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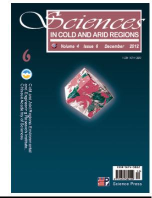

Methods for extraction of microorganism DNA from glacier surface snow

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ABSTRACT

In order to thoroughly investigate the diversity of glacier microorganisms, four DNA extraction methods with different lysis patterns were tested and two screened methods (the Bosshard-Bano method and the Zhou method) were optimized for the most effective form of the filter membrane (cut vs. uncut), the DNA extraction method, and the precipitation method. The two optimized methods were then compared with the commercial Mo-Bio DNA extraction kit, and the results showed that the kit was generally suitable for extraction of microorganism DNA from glacier surface snow. Procedurally, it was found that a modified Bosshard-Bano method (*i.e.*, cutting the filter membrane into pieces, using a specific lysis pattern [lysozyme (5 mg/mL)-protease K (1 mg/mL)-CTAB (1%)-SDS (1%)], performing the extraction only once by chloroform-isoamyl alcohol (24:1), and conducting DNA precipitation by pure ethanol) was also an effective and less expensive method for extraction of microorganism DNA from glacier surface snow.

Keywords: glacier surface snow; extraction of microorganism DNA; optimizing method

1. Introduction

Microorganisms are a primary life form in low-temperature environments. They are deposited in glaciers through atmospheric circulation and precipitation (Castello *et al*., 1999; Sattler *et al*., 2001; Priscu *et al*., 2006). Glacier microorganisms can influence surface albedo and the hydrochemistry of precipitation, and serve as primary producers in material cycles and energy flows of glaciers (Tomas and Duval, 1995; Sharp *et al*., 1999; Tranter *et al*., 2002; Skidmore *et al*., 2000, 2005). The community structure and diversity of culturable microorganisms in ice cores and snow pits have been studied by traditional culture methods (Christner *et al*., 2001; Zhang *et al*., 2003; Miteva *et al*., 2004; Xiang *et al*., 2005; Zhang *et al*., 2007; Zhang *et al*., 2007a; Ma *et al*., 2009). However, it is estimated that only 1% of the microorganisms in environmental samples can be isolated (Amann *et al*., 1995; Schloss and Handelsamn, 2003), so accurate environmental microbial study is hampered. Therefore, since the mid 1980s, many microbiologists have investigated the amount, community structure, and function of environmental microorganisms by culture-independent methods (Amann *et al*., 1995), such as molecular hybridization, electrophoresis (denaturing gradient gel electrophoresis, DGGE; and temperature gradient gel electrophoresis, TGGE), high-throughput sequencing, and so on, but their uses in the study of environmental microorganisms are based on the high DNA yield and quality.

DNA extraction of environmental microorganisms involves sample pretreatment, cell lysis (physical lysis, chemical lysis, enzyme lysis, and the combining of different lysis patterns), and extraction, precipitation, and purification of

DNA. Many biotechnology companies have produced various DNA extraction kits for environmental microorganisms. Compared with traditional extraction methods, using DNA extraction kits is time- and labor-saving, making them suitable for extracting DNA from scores of microbial samples. However, DNA extraction kits are expensive, so their application is limited. To date, there is no single DNA extraction method suitable for all environmental microbial samples. The biomass of glacier microorganisms is low and Gram-positive bacteria are the dominant group (Xiang *et al.*, 2006; Miteva, 2008), so it is very difficult to establish a high-quality DNA extraction method. This paper reports on certain optimized DNA extraction methods and compares them with a typical commercial kit, and makes recommendations for their use in the study of glacier microorganisms.

2. Material and methods

2.1. Bacterial strains

Gram-positive bacteria (*Mycobacterium* spp., *Clavibacter* spp., *Pseudonocardia* spp., and *Nocardioides* spp.) isolated from snow samples and stored at our laboratory were cultured with LB medium, at 25 °C and 180 rpm, and used for DNA extraction.

2.2. Snow samples

In late April and early June 2011, surface snow samples were collected at the No. 12 Glacier in Laohugou Valley, Qilian Mountains (39°26′N, 96°33′E; 4,600 m a.s.l.) by a technician wearing clean clothes, a mask, and PE gloves, and were placed into sterile bottles. The snow samples were transported to the laboratory and stored at <15 °C temperature.

