CHAPTER 5

Discussion

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5.1 Overview

A key step for culture-independent nucleic acid approaches is the direct extraction of nucleic acids from environmental matrices. The parameters critical to effective recovery of nucleic acids include the efficiency of cell lysis, efficiency of nucleic acid recovery after lysis, and purification from contaminating humic acid-like organic matter (Hurt *et al.*, 2001). The efficiency of the extraction is of equal importance. High DNA yields are important to enable low detection limit and to ensure that the DNA sample is representative of the soil gene pool (Bürgmann *et al.*, 2001). This is increasingly important as molecular ecology focuses more on quantitative and activity-related analyses. Extraction methods failing to lyse certain cell types or cells in protected soil habitats would introduce bias into the subsequent analyses. Although many methods have been published and successfully used, the effective recovery of nucleic acids from environmental samples, particularly from soils, is still a challenge. This study was successful in developing a rapid method to extract and purify PCR-ampliable method DNA from mangrove soil.

5.2 DNA extraction methods

Many published direct lysis method for extracting DNA from soil and sediments are available, but only eight direct lysis methods have been selected in this study (Table 3.1; section 3.3.1.1). These methods were selected because they are all simple to perform, do not require toxic reagent/materials, inexpensive and utilize commonly available reagent/materials in the lab. None of the methods selected had used mangrove soil as samples. The eight selected published DNA extraction methods differed in the processing time and amount of sample required to process soil (Table 5.1). The methods required between 2.2 to 6.8 h processing time and 0.1 to 10 g of soil to obtain microbial DNA.

Table 5.1 Analysis of processing time and amount of samples used.

Direct lysis method	Time (h)	Soil weight (gram)
1. Selenska and Klingmuller, 1991	*3.0	2
2. Tsai and Olson, 1991	*3.9	1
3. Tebbe and Vahjan, 1993	2.6	5
4. Yeates *et al.*, 1997	6.8	10
5. Picard *et al.*, 1992	2.2	0.1
6. Zhou *et al.*, 1996	*4.8	5
7. Cho *et al.*, 1996	3.2	5
8. McDonald *et al.*, 1999	*6.7	2

Note:
* DNA precipitation and incubation requires overnight (24 h)

All the DNA extraction methods were compared based on their DNA yield and purity. Among the eight published direct lysis method, Method 7 (Cho *et al.*, 1996) produced better DNA yield; which was 3.03330 µg/µl (Table 3.2; section 3.3.1.2). This, could be explained by the use of extraction buffer containing high concentration of lysozyme (20 mg/ml), SDS solution and phenol/chloroform/isoamyl (25:24:1).

In all cases, DNA samples obtained were of low purity, apparently because of contamination with protein and humic materials. DNA extracted using Method 1 (Picard *et al.*, 1992) had a significantly higher. This could possibly be explained by the usage of extraction buffer containing polyvinylpolypyrrolidone (PVPP) which was used to remove humic acids and other phenolic impurities by adsorption.

Therefore, four different extraction procedures were identified on the basis of (i) extraction buffer; (ii) mechanical cell lysis; (iii) chemical and enzymatic cell lysis; and (iv) removal of cell fragment were compared with expected DNA yields.

Eight extraction buffers for DNA lysis from mangrove soil were tested. Extraction buffer from EB 5 (Picard *et al.*, 1992) produced better DNA yield (Table 3.4). This was possibly because the extraction buffer from EB 5 contained Tris and EDTA to protect the DNA from nuclease activity, sodium chloride to provide a dispersing effect to the solution, and polyvinylpolypyrrolidone (PVPP) (Picard *et al.*, 1992).

Three types of mechanical cell lysis were tested. These mechanical cell lyses methods were vortex, rotex and sonication. In comparing the different mechanical cell lysis, DNA extraction using sonication at 2 min produce better DNA yield; which was 0.46710 µg/µl (Table 3.5). According to Moré *et al.*, (1994), mechanical cell lysis is effective for cell disruption. However, violent and thorough

treatment to disrupt cells may damaged the DNA (Picard *et al.,* 1992). Sonication is an efficient method of releasing bacteria attached to soil aggregates (Porteous and Armstrong, 1997; Frostegård *et al.*, 1999). Sonication was described as the most efficient means of releasing the natural bacterial population attached to soil aggregates (Ramsay, 1984). This indicates that sonication could be effective not only in extracting DNA but also in releasing protected bacteria, making them available for subsequent lytic treatments.

