomic DNA from unknown bacteria effectively. In this study, we used *B. pyrrocinia* JK-SH007 (Gram-negative bacteria) and *B. pumilus* HRl0 (Gram-positive bacteria) to test the universality of our DNA extraction protocol. The results showed that our

method can successfully isolate genomic DNA from both Gram-positive and Gram-negative bacteria (Fig. 2a). The A260/280 ratio ranged from 1.83 to 1.96, and the A260/230 ratio was higher than 2.0, which suggested that the extracted DNA were free of proteins and polyphenolic/polysaccharide compounds. The DNA yield ranged from 0.71 to 1.78 mg/g (wt) of *B. pyrrocinia* JK-SH007 and 0.66 to 1.31 mg/g (wt) of *B. pumilus* HRl0 (Table 1).

Furthermore, the results also indicated that the genomic DNA could be isolated efficiently from other organisms as well as bacteria. Various organisms, including *B. cinerea* representing fungi and *B. xylophilus* representing nematodes, were used to verify the universality of our DNA extraction protocol, and the genomic DNAs were successfully isolated from these organisms (Fig. 2b). Moreover, the A260/280 ratio ranged from 1.81 to 1.94 and the A260/230 ratio ranged from 2.0 to 2.24, which showed that the isolated DNA was of high quality without proteins, carbohydrates, RNA, and other contaminating substances. The yields of DNA ranged from 0.51 to 1.09 mg/g (wt) of *B. cinerea* and 0.43 to 0.85 mg/g (wt) of *B. xylophilus* (Table 1), suggesting that this method has great potential for extracting genomic DNA from multiple organisms at the same time.

In addition, the conventional enzymatic lysis method [16], lysing the cells using proteinase K, was used to extract DNA from *B. pyrrocinia* JK-SH007 (Gram-negative bacteria) and *B. pumilus* HRl0 (Gram-positive bacteria). Their DNA yields were 0.73 mg/g and 0.38 mg/g, respectively.

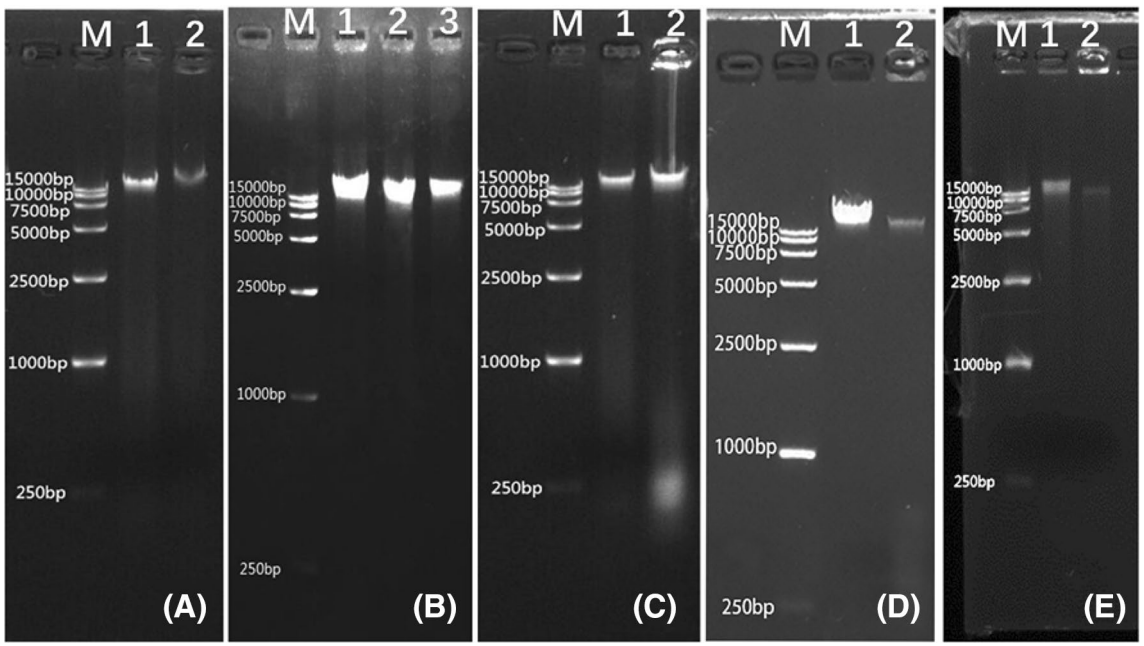

Fig. 2 The analysis of agarose gel electrophoresis of undigested genomic DNA: **a** genomic DNA extraction of Gram-positive and Gram-negative bacteria by our method; Line 1. The genomic DNA of *B. pyrrocinia* JK-SH007; Line 2. The genomic DNA of *B. pumilus* HRl0, **b** genomic DNA extraction from different organisms by our method; Line 1. The genomic DNA of *B. xylophilus;* Line 2. The genomic DNA of *B. pyrrocinia* JK-SH007; Line 3. The genomic DNA of *B. cinerea,* **c** comparing the CTAB–SDS method with our method for extracting DNA from *B. pyrrocinia* JK-SH007; Line 1. The genomic DNA extracted by our method; Line 2. The genomic DNA extracted by the CTAB–SDS method, **d** comparing the enzymatic lysis method with our method for extracting DNA from *B. pyrrocinia* JK-SH007; Line 1. The genomic DNA extracted by our method; Line 2. The genomic DNA extracted by the enzymatic lysis method, **e** comparing the genomic DNA extracted by the Grinding-CTAB method with our method; Line 1. The genomic DNA of *B. cinerea* extracted by our method; Line 2. The genomic DNA of *B. cinerea* extracted by the Grinding-CTAB method