2.3. DNA extraction of glacier microorganisms

A total of 1.5 mL of cultured medium of every bacterial strain was added successively into a single 2 mL centrifugal tube and centrifugalized at 8,000 rpm for 15 min. Afterwards, the bacterial colonies were collected and their DNA was extracted according to the following four methods. Every method had three repetitions.

2.3.1 DNA extraction according to the Bosshard-Bano method

We followed the Bosshard-Bano method with one minor modification (Bosshard *et al.*, 2000; Bano and Hollibaugh, 2002). Bacterial colonies were resuspended in 200 μL solubilization buffer (50 mM sucrose, 25 mM Tris, 10 mM EDTA, pH 8.0, 1.5 M NaCl, 1% CTAB), and 50 μL of lysozyme (20 mg/mL) in centrifuge tubes, and the samples were incubated in a 37 °C water bath for 1.5 hours. Then 10 μL of proteinase K (20 mg/mL) and 15 μL of 20% SDS were added, and the samples were incubated in 37 °C and 55 °C water baths for 30 min and 2 hours, respectively. Afterwards, pre-heated CTAB/NaCl (10% CTAB and 0.7 M NaCl) was added and the samples were incubated in a 65 °C water bath for 30 min. The supernatants were collected after centrifugation at 8,000 rpm for 15 min and transferred into 2 mL centrifuge tubes. Supernatants from the extractions were combined with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifugalized at 12,000 rpm for 10 min. The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at −20 °C overnight. The pellet of crude nucleic acids was obtained by centrifugation at 12,000 rpm for 20 min, washed twice with 75% ethanol, and dried out at room temperature. The nucleic acids were resuspended in TE, giving a final volume of 20 μL.

2.3.2 DNA extraction according to the Liu method

Based on the Liu method (Liu *et al.*, 2009), bacterial colonies were resuspended in 1 mL GTE buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose, 20 mg/mL, pH 8.0) and the samples were incubated in a 37 °C water bath for 2 hours. Then 10 μL of proteinase K (20 mg/mL), 140 μL of NaCl (5 M), and 55 μL of 20% SDS were added and the mixture was incubated at 65 °C for 1.5 hours. After extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) and being centrifugalized at 10,000 rpm for 15 min, DNA in the aqueous phase was precipitated with an equal volume of isopropanol at −20 °C overnight. The pellet of crude nucleic acids was obtained by centrifugation at 12,000 rpm for 20 min, washed twice with 75% ethanol, and dried out at room temperature. The nucleic acids were resuspended in TE, giving a final volume of 50 μL.

2.3.3 DNA extraction according to the Zhou method

Based on the Zhou method (Zhou *et al.*, 1996), bacterial colonies were resuspended in 650 μL of DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% CTAB), and 5 μL of lysozyme (10 mg/mL) in centrifuge tubes, by horizontal shaking at 225 rpm for 20 min at 37 °C. Then 5 μL of proteinase K (10 mg/mL) was added and shaken for 20 min. Afterwards, 75 μL of 20% SDS was added, and the samples were incubated in a 65 °C water bath for 2 hours with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 8,000 rpm for 15 min and transferred into 2 mL centrifuge tubes. Supernatants from the extractions were combined with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) and centrifugalized at 10,000 rpm for 15 min. The aqueous phase was precipitated with 0.6 volume of isopropanol at room temperature overnight. The pellet of crude nucleic acids was obtained by centrifugation at 12,000 rpm for 30 min, washed twice with 75% ethanol, and dried out at room temperature. The nucleic acids were resuspended in

sterile deionized water, giving a final volume of 50 μL.

2.3.4 Freezing-thawing method

Bacterial colonies were resuspended in 650 μL of DNA extraction buffer and subjected to freezing-thawing three times (Benjamin *et al.*, 2010) and were then processed according to the Zhou method.

2.4. Form of the filter membrane (cut vs. uncut) and optimizing the DNA extraction and precipitation methods

(1) After glacier surface snow was melted at 4 °C (Table 1), about 800 mL of melted water was averagely filtrated into four pieces of 0.22 μm filter membrane. Then two pieces of filter membrane were cut and the others were uncut. Afterwards, each piece of filter membrane was put in a separate 2 mL centrifuge tube with 200 μL of solubilization buffer. The subsequent procedures of DNA extraction followed the Bosshard-Bano method.

