

REVIEW

Skim Milk Drastically Improves the Efficacy of DNA Extraction from Andisol, a Volcanic Ash Soil

Yuko TAKADA HOSHINO* and Naoyuki MATSUMOTO

Department of Biological Safety, National Institute for Agro-Environmental Sciences (NIAES)
(Tsukuba, Ibaraki 305–8604, Japan)

Abstract

There is an increasing interest in the extraction of DNA from soil, since molecular techniques facilitate the analysis of the microbial community including unculturable microorganisms. Though various methods of direct DNA extraction have been devised, it still remains difficult to extract DNA from some soils. We developed an improved method to extract DNA from such “recalcitrant soils”. Andisol, volcanic ash soils are recalcitrant in this context. Skim milk increased the efficacy of DNA extraction from such soils, when added to the extraction buffer as an adsorption competitor to soil particles. This method is applicable to molecular community analysis of soils which strongly adsorb DNA.

Discipline: Agricultural environment

Additional key words: environmental DNA, microbial community analysis, molecular methods, unculturable microorganisms

Introduction

Soil microorganisms have important roles in agricultural fields. They are responsible for the principal mineralization reactions that recycle nutrients and degrade environmental pollutants, and for the suppression of soilborne plant diseases¹¹. There are, however, many difficulties in understanding the whole soil microbial community. First, soil microorganisms live in a complex community. Second, there has been no appropriate method to study the complexity. Traditionally, investigations on soil microbial community were mainly based on isolation and laboratory cultivation. This method is both time- and labor-consuming and applicable to less than 1% of microorganisms present in soil that are readily culturable¹. Enrichment media used to culture microorganisms in the laboratory are inherently selective for a small portion of the whole microbial community.

Molecular techniques that do not depend on culturing have recently been developed to characterize microbial communities in various environments^{23,24}. These methods rely on direct DNA extraction from environmental samples. Such DNA is called environmental DNA or eDNA. eDNA is useful for (i) the detection of

specific genes present in pathogens and genetically modified organisms, (ii) the analysis of microbial community including unculturable microorganisms, and (iii) the search for new functional genes for industrial application.

Investigations on eDNA have been increasing. However, a technical problem has remained unsolved to extract DNA from soil. Because of its complexity and diversity, direct DNA extraction from soil is more difficult than from other environmental samples such as water. DNA extraction especially from Andisol, a volcanic ash soil is known to be very difficult. Here, we introduce an improved method using skim milk for DNA extraction from such recalcitrant soils.

An improved method for DNA extraction from Andisol

1. Methods for DNA extraction from soils

Many protocols for DNA extraction from soil have been developed²¹. These procedures may be divided into two groups. In one approach, the microbial cell fraction is first separated from soil particles and then lysed to purify DNA²⁶. The other approach includes *in situ* cell lysis followed by DNA purification²⁰. Both approaches have advantages and disadvantages in terms of yield and

*Corresponding author: fax +81–29–838–8199; e-mail yuko422@affrc.go.jp

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purity of DNA for molecular analysis and the extent of bias through extraction. The cell separation procedure causes low DNA yield, biases in the collection of microorganisms, and requires time and effort. The direct *in situ* lysis method is superior in this respect^{15,25} and has widely been used.

There are various kinds of microorganisms in soil. Their cell wall varies from one species to another in fragility. For example, Gram-negative bacteria tend to disintegrate more easily than Gram-positive bacteria. To investigate a whole microbial community, it is necessary to disrupt them unbiased. The majority of the direct extractions is the combination of chemical and/or enzymatic treatments and physical procedures such as freeze-thaw cycles²⁷, freezing in liquid nitrogen followed by grinding²⁸ or bead-beating¹⁸. Bead-beating is most effective in cell disruption^{12,17,18} and commercially available kits include this step². In these procedures, about 500 mg of soil are shaken hard with small glass beads in buffer including detergents for several tens of seconds. Microbial cells are disrupted within the soil matrix and nucleic acids are released from lysed cells. DNA is, then, recovered and purified.