Comparison of DNA extraction using three reagents, which were SDS, lysozyme and proteinase K was also performed. From the results (Table 3.6), in terms of DNA yield, lysozyme produced the most satisfactory DNA yields (0.35055 µg/µl). A final component of many DNA extraction techniques is enzymatic lysis. Lysozyme (Bruce *et al.*, 1992; Erb and Wagner-Dobler 1993; Herrick *et al.*, 1993; Holben, 1994; Rochelle *et al.*, 1992; Tebbe and Vahjen, 1993) have been employed to promote cell lysis, and digestion is the most widely used procedure. Because of a lack of comparative studies, it is unclear what effect the addition of an enzymatic lysis step has on DNA yield (Miller *et al.*, 1999).

Comparison of DNA extraction using reagents for removal of cell fragment used Tris-HCl (pH 8.0)-saturated phenol, saturated phenol-chloroform (1:1), phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). Tris-HCl (pH 8.0)-saturated phenol produced the best DNA yields (4.60005 µg/µl). Many authors have used solvents such as phenol, phenol–chloroform, chloroform–isoamyl alcohol (Ogram *et al.*, 1987; Selenska and Klingmüller, 1991; Tsai and Olson, 1991, 1992; Osborn *et al.*, 1993; Smalla *et al.*, 1993; Tebbe and Vahjen, 1993; Tsai *et al.*, 1993) for removal of cell fragment.

Using results obtained from the method analysed, a new method was developed. It was named the Soil DNA Direct Lysis Method. The new method utilized extraction buffer from Picard *et al.* (1992) as the extraction buffer, lysozyme as a enzymatic cell lysis, sonication as a mechanical cell lysis and Tris-HCl [pH 8.0]-saturated phenol (1:1) proved to yield higher DNA efficiency (Table 3.8).

Compared to other published direct lysis methods, Soil DNA Direct Lysis Method is relatively simple, easy to perform and rapid to obtain crude DNA (Table 5.2). Furthermore, the separation of DNA from soil was considerably faster than all the other methods, which means more samples could be handled, and no aerosols were formed during separation.

Table 5.2 Analysis of processing time and amount of samples used using Soil DNA Direct Lysis Method.

New Direct lysis method	Time (min)	Amount of sample used (gram)
Soil DNA Direct Lysis Method	5	0.2

5.3 DNA purification methods

It is often more difficult to purify microbial DNA from soil than from other environments such as water (Porteous and Armstrong, 1991). The humic acids and phenolic compounds in soil or sediment are difficult to remove, making DNA purification, a critical step. Their presence is revealed by the brownish color of the recovered DNA extract. Tebbe and Vahjen (1993) found that 0.2–1% of soil humic acids were extracted with nucleic acids from soils containing 1–1.97% total organic carbon (TOC). In fact, humic acids have physicochemical properties similar to those of nucleic acids (reviewed in Harry *et al.*, 1999), so that they can compete with nucleic acids for adsorption site during the purification step when minicolumns are used. These contaminants can decrease the efficiency of DNA/DNA hybridization (Steffan, 1988; Tebbe and Vahjen, 1993), or inhibit certain restriction endonucleases (Porteous and Armstrong, 1991; Tsai and Olson, 1991; Jacobsen and Rasmussen, 1992). They could also inhibit *Taq polymerase*, the key enzyme in the PCR, by chelating Mg^{2+} ions. Tebbe and Vahjen (1993) reported that 0.08 µg/ml humic acids is sufficient to inhibit the most sensitive *Taq polymerase* while 0.5–17 µg/ml could inhibit restriction enzymes.

Table 3.9 showed the analysis of four selected published DNA purification methods. These methods were selected because all the methods are simple, did not require toxic reagent/materials, inexpensive and utilize usual reagent/materials in the lab. None of the methods selected used mangrove soil as samples. Four selected published DNA purification methods differed in time of sample required to process supernatant containing microbial DNA (Table 5.3). DNA purification method required 2 to 4.5 h of supernatant containing DNA to obtain pure microbial DNA.

Table 5.3 Analysis of processing time of samples with five published purification method.

Method	Time (h)
1. Steffan and Atlas, 1998	* 1
2. Yeates *et al.*, 1997	4.5
3. Großkopf *et al.*, 1998	3.8
4. Miller *et al.*, 1999	*0.5

Note:
* DNA precipitation and incubation required overnight (24 h)

In this study, four published DNA purification methods were selected and evaluated. All DNA purification methods were compared based on DNA yield and purity. Among the four DNA purification methods, Method 3 (Yeates *et al.*, 1997) produces better DNA yield; which was 0.06870 µg/µl (Table 3.10). In all cases, DNA samples obtained were of low purity, apparently because of contamination with protein and humic materials. Method 4 (Großkopf *et al.*, 1998) produces low contamination of protein materials; which was 1.61046. This was possibly because this method was purified by two rounds of precipitation with isopropanol. Method 2 (Yeates *et al.*, 1997) produces low contamination of humic acid; which was 0.79368. This was possibly because this method uses phenol/chloroform (25:24) and chloroform/isoamyl alcohol (24:1).