(2) About 1,100 mL of melted water was averagely filtrated into six pieces of 0.22 μm filter membrane. Then each piece of filter membrane was put in a separate 2 mL centrifuge tube with 200 μL of solubilization buffer. Three methods were then used for DNA extraction: FL [the first extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and the second extraction with chloroform-isoamyl alcohol (24:1)]; LII [extraction with chloroform-isoamyl alcohol (24:1) twice]; and LI [extraction with chloroform-isoamyl alcohol (24:1) once]. The other procedures of DNA extraction followed the Bosshard-Bano method.

(3) About 600 mL of melted water was averagely filtrated into four pieces of 0.22 μm filter membrane. Then each piece of filter membrane was put in a separate 2 mL centrifuge tube with 200 μL of solubilization buffer. Two deposition methods were used for DNA precipitation: Y (deposition with three volumes of pure ethanol), and B (deposition with 2/3 volume of isopropanol). The other procedures of DNA extraction followed the Bosshard-Bano method.

Each of these treatments was duplicated, and the DNA yield of the first water bath and the mixture DNA yield of the second and third water bath were calculated.

Table 1 Optimizing for form of filter membrane (cut vs. uncut), DNA extraction method, and precipitation method

Treatment	Filtrated volume (mL)	Form of filter membrane	Extraction method	Precipitation method
J	200	Cut filter membrane	Same as LII	Same as B
W	200	Uncut filter membrane	Same as LII	Same as B
FL	180	Same as W	First extraction with phenol- chloroform-isoamyl alcohol (25:24:1); second extraction with chloroform-isoamyl alcohol (24:1)	Same as B
LII	180	Same as W	Extraction with chloroform- isoamyl alcohol (24:1) twice	Same as B
LI	180	Same as W	Extraction with chloroform- isoamyl alcohol (24:1) once	Same as B
Y	150	Same as W	Same as LII	Precipitation with three volumes of pure ethanol
B	150	Same as W	Same as LII	Deposition with 2/3 volume of isopropanol

2.5. Comparing the optimized extraction methods with kit extraction

The optimized Bosshard-Bano method and the Zhou method were named ZE and ZH, respectively. About 600 mL of melted water was averagely filtrated into four pieces of 0.22 μm filter membrane. Then DNA was extracted with a Mo-Bio kit (PowerWater DNA Isolation Kit, No. 14900-50-NF, Mo-Bio Laboratories, Carlsbad, CA, USA), the ZE and ZH methods were applied, and the better method of glacier microorganism DNA extraction was determined.

2.6. The quantity of microbial DNA

The DNA concentration and optical density values at 230, 260, and 280 nm were estimated with an ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA), and DNA purity was assessed with A260/A230 (DNA/ humic acid) and A260/A280 (DNA/protein).

2.7. PCR amplification

DNA extracted with the Mo-Bio kit and the ZE and ZH methods were amplified by PCR using the oligonucleotide primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1507-1492r (5'-CGGTTACCTTGTTACGACTT-3') (Whitaker *et al.*, 2003). PCR was carried out in a final volume of 25 μL using 4-μL template DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM each primer, and 1 U Taq polymerase (MBI). To prevent contamination, a negative parallel

amplification was established by autoclaved deionized water. Reactions were performed with the following cycling parameters: 94 °C for 1 min for an initial denaturation, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min, and a final incubation at 72 °C for 10 min. The products of PCR amplification were estimated with 1.0% agarose gel electrophoresis.

3. Results and discussion

3.1. Comparison of different DNA extraction methods

The A260/A280 ratios of the four methods ranged from 1.8 to 2.0 (Table 2), which indicated that the extracting effects of phenol and chloroform were good; their A260/A230 ratios were >2 (Manchester, 1995; Small *et al*., 2001), which indicated that there were few residuals of reagents and impurities. The discrepancy of the DNA yields was basically attributable to the different lysis patterns, because our experimental samples were mixtures of pure cultures, not environmental samples containing humic acid and so on.

Gram-positive bacteria are the dominant group of glacier bacteria. They adapt to extreme low-temperature environments with the complex structure of their cell walls (Miteva, 2008), so we tested four methods with different lysis patterns for extracting DNA from the mixture of Gram-positive bacteria. In lysis patterns, lysozyme and protein K can dissolve cell walls and protein, respectively. CTAB and SDS can decompose the lipid bilayer, and repeated freezing-thawing can break down the cell structure.