Thus, the bases of DNA extraction methods have been established. However, these methods do not necessarily apply to all types of soil. Volcanic ash soils are, above all, recalcitrant in this context. DNA extraction from soil is difficult because soils have a complex matrix including a variety of substances and their properties are extremely diverse. Modifications are required to adjust to different soil types²⁹.

2. Reason for the difficulty in DNA extraction from Andisol⁷

Andisol, a volcanic ash soil, is widely distributed all over the world, especially in the circum-Pacific Ring of

Fire, East Africa and the Mediterranean. In a type of Andisol including allophane as a clay mineral, it has been difficult to extract DNA by various methods so far reported.

Seven Andisol samples taken from fields and a forest all over Japan (Table 1, Fig. 1) were subjected to DNA extraction, using a commercially available DNA extraction kit, FastDNA Spin kit for soil (Q-BIOgene) according to the manufacturer's protocols. In five of them, extracted DNA was too scanty to detect by agarose electrophoresis following ethidium bromide staining (Fig. 2-A), and subsequent PCR amplification of bacterial 16S rDNA was unsuccessful (Fig. 2-B).

To identify the reason for the difficulty, we started experiments first by determining microbial community size in soil samples. In a field soil sample of NIAES (soil 1), the number of microbial cells, as determined by epifluorescence microscopy using DAPI staining, was over 10^9 cells g^{-1} dry wt soil and the colony forming units by plate count was about 10^6 cells g^{-1} dry wt soil. These results agreed with those from previous reports^{13,29}. In addition, microscopic observations showed that the efficacy of cell disruption by bead-beating was 61%. The extract from this soil sample was expected to contain 1–4 μg DNA g^{-1} dry wt soil, but genomic DNA was not detected by agarose gel electrophoresis. Then two other lysis procedures were applied: (i) a combination of bead-beating and subsequent heating at 60°C for 10 min, and (ii) bead-beating with extraction buffer containing phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0). Genomic DNA was not detected from these extracts by agarose electrophoresis. We examined the recovery of DNA of ca. 500 bp which had been added to the soil sample. After bead-beating of the soil with additional DNA, DNA of ca. 500 bp and its degraded fragments could not be detected in the supernatant even at 1,000 μg of DNA

Table 1. Chemical and physical properties of soils used for DNA extraction

Soil no.	Origin	Soil taxonomy ^{a)}	Soil texture	pH (H ₂ O)	Organic C content (g kg ⁻¹)	P retention (%)
1	Spinach field, Ibaraki	Dystric-Silic Andisol	light clay	5.46	83.419	83
2	Conserved forest, Ibaraki	Dystric-Silic Andisol	light clay	4.84	149.43	84
3	Apple orchard, Aomori	Silic-Eutrisilic Andisol (Dystric)	sandy clay loam	6.08	122.893	75
4	Vegetable field 1, Fukushima	Dystric-Silic Andisol	light clay	6.20	78.795	71
5	Vegetable field 2, Fukushima	Haplic-Dystric Cambisol	clay loam	6.02	23.239	65
6	Upland crop field, Kumamoto	Dystric-Silic Andisol	heavy clay	5.59	117.283	82
7	Paddy field, Kumamoto	Silic-Eutrisilic Andisol (Dystric)	heavy clay	6.38	119.425	91

a): According to the world reference base (WRB) for soil resources classification.

g^{-1} soil. Considering these results, the failure in DNA extraction from soil 1 was ascribed to the adsorption of DNA to soil. DNA adsorption to soil particles is the main factor reducing DNA extraction efficiency^{3,20,22}.