Proteins can be salted-out using saturated salt solutions, either sodium chloride (Holben *et al.*, 1988; Selenska and Klingmüller, 1991), potassium chloride (Torsvik *et al.*, 1990), ammonium acetate (Steffan and Atlas, 1988; Pitcher *et al.*, 1989; Knaebel and Crawford, 1995; Xia *et al.*, 1995), potassium acetate (Hilger and Myrold, 1991; Porteous and Armstrong, 1991; Smalla *et al.*, 1993) or sodium acetate (Holben *et al.*, 1988). The proteins precipitate during the centrifugation at

low speed and the nucleic acids are recovered in the supernatant. Harry *et al.* (1999) recommended deproteinisation in NaCl, because it allows soil particles to precipitate with the cell fragments and proteins, giving cleaner DNA without using toxic organic solvents. The effect of reagents NaCl, NH_4Ac, KaAc and NaAc for removal protein contamination were evaluated (Table 3.11). In this study, KaAc had a significantly for removal protein contamination.

When added in the first step of the procedure for organic rich samples, some products can improve the purity of the recovered DNA by complexing humic compounds. These products are polyvinylpolypyrrolidone (PVPP) (Holben *et al.*, 1988; Steffan *et al.*, 1988; Porteous and Armstrong, 1991; Rochelle *et al.*, 1992; Bakken and Lindahl, 1995; Gray and Herwig, 1996; Rheims *et al.*, 1996) and cetyltrimethylammonium bromide (CTAB) (Knaebel and Crawford, 1995; Saano *et al.*, 1995; Xia *et al.*, 1995; Lee *et al.*, 1996; Zhou *et al.*, 1996; Chandler *et al.*, 1997; Porteous *et al.*, 1997). In spite of the addition of these compounds, numerous investigators showed that the obstinacy of contaminants which inhibited the activity of certain restriction enzymes (Porteous and Armstrong, 1991; Rochelle *et al.*, 1992). Purification steps are then required. Both CTAB and PVPP can effectively remove humic materials, but unlike PVPP, CTAB resulted in DNA loss (Table 3.12). In this study, 20% (w/v) CTAB had a significantly for removal humic asid contamination. From the results of precipitation of nucleic acids using ethanol, Isopropanol and PEG 6000, it was clear that the usage of PEG 6000 provide the best DNA yield (Table 3.13).

The purity of the extracted DNA varied as determined by the ratio of A_{230}, A_{260} and A_{280}. In all cases, DNA samples obtained were of low purity, apparently because of contamination with protein and humic materials. Nucleic acid

precipitation using ethanol had the lowest amount of contamination from protein materials (1.06308), and humic materials (0.45584).

Nucleic acids are concentrated by precipitating them with ethanol, isopropanol or polyethyleneglycol (PEG) (Ogram *et al.,* 1987; Bruce *et al.,* 1992; Xia *et al.,* 1995; Porteous and Armstrong, 1997). PEG or isopropanol can be used instead of ethanol, and this reduces the total volume of the sample (0.54 vol. of isopropanol or 0.5 of PEG instead of 2.5 vol. of ethanol). Porteous (1997) considered that alcoholic precipitation (contrary to PEG precipitation) favored the co-precipitation of humic acids. Cullen and Hirsch (1998) compared precipitation with ethanol, isopropanol and PEG 6000. Ethanol yielded lower recovery of DNA and more humic substances than PEG 6000 or isopropanol. PEG 6000 provided good yields, but had to be removed by phenol extraction as it might interfere with PCR. Unfortunately, this extra step reduced the final DNA yield. The authors, therefore, recommended using ethanol, which provided good yield without increasing the contamination of protein and humic acid.

A new method was developed based on the results presented in during purification. It is named Soil DNA Purification Method (Table 3.14). Microbial DNA was purified using Soil DNA Purification Method and was analysed spectrophotometrically. DNA concentration obtained was 0.03795 µg/µl, respectively. The $OD_{260}/_{280}$ and $OD_{260}/_{230}$ ratios were 1.18653 and 0.90947, respectively. These low ratios indicated that the DNA extracts probably contained protein and humic material (Steffan *et al.*, 1988).