Table 2 Yield and purity of DNA extracted by four methods

Method	DNA yield (ng/μL)	A260/A280	A260/A230
Zhou	155.230±82.792	1.953±0.021	2.110±0.120
Freezing-thawing	162.570±94.330	1.920±0.044	2.037±0.122
Bosshard-Bano	202.070±38.511	1.950±0.010	2.090±0.017
Liu	49.650±12.092	1.935±0.021	2.225±0.035

Our results showed that, first, the DNA yield of the Bosshard-Bano method was higher than the other three methods, but not significantly different from freezing-thawing and the Zhou method (p>0.05), which indicates that freezing-thawing does not significantly increase DNA yield. Leff and others also discovered that freezing-thawing did not influence the DNA extraction from sediment (Moré *et al*., 1994; Leff *et al*., 1995). Second, the DNA concentrations of the Liu method were lower than the other three methods and significantly lower than the Bosshard-Bano method, which is probably attributed to there being no CTAB in the lysis pattern of the Liu method. CTAB has not only the function of removing humic acid but also a lysis function similar to SDS (Lee *et al*., 1996; Fortin *et al*., 2004). Finally, the lysis patterns of the Bosshard-Bano and the Zhou methods, namely, lysozyme-protein K-CTAB-SDS, were more effective.

3.2. Form of the filter membrane (cut vs. uncut) and optimizing the DNA extraction and precipitation methods

The mixture DNA yield of the second and third water bath accounted for 25%–37% of the total DNA yield (Table 3). Repeated extractions were beneficial, since small amounts of DNA were still collected after the second and even the third water bath. The repeated extractions were also effective for soil microorganisms (Zhou *et al*., 1996). Regarding the filter membranes, we found that cutting the filter membrane can significantly increase DNA yield (p<0.01). For example, the DNA yield of treatment J was 2.6 times higher than treatment W but its A260/A280 ratio was lower than treatment W, which indicated that the removing effect was probably influenced by a small amount of membrane fragments dissolving in the extraction buffer.

Regarding the DNA extraction method, the DNA yield of treatment LI was evidently higher than treatments LII and FL; the DNA yields of the latter treatments were similar, which indicated that repeated extraction can decease the DNA yield. The A260/A280 ratio of treatment FL, about 2.0, was higher than that of LII and LI. This suggests that treatment FL is appropriate for complex environmental samples, whereas treatment LI is appropriate for ice and snow samples with low biomass.

Regarding the DNA precipitation method, the DNA yield of treatment Y was evidently higher than treatment B, and the A260/A280 ratio of the former was higher than the latter (p<0.05), suggesting that precipitation with ethanol is more effective than isopropanol for extracting DNA from glacier microorganisms.

3.3. Comparing the optimized extraction methods with kit extraction

The DNA yields of the ZE, the Mo-Bio kit, and the ZH methods were, respectively, about 0.034, 0.020, and 0.015 ng/μL (Table 4), but no significant differences were observed among them (p>0.05). The DNA yields of these three extraction methods used for surface snow from No. 12 Glacier in Laohugou Valley were far higher than those of the high-salt method used for snow samples from another glacier (Shang, 2009). This is probably because these three methods are more effective than the high-salt method, and also because the microbial amounts and community structures of the different glaciers are distinct (Zhang *et al*., 2007b; Xie *et al*., 2009).

Table 3 The effects of filter membrane (cut vs. uncut), extraction method, and precipitation method on DNA yield

Treatment	The DNA yield of the first water bath (ng/μL)	The mixed DNA yield of the second and third water bath (ng/μL)	The total DNA yield (ng/μL)	The percentage of mixed DNA yield in total DNA yield (%)	A260/A280	A260/A230
J	0.130±0.075	0.043±0.018	0.173±0.093	25	1.610±0.077	0.713±0.265
W	0.052±0.004	0.024±0.001	0.076±0.005	32	1.745±0.045	0.603±0.176
FL	0.046±0.006	0.026±0.001	0.072±0.007	36	1.915±0.233	0.528±0.178
LII	0.051±0.000	0.030±0.004	0.081±0.004	37	1.733±0.188	0.540±0.143
LI	0.076±0.023	0.030±0.003	0.106±0.026	28	1.748±0.225	0.595±0.174
B	0.006±0.004	0.002±0.001	0.008±0.005	29	1.737±0.083	0.505±0.368
Y	0.031±0.014	0.015±0.001	0.046±0.015	33	1.710±0.629	0.978±0.312

Note: Table values are mean ± standard deviation (n=2).