3. Skim milk enables DNA extraction from Andisol⁷

To improve DNA recovery, the FastDNA Spin kit protocol was modified to prevent DNA from binding to soil particles. Then, skim milk or RNA was added as an adsorption competitor. Skim milk was known as a carrier to minimize the degradation and adsorption of nucleic acids to soil^{4,10,28}. RNA competes with DNA for the adsorption sites on the soil particles³. Ten, 40 or 100 mg of skim milk or 4, 10 or 40 mg of yeast RNA g^{-1} soil were added to soil 1 on bead-beating. Genomic DNA was detected when more than 10 mg skim milk or more than 10 mg RNA were added (Fig. 3-A). DNA could successfully be extracted from other recalcitrant soil samples with 40 mg of skim milk g^{-1} soil (Fig. 2-A). The addition of skim milk improved the efficiency even in soils 2 and 4 from which DNA could be extracted without skim milk (Fig. 2-A). PCR products of the expected size were amplified from all extracts with skim milk (Fig. 2-B). PCR products of the expected size were amplified from all extracts with skim milk (Fig. 2-B).

Resultant extracts were suitable for PCR and no other purification procedures were needed.

Though a higher amount of DNA was extracted with RNA than with skim milk, extracts with RNA contained

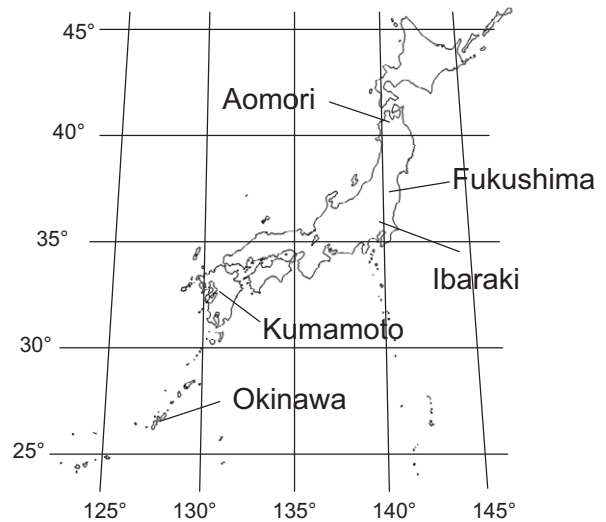


Fig. 1. Locations of sampling sites of soil samples

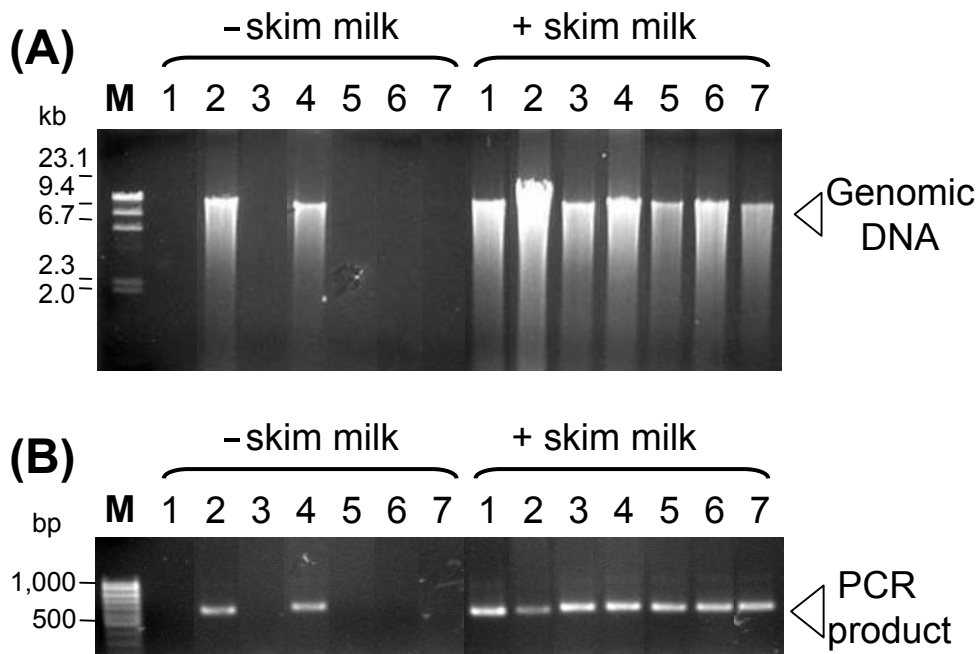


Fig. 2. Agarose gel electrophoresis of DNA and PCR products extracted from sample soils (1–7)