The Soil DNA Purification Method produces pure DNA compared to other published DNA purification methods tested. Also, protein and humic acid contaminants were removed from the microbial crude DNA and good recovery was

achieved with mangrove soil. Furthermore, Soil DNA Purification Method is very rapid (Table 5.4) and less expensive.

Table 5.4 Analysis of processing time of samples used using Soil DNA Purification Method.

Method	Time (min)
Soil DNA Purification Method	*6

Note:
*DNA precipitation and incubation required 1 h

5.4 PCR amplification of extracted and purified mangrove soil microbial DNA

PCR which is a sensitive tool for detecting and characterizing organisms from small amounts of DNA has been widely applied. The DNA-based approach often consists of amplification by PCR of the ribosomal RNA genes (rDNA) from the whole bacterial or fungal community DNA. The use of PCR template of the bulk DNA that is a mixture of DNA extracted from diverse microorganisms generates rDNA products differing in sequence but generally not in size. The analysis consists of discriminating between these PCR products to obtain a survey of the microbial communities (Roose-Amsaleg *et al.*, 2001).

Table 4.1 shows the analysis of PCR protocols used in this study. Protocol 1 (McDonald *et al.*, 1999), Protocol 2 (Kuske *et al.*, 1998), Protocol 3 (LaMontagne *et al.*, 2002), Protocol 4 (Hurt *et al.*, 2001) were published PCR protocol while, Protocol 5 (Standard Method), was obtained directly from Ian. M. Head (personal communication). Five selected published PCR protocols differed in time of sample required to amplify microbial DNA (Table 5.5).

Table 5.5 Analysis of processing time of samples used using selected published PCR protocols.

Protocol	Time (h)
1. McDonald *et al.*, 1999	3.11
2. Kuske *et al.*, 1998	2.14
3. LaMontagne *et al.*, 2002	1.56
4. Hurt *et al.*, 2001	4.11
5. Standard Method	2.22

All PCR protocol was conducted using crude microbial DNA from mangrove soil. Two PCR primer sets were used; pA and pH to amplify 16S rRNA gene and degradative gene specific primers to amplify related region of a particular degradative gene.

Standard Method was chosen from five PCR protocol tested. This PCR protocol yields of PCR product in less time and instead of frequently used in our lab.

Figure 4.2 shows the yield of PCR products using purified crude soil microbial DNA from five purification methods. From the results, purified crude soil microbial DNA from Method 2 (Yeates *et al.*, 1997) and Soil DNA Purification Method yield PCR product of the expected size 1.5 kb, while other purified crude soil microbial DNA from published method failed to yield any product. This may be because crude soil microbial DNA from Method 2 and Soil DNA Purification Method was sufficiently pure PCR amplified.

In addition to primers sets pA and pH, specific primers designed for the detection of alkane or naphthalene degradation genes were also tested.

The primers were used with crude soil microbial DNA produced by Soil DNA Purification Method (see section 3.3.4) and amplified using Standard Method (PCR protocol).

From the results obtained, all PCR amplifications failed, except PCR amplification using primer sets alkM and alkM' (Figure 4.4; lane 7).

5.5 Future experiment to be conducted

Future experiments should be concentrated on optimizing the PCR amplification technique, either by combination with dot blot hybridization and phosphorimaging or by inclusion of internal standards in the PCR, which seemed to be promising ways of estimating the size of a targeted population. In addition, cloning and sequencing would lead to the identification of microorganisms in the soil mangrove. Nucleic acid microarrays or DNA chips, represent the latest advancement in molecular technology, providing unparalleled opportunities for multiplexed detection of nucleic acids. These studies should lead to a better understanding of the biodiversity of microorganisms in the mangrove soil.

5.6 Conclusion

The major goal of DNA extraction procedures is to obtain the greatest DNA yield, and hence the most representative DNA, from the microbial community. Total extracted DNA should provide a less biased view of soil microbial communities than do culture-based microbiological methods. However, all these extraction methods suffer from shortcomings, such as incomplete cell lysis, DNA sorption to soil surfaces, extraction of enzyme inhibitors from the soil, and the loss, degradation or damage of DNA. Moreover, each purification step, such as repetitive procedures to purify the DNA prior to any molecular DNA study, inevitably induced DNA loss.

This study was successful in developing methods for DNA extraction and purification. The proposed method for the preparation of DNA from soil and for PCR is as follows: SDS-based extraction using extraction buffer containing

lysozyme and CTAB, freeze and thaw cycles, phenol-chloroform extraction and PCR with reaction buffer containing BSA. This procedure will be a rapid and simple method for molecular microbiological study of soil environments.

In summary, this study shows that nucleic acid based techniques provide rapid and valuable tool to amplify microbial DNA from mangrove soil.