Table 4 Yield and purity of the DNA extracted by the optimized methods and the Mo-Bio kit

Method	DNA yield (ng/μL)	CV (%)	A260/A280	CV (%)	A260/A230	CV (%)
ZH (Zhou method)	0.015±0.010	67	2.005±0.530	26	0.820±0.184	22
ZE (modified Bosshard-Bano method)	0.034±0.014	41	1.930±0.311	16	0.840±0.010	12
Kit	0.020±0.008	40	1.815±0.091	5	1.145±0.011	10

Note: Table values are mean ± standard deviation (n=3).

The A260/A280 ratios of the ZH, ZE, and kit methods ranged from 1.8 to 2.0, which indicated that optimized ZE and ZH methods and the kit method can entirely remove protein from a glacier surface snow sample. The A260/A230 ratios of these three methods were <2, which indicated that the DNA extracted by these three methods was contaminated by humic acid and so on. The A260/A230 ratio of the kit method was higher than the other two methods.

As shown in Figure 1, the 16S rDNA amplification of DNA extracted by these three methods showed that not all repetitions of DNA extracted by the ZH method produced PCR amplification. Also, the band amplified from one repetition of DNA extracted by the ZE method was light and the other two bands were weak. The amplification effect of DNA extracted by the kit method was best and all of its repetitions had light amplified bands, which was probably due to the higher purity of the DNA extracted by the kit method.

The repeatability of the DNA extraction method can be estimated by the coefficient of variation of inter-repeats through comparing parameter variations of DNA extracted by different methods (Ge, 2000). The coefficients of variation of the DNA yield and the A260/A280 ratio and A260/A230 ratios of the three repetitions of the kit method were lower than those of the other two methods, which suggest that the repeatability of the kit method was best. The coefficients of variations of the DNA parameter of the ZE method, except the A260/A280 ratio, approached that of the kit method, suggesting that the ZE method is also an appropriate method for DNA extraction from glacier microorganisms.

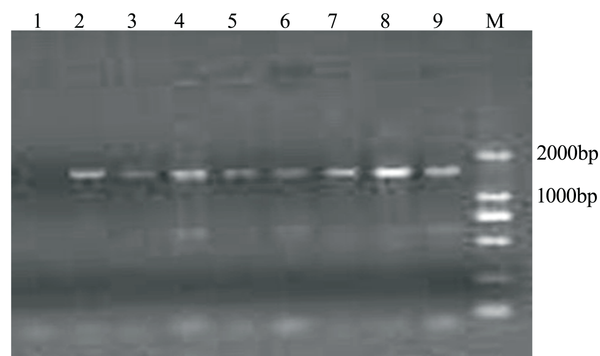

Figure 1 Agarose gel electrophoresis of PCR amplified 16S rDNA gene of bacteria from glacier surface snow by three methods. 1–3: ZH (Zhou) method; 4–6: ZE (modified Bosshard-Bano) method; 7–9: Kit method. Model DL2000 DNA Marker (ShineGene Molecular Biotech, Inc., Shanghai, China)

4. Conclusion

Four DNA extraction methods with different lysis patterns were used for extracting the DNA of glacier microorganisms (Gram-positive bacteria) cultivated in our laboratory, and the DNA yields of these four methods were estimated. The lysis patterns of the Bosshard-Bano and Zhou methods (*i.e.*, lysozyme-protein K-CTAB-SDS) were better than those of the freezing-thawing and the Liu methods. Procedurally, cutting the filter membrane, extracting with chloroform only once, and precipitating with ethanol were shown to be more favorable for DNA extraction of glacier

microorganisms. Comparison of these optimized traditional extraction methods with the Mo-Bio kit showed that, based on DNA parameters and PCR amplification, the kit method is suitable for study of microbial diversity in glacier surface snow, while the Bosshard-Bano method is also an effective and less expensive glacier microorganism DNA extraction method.

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