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IMPROVEMENT OF DIRECT EXTRACTION AND DETECTION OF BACTERIAL DNA FROM SOIL

Miss Thanwalee Sooksa-nguan

A Thesis Submitted In Partial Fulfillment of the Requirements

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IMPROVEMENT OF DIRECT EXTRACTION AND DETECTION OF BACTERIAL DNA FROM SOIL

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การพัฒนาความรู้ทางอณูชีววิทยาได้ถูกนำมาใช้ในการศึกษาเกี่ยวกับกลุ่มของจุลินทรีย์ในสิ่งแวดล้อมอย่างแพร่หลาย โดยเฉพาะอย่างยิ่งในกลุ่มจุลินทรีย์ที่ยากแก่การแยกเชื้อมาเพาะเลี้ยงให้ห้องทดลอง ในการศึกษาครั้งนี้ได้ทำการพัฒนาวิธีการสกัดดีเอ็นเอจากจุลินทรีย์ที่มีอยู่ในดินโดยตรง พบว่าการสกัดดีเอ็นเอโดยตรงจากดินด้วยวิธีที่ใช้ไลโซไซม์ และโปรทีเนส-เค ตามด้วยสารละลายด่างสามารถสกัดดีเอ็นเอได้ในปริมาณสูง ดีเอ็นเอบริสุทธิ์ที่สกัดได้มีปริมาณ 4 ไมโครกรัมต่อกรัมดินนาข้าว (น้ำหนักแห้ง) ซึ่งเมื่อใช้โปรทีเนส-เคจะให้ประสิทธิภาพในการทำลายเซลล์ของจุลินทรีย์ได้ 82.34% การสกัดโดยวิธีนี้ใช้เวลาเพียง 5 ชั่วโมง โดยจากตัวอย่างที่มีสารประกอบอินทรีย์ในช่วง 0.73 ถึง 62.77% สารประกอบอินทรีย์เหล่านี้จะปนเปื้อนอยู่ในสารละลายดีเอ็นเอที่สกัดได้จากตัวอย่างที่ได้ และสามารถกำจัดออกโดยอาศัย Agarose gel electrophoresis และ Sephacryl S-300 column chromotography จากนั้นใช้เป็น Template ด้วยเทคนิค PCR โดยใช้ RISA primer จากการศึกษาผลของการยับยั้งสารอินทรีย์ที่ปนเปื้อนมากับสารละลายดีเอ็นเอต่อการยับยั้งกระบวนการ PCR ผลพบว่าสารอินทรีย์ที่มีอยู่จะยับยั้งการทำงานของเอ็นไซม์ในกระบวนการ PCR ในระดับหนึ่งและยับยั้งมากขึ้นเมื่อมีในปริมาณที่มากขึ้น ในการศึกษานี้ยังได้ใช้ RISA primer เพื่อศึกษาความหลากหลายของลักษณะดีเอ็นเอจากแบคทีเรียที่ทำการสกัดจากแหล่งดินต่าง ๆ อีกด้วย ในส่วนของประสิทธิภาพความไวในการตรวจสอบพบว่าเมื่อใช้ *Pseudomonas aeruginosa* ที่ปลูกเชื้อลงไปในตัวอย่างต่าง ๆ ที่ผ่านการฆ่าเชื้อพบว่าประสิทธิภาพในการตรวจสอบจำนวนของเซลล์เท่ากับ 1 , 10, 10^3, 10^4 และ 10^5 เซลล์ต่อกรัมของตัวอย่างที่มีปริมาณสารอินทรีย์ปนเปื้อน 0.73% (ดินร่วนปนทราย), 1.07% (ดินเหนียว), 3.38 (ดินทราย), 4.25% (ดินร่วน) และ 62.77% (พีท) ตามลำดับ

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2543

ลายมือชื่อนักศึกษา ……..
ลายมือชื่ออาจารย์ที่ปรึกษา ……..
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The development of molecular biology is allowed scientists to realize that microbial populations in the natural environment are much more diverse than microorganisms, so far isolated in the laboratory. In this study, a rapid method for the direct extraction of DNA from soil was developed. The methodology was developed by using lysozyme and proteinase-K followed by alkali treatment. This approach provides relatively highest yield of DNA recovery. Purified DNA was 4 μg per gram soil (dry weight) sample collecting from rice field. This rapid procedure took at least 5 hours for completion. Extraction efficiency was evaluated based on percentage of bacterial survival. When applied proteinase-K in this extraction protocol the bacterial cell destruction efficiency was 82.34%. By using agarose gel electrophoresis followed by Sephacryl S-300 column chromatography able to separate organic matter and other enzyme inhibitors for extracted DNA in samples which contained high concentrations of organic matter (range between 0.73 to 62.77%). Then it was used as a template in order to determine the inhibitory effect on PCR condition. It was found that the inhibitory effect was increased when using higher amount of DNA template. Moreover, RISA primer was also used to demonstrate the DNA diversification patterns from various soil samples. To determine the detection limit or sensitivity of this system, various numbers of certain amounts of *Pseudomonas aeruginosa* were inoculated into sterilized soil samples prior to direct extraction from soil. It was found that the detection limits of this system were 1, 10, 10^3, 10^4, and 10^5 cells per gram soil (dry weight) that contained the amount of organic matter of 0.73% (loamy sand), 1.07% (clay), 3.38% (sand), 4.25% (sandy clay loam), and 62.77% (peat), respectively.