FastPrep was used for extracting DNA from soils (A). PCR products from these extracts were amplified with bacterial 16S rDNA universal primer set (338f and 907r) (B) when amended with (+) or without (–) 40 mg of skim milk g^{-1} soil. Numbers show soil samples listed in Table 1. M: Molecular marker (A: λ /Hind III digest, B: 100 bp ladder).

most of the RNA added (Fig. 3-A). Then they were treated with RNase and subsequently with phenol and chloroform. The band intensity of genomic DNA decreased after the treatment (Fig. 3-A). Moreover, the extracts with RNA were darker, due presumably to co-extracted humic substrates which inhibited PCR (Fig. 3-B). Soil harbors a variety of substances that inhibit molecular techniques such as PCR, restriction enzyme digestion, and hybridization. It is difficult to remove all humic substances from soil DNA extracts²². Removing RNA and concomitant purification procedures are time-consuming and expensive. The use of skim milk is more suitable for PCR (Fig. 3-B) and cost-effective.

The contamination of extraneous DNA from skim milk was negligible; genomic DNA was not detected from skim milk, and subsequent PCR of 16S rDNA did not produce any appreciable band. Moreover, skim milk did not affect DGGE patterns when used during the DNA

extraction step. These results indicate that skim milk does not affect the quality of DNA for microbial community analysis.

The combination of the commercial kit and skim milk was most effective in extracting PCR-suitable DNA from a wide range of recalcitrant soils. In the case of red soils with organic matter in Okinawa, such as Kunigashira Marji (red and yellow soil, Acrisols), Shimajiri Marji (dark red soil, Nitisols) and Jargaru (gray tableland soil, Vertisols), the addition of skim milk also increased the efficacy of DNA extraction (Miyamaru, N., Okinawa Prefectural Agricultural Experiment Station, personal communication). Some reports showed that competitors such as skim milk were effective in clay-rich soils^{3,4,28}. Soil properties such as high clay content and low organic matter content may contribute to high adsorption of DNA. The skim milk method is effective for soils which are difficult to extract DNA because of their high adsorp-