Table 1 DNA yield from different organisms in this study

Organisms	Range of DNA yield (mg/g)	Range of 260/280	Range of 230/260
B. pyrrocinia JK-SH007	0.71–1.78	1.85–1.96	2.01–2.22
B. pumilus HRl0	0.66–1.31	1.83–1.94	2.03–2.18
B. cinerea	0.51–1.09	1.87–1.94	2.11–2.24
B. xylophilus	0.43–0.85	1.81–1.93	2.0–2.13

When proteinase K is not added, the DNA were not isolated from *B. pyrrocinia* JK-SH007 and *B. pumilus* HRl0. However, the DNA extraction was successful when using our method (no proteinase K and lysozyme), The DNA yields were 1.28 mg/g and 0.97 mg/g, respectively.

In terms of grinding treatment, *B. cinerea* (filamentous fungi) and the *B. xylophilus* (nematode) DNA were extracted through the traditional grinding with the CTAB method [17]. The yield of DNA from *B. cinerea* was 0.0663 mg/g, while yield was 0.0169 mg/g without the grinding process. Moreover, the yield of DNA from *B. xylophilus* was 0.02142 mg/g, whereas the DNA was not obtained without grinding treatment. However, when using the method (without grinding) described in this report, the DNA extraction was successful. The DNA yields were 0.63 mg/g and 0.54 mg/g, respectively. Therefore, when compared with the classic Grinding-CTAB method (Fig. 2e), the quality and yield of the extracted DNA in this study have obviously improved. The method described in this report overcomes the loss of samples during the grinding process, especially for small number of samples, which might be the reason for the low DNA yield from the traditional method.

It is well-known that *Burkholderia* contains a significant number of polysaccharides [19], which could be one of the major obstacles for effective DNA isolation. Additionally, compared with the enzymatic lysis and CTAB–SDS methods, the yield of genomic DNA from *B. pyrrocinia* JK-SH007 using our method exhibited the highest yield (100%, 1.78 mg/g), followed by the CTAB–SDS method (87%, 1.35 mg/g) and the enzymatic lysis method (56%, 0.99 mg/g). Moreover, our method performed well in extracting genomic DNA from polysaccharide-producing bacteria (*B. pyrrocinia* JK-SH007) (Fig. 2b). According to the results from agarose electrophoresis, the lane loading the DNA samples extracted by our method was almost free of impurities, while the lane loading the samples obtained by the CTAB–SDS method (Fig. 2c) and the enzymatic lysis method (Fig. 2d) showed a certain amount of impurities.

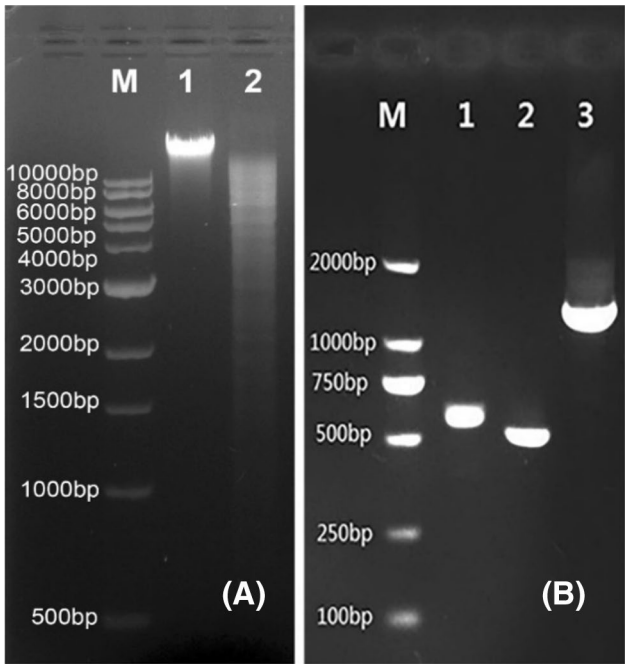

Fig. 3 The validation of DNA quality: **a** The genomic DNA digested by EcoRI by our protocol. M, 1 kb DNA Ladder molecular weight marker; Line 1. The DNA of *B. pyrrocinia* JK-SH007 with the treatment of reaction buffer of EcoRI; Line 2. The DNA of *B. pyrrocinia* JK-SH007 with treatment of EcoRI restriction enzyme, **b** the validation of DNA quality by PCR amplification; Line 1. The ITS-rDNA of *B. xylophilus;* Line 2. The ITS-rDNA of *B. cinerea;* Line 3. The 16S-rDNA of *B. pyrrocinia* JK-SH007