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา ……..
ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่ปรึกษา ……..
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Finally, thanks to myself for not discourage during the work.

Thanwalee Sooksa-nguan

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LIST OF ABBREVIATIONS

bp	base pair	^{o}C	degree Celsius
cm	centimeter	e.g.	for example
et al.	et alia (and others)	g	gram
Fig.	Figure	min	minute
hr.	hour	ml	milliliter
mg	milligram	nmol	nanomole
µl	microliter	µg	microgram
ng	nanogram	mM	millimolar
%	percent	rpm	round per minute
pp	page	UV	ultraviolet

CHAPTER 1
INTRODUCTION

The conventional microbiological approaches as microscopic observation or cultivation have been widely used for identifying bacteria in the natural environment, eventhough these methods are thought to be insufficient for these purposes. Problems associated with microscopic observation are that (i) the morphology of bacterial cells is generally too simple to serve as a basis for sound identification and to allow reliable classification and (ii) microorganisms may adopt different morphologies under different physiological conditions (Roszak and Colwell, 1987). Cultivation methods, e.g., viable plate count and most-probable-number (MPN) techniques, have been used for quantification of active cells in environmental samples. However, because the medium used in these methods always selects for certain organisms, the results are always biased toward these organisms (called cultivation bias). In addition, some bacterial cells may be viable but not be able to replicate under stress conditions (Oliver et al., 1991; Roszak et al., 1987). These problems have been realized by the observations that direct microscopic counts of bacteria in aquatic and soil habitats exceed viable plate counts by several orders of magnitude (Ferguson et al., 1984; Jones, 1977; Torsvik et al., 1990). In most cases, conventional cultivation methods can detect (and hence recover) only a small fraction of the microbial consortia.

The environmental sample as soil represents a highly heterogeneous environment consisting of solid, liquid and gaseous phases. The dominating soil solid phase is composed of inorganic (sand, silt and clay) and organic (humic matter) materials, which are to varying degrees complexes with one another. The soil biota, including soil microorganisms such as bacteria, fungi and protozoa, are known to inhabit different sites in the soil pore matrix. Organisms associate in particular with soil solids, e.g. clay/organic matter complexes, in soil pores conductive to their survival. Although bacteria are the most abundant life forms on earth, knowledge of microbial community structures and population dynamics is still minimal. An estimated 80 to 90% of

microorganisms in soil are as yet unidentified (Amann et al., 1994). Due to a wealth of information is available about many microorganisms isolated from soil by culturing techniques. The classical plate count, which is the most direct enumeration method for microbes, is of fairly limited value. The efficiency of recovery of species is likely to be poor. Actually, the nature of a substantial part of the soil microbiota is essentially unknown because of their unculturability. 'Unculturable' cells are microorganisms that are phylogenetically similar or identical to the culturable minority but in a physiological state that makes them recalcitrant to culturing (Rondon et al., 1999). Selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat. Furthermore, many microorganisms are bound to sediment particles and are thus not detected by conventional microscopy.

The advent of new methods redefines the scope. This has been the case for microbial ecology in the last two decades, when microbiologists started to use molecular ecological methods, particularly that known as the 16S rRNA framework (Amann et al., 1995; Olsen et al., 1986; Pace et al., 1986), for analyzing natural bacterial populations.

Molecular biological techniques offer new opportunities for the analysis of the structure and species composition of microbial community without cultivation. The DNA is extracted directly from a microbial consortium, so that the cultivation bias is eliminated. The extraction and analysis of total microbial community DNA from soil by using the DNA technique is useful for several purposes (Trevors and Van Elsas, 1989). First, it provides insight in the prevalence of specific genes in microbial communities in the soil ecosystem, possibly resulting in a better understanding of natural selection of specific microbial groups under the influence of soil conditions. Secondly, by using 16S/18S or 23S/25S ribosomal DNA sequences as 'signature molecules' (biomarkers), overall community DNA analysis may help to describe microbial communities in terms of their population structure (Gerard et al., 1993; Holger et al., 1997; Schwieger and Tebbe, 1998; Virginia et al., 1999). This can be achieved by applying temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) to PCR products generated with sets of conserved primers, resulting in a type of community structure data which were not obtainable

previously (Ferris et al., 1996; Gerard et al., 1993, Holger, et al., 1997). The advantages of the use of this gene are: (i) all organisms harbor this gene, and their evolutionary relationships can be deduced (Woese, 1987), (ii) a large number of sequences of different organisms are stored in databases (Maidak et al., 1999), (iii) universal PCR primers can be designed using sequences in several highly conserved regions, and (iv) bacterial cells can be detected by *in situ* hybridization targeting abundant ribosomes in cells. Using the 16S rDNA sequences, bacteria are classified into the phylogenetic groups proposed by Woese and the identification of natural populations follows this phylogenetic classification. Finally the application of microbial community DNA extraction methodologies allows investigations on the nature of non-culturable cells, which are known to abound in soil.