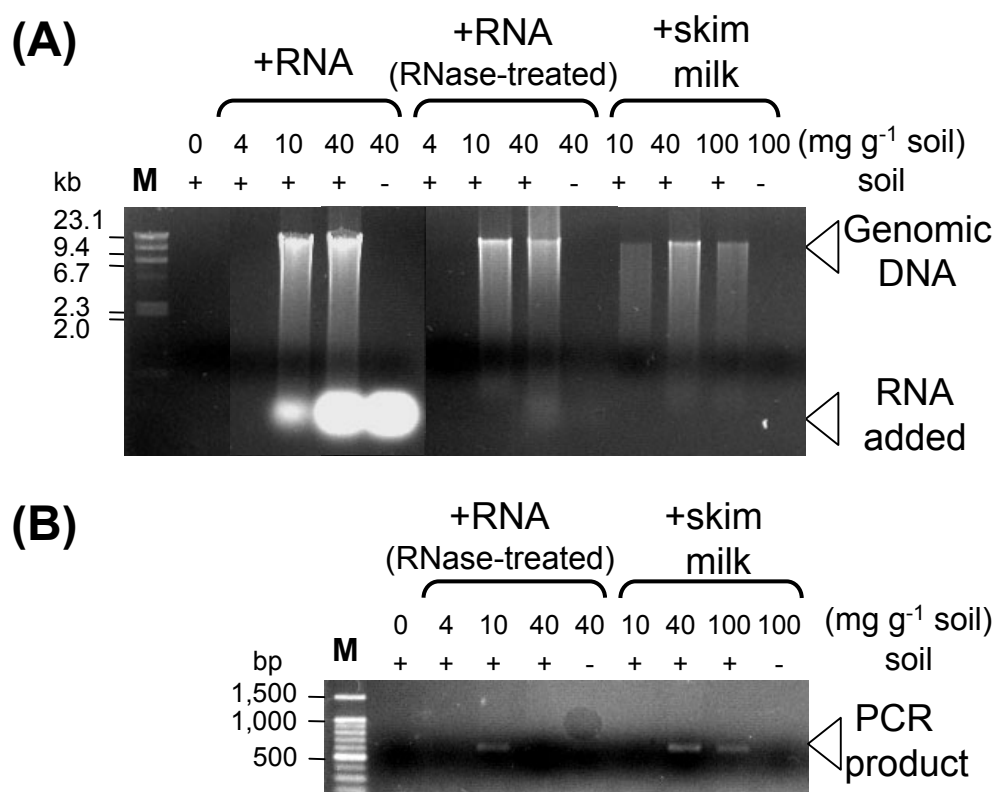


Fig. 3. Agarose gel electrophoresis of DNA and PCR products extracted from soil 1

FastDNA Spin kit was used for extracting DNA from soil (A). PCR products from these extracts were amplified with bacterial 16S rDNA universal primer set (B) when amended with RNA at a rate of 4, 10 or 40 mg g⁻¹ soil or with skim milk at a rate of 10, 40 or 100 mg g⁻¹ soil. When RNA was used, the extracts were treated with RNase after extraction. The extracts from 50 mg of skim milk or 20 mg RNA per 1,100 µL of extraction buffer (corresponding to 100 mg of skim milk or 40 mg RNA g⁻¹ soil respectively) without soil by FastDNA Spin kit for soil were also used as controls. +: 500 mg of soil 1 was used, -: extraction buffer without soil was used. M: Molecular marker (A: λ/Hind III digest, B: 100 bp ladder).

tion to soil particles. Our preliminary data showed that skim milk addition did not increase the amount of extracted DNA from sandy soil. However, we were unable to identify specific factors, determining the efficiency of DNA extraction.

Future prospect

Many molecular methods have been developed to analyze DNA extracted directly from soil. In particular, the PCR technique is widely employed. Specific primer sets allow us to detect and to monitor the genes carried by pathogens²⁸ or genetically modified organisms. Universal primer sets, on the other hand, amplify certain genes (ribosomal RNA genes in many cases) of the whole bacteria or eukaryotes to reveal the structure of the microbial community⁶.

PCR products amplified with a universal primer set for ribosomal RNA genes (rDNA) are the mixtures of rDNA amplicons derived from various kinds of microorganisms. The resulting mixture can be cloned into an appropriate vector for sequencing. Sequence similarity is searched using the internet, such as the BLAST network service of the GenBank database to identify the nearest relatives of the sequenced genes. Some electrophoresis techniques can also be used to separate PCR products. For example, DGGE (denaturing gradient gel electrophoresis) can separate gene amplicons that have similar lengths but different melting point and hence different primary sequences¹⁹. TGGE (temperature gradient gel electrophoresis), SSCP (single-strand conformation polymorphisms)¹⁴ and T-RFLP (terminal restriction fragment length polymorphisms)¹⁶ are the alternative methods. These electrophoresis techniques make it possible to analyze many samples at a time and facilitate monitoring the change of microbial community structure and the estimation of their diversity.

Molecular methods, including PCR amplification may, however, incite biases through primer specificity and amplification of different targets, preventing full recognition of microbial diversity⁹. Thus, new approaches without PCR, such as direct DNA cloning⁵ or DNA microarray⁸ have been devised. Genomic libraries derived from eDNA are termed metagenomic libraries⁵. Such metagenomic libraries can be used to identify novel genes from unculturable microorganisms that are significantly responsible for ecosystem processes. They are effective for isolating enzymes involved in biosynthesis of novel pharmaceuticals or other industrial uses in addition to elucidating the real microbial community structure. Recently, oligonucleotide microarrays (microchips) have been used widely in molecular biological studies

and have shown a great potential for environmental diagnostics. In conclusion, nucleic acid extraction methods should further be improved to recover longer DNA fragments or mRNA for the above purposes.

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