Table 2 The time needed for extracting genomic DNA by different methods

Methods	Time (h)	References
Freeze–thawing cycle method	1.5–2	The study
CTAB-grinding method	2–3	[17, 20]
CTAB–SDS method	3–3.5	[18, 21]
Enzymatic lysis method	2–3	[16, 18]

DNA quality verification

The quality of isolated DNA was validated by restriction digestion and PCR amplification. It was found that the genomic DNA could be digested by the restriction enzyme EcoRI (Fig. 3a). The results suggested that this method can provide the DNA required for biological research involving sequencing, Southern blot hybridization, probe-based RFLPs, etc. Furthermore, the extracted DNA was directly used as the template for PCR, and the results of ITS rDNA and 16S rDNA suggested that the quality of DNA was outstanding (Fig. 3b). Moreover, only 10–20 ng of DNA was needed for PCR amplification in a 20 µL reaction mix. These results also suggested that the isolated DNA by this method was pure, high quality and suitable for further molecular experiments.

Discussion

In the present study, we simplified and optimized the steps for DNA extraction. Compared with other protocols, our method is simpler, easier to operate, and without cumbersome steps. Table 2 shows that the DNA isolation takes only eight steps and less time than other methods (1.5 to 2 h in our protocol vs. 2 to 3.5 h in other protocols). Furthermore, the use of freeze–thawing cycles (Fig. 1) increases the efficacy of extraction by breaking down and disintegrating the

cell wall to a higher degree, releasing more cell contents. Compared with the grinding treatment, our method avoids the loss of the sample along with the reduction in the yields of DNA. Compared with enzymatic lysis, proteinase K and lysozyme are not required. Thus, our method is more concise, cost-effective, and beneficial to large scale extractions.

If pure DNA is not required or the DNA is not used immediately (i.e., RNA is not stable and easy to be degraded, even stored at − 20 °C), the third step in our method may be skipped (not adding RNase). Instead, 0.1 g of micro-organisms could be mixed with 50 µL of ddH_2O, 400 µL of extraction buffer, 50 µL of 5 M NaCl, and 100 µL of 10% SDS, then go through the freeze–thawing cycles three times. This simplified protocol is even faster and easier to operate than our method described above. It suggested that the simplified protocol is advantageous in the experimental process.

The genomic DNA of *B. pyrrocinia* JK-SH007 (Gram-negative bacteria), *B. pumilus* HRl0 (Gram-positive bacteria), *B. cinerea* (Filamentous fungi), and *B. xylophilus* (nematodes) were successfully isolated by using the protocol presented. The results suggested that this is a promising DNA extraction method for a wide range of micro-organisms. Furthermore, it has outstanding performance in the application of isolating DNA from *B. pyrrocinia* JK-SH007 which produces a lot of polysaccharides. These also showed that our protocol also has excellent potential for extracting DNA from polysaccharide-producing bacteria.

The DNA quality was estimated by measuring the absorbance ratio of 260/280 nm which varied between 1.81 and 1.96. We also evaluated the quality of the extracted DNA through agarose gel electrophoresis. Figure 1 showed the results of the extracted DNA run on a 1% agarose gel, stained with GelRed and visualized with UV light. The DNA quality in our study was higher than the classic method [22], which means our DNA was almost free of proteins, carbohydrates, phenols, RNA, and other substances. We also obtained very high DNA yields with our procedure in several different micro-organisms. The average yield was approximately 1 mg DNA/g of the bacteria, and the yield is higher than the classic method which yields about 0.1 mg/g [23]. Therefore, our method is convenient and highly efficient.

DNA can also be directly used as the template for PCR, and the results of ITS rDNA and 16S rDNA in this study suggest that the quality of our DNA was excellent. In cases even with a small amount of template, the pure DNA performed well in PCR and produced a good yield. In order to confirm whether the quality of the DNA satisfies the requirements for sequencing, Southern blotting and RFLPs, we also performed DNA enzyme digestion experiment tests on *B. pyrrocinia* JK-SH007 with the restriction enzyme EcoRI. We found that a small amount of DNA extracted with our method can also satisfy the requirements for restriction endonuclease digestion.

Conclusion

In summary, we have reported a new extraction method for genomic DNA that is generally applicable to Gram-positive bacteria, Gram-negative bacteria, filamentous fungi, and nematodes which can overcome the 'one method for one organism' limitation in molecular biology research. The purified DNA performed well in PCR and restriction endonuclease digestion which suggested that the method has the potential for application for further sequencing, Southern blotting and probe-based RFLPs. It provides relatively simpler steps and a shorter extracting time with a higher purity of genomic DNA that is almost free from proteins, carbohydrates, phenols, RNA, and other contaminating substances.

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Data availability The data used to support the findings of this study are included in the article.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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