Since these molecular methods are capable of detecting microbial populations that are hardly detected by conventional culture-dependent methods (Nold et al., 1996; Sievert et al., 1999; Wagner et al., 1993), researchers have started to apply them for analyzing microbial consortia in environmental biotechnological processes. Several newly emerging methods for monitoring specific microbial genotypes in environmental samples and for analyzing microbial community structure at the genetic level, which do not require the culturing of the microorganisms from the samples, depend upon the efficient recovery of DNA as an essential part of the procedures. These methods are advantageous because they avoid problems associated with enumeration procedures on the basis of culturing organisms from environmental samples (Roszak et al., 1987). Two different approaches have been proposed for the recovery of DNA from environmental samples.

Cell extraction method

Cell extraction method is the separation of microbial cells from soil particles, followed by subsequent cell lysis and extraction to obtain total bacterial community DNA from soil (Holben et al., 1988). It was initially reported by Faegri et al. in 1977; Torsvik and Goksoyr in 1978 for recovering bacterial DNA from soil; the methodological approach involves the separation of bacterial cells from the soil particles by differential centrifugation followed by lysis

and subsequent separation of bacterial DNA or RNA from soil organic carbon by a reries of chromatographic separations. As with most first generation procedures, this was time consuming and inefficient; the original procedure required sample sizes of 60-90 g and at least 3 days for completion (Trosvik, 1980). Holben and colleagues (1988) used this basic approach, but modified the procedure by using polyvinylpolypyrrolidone (PVPP) to remove soil organic matter from cell preparations as well as to simplify purification of recovered DNA by using the hydroxyapatite column chromatography with repetitive cesium chloride density gradient ultracentrifugation purification steps. This approach showed the recovery about 33% of the bacterial cells from soil and that the recovered DNA was of sufficient purity to perform gene probe detection of specific genotypes in the soil bacterial community and to perform restriction enzyme and Southern blot analysis. However, the disadvantage of this methodology is inefficient of separation of bound cells or DNA from soil particle.

Direct DNA isolation method

Second generation methods are streamlined and require smaller sample sizes, thereby allowing simultaneous processing of multiple samples (Kuske et al., 1998; Tsai and Olson, 1991). Most current procedures are based on the direct lysis of cells in the presence of the soil (rather than first separating cells from sand, silt and clay particles), followed by extraction of nucleic acids from soils the methods used often include various combinations of the following basic elements: physical disruption, chemical lysis, and enzymatic lysis.

Four different physical disruption techniques, freeze-thawing (Hugenholtz et al., 1998; Kuske et al., 1998; Tsai et al., 1991), bead mill homogenization (Kuske et al. 1998; Liesack and Stackebrandt, 1992; Steffan et al., 1988), ultrasonication (Picard et al., 1992), and grinding under liquid nitrogen (Volossiouk et al., 1995; Zhou et al., 1996), have been described. However, freeze-thaw disruption and bead mill homogenization are the most favourable. It is well established that bead mill homogenization yields more DNA than freeze-thaw disruption yields (Kuske et al., 1998, More et al., 1994). The drawbacks to bead mill homogenization include the fact that larger amounts of contaminating humic acids are recovered (Leff et al., 1995; Ogram et

al., 1987; Smalla et al., 1993) and the fact that, in some instances, the DNA is sheared (Leff et al., 1995). The chemical lysis procedures used in the methods that have been described also vary, but the lysis mixtures can be categorized into mixtures that contain detergent (either sodium dodecyl sulfate [SDS] [Kuske, 1998; Ogram, 1998; Zhou et al., 1996] or Sarkosyl [Holben, 1994; Smith and Tiedje, 1992]), mixtures that contain NaCl, and mixtures that contain various buffers (usually Trisor phosphate, pH 7 to 8). The modifications of the basic chemical lysis techniques include high-temperature (60^{o}C to boiling) incubation (Kuske et al., 1998; Smith et al., 1992), a phenol (Smalla et al., 1993, Tsai et al., 1991) or chloroform (Herrick et al., 1996) extraction step, and incorporation of chelation agents (EDTA and Chelex 100) to inhibit nucleases and disperse soil particles (Jacobsen and Rasmussen, 1992). The efficacy of diverse chemical lysis components remains largely unknown since only overall DNA recovery after cell lysis and subsequent purification is reported. Furthermore, the cellular lysis efficiency at each step of a protocol is reported only rarely (More et al., 1994). A final component of many DNA extraction techniques is enzymatic lysis. Lysozyme (Herrick et al., 1993; Tsai et al., 1991.), proteinase (Zhou et al., 1996), achromopeptidase (DeGrang and Bardin, 1995), and pronase E (Holben, 1994) have all been employed to promote cell lysis, and lysozyme digestion is the most widely used procedure.

The direct DNA isolation from soils or sediments and another complex environmental matrices (Holben et al., 1988; Holger et al., 1997; Ogram et al., 1987) which involves the initial separation of microorganisms from the environmental matrix prior to lytic release of DNA. Another approach, which refers to as the direct lysis method, has been developed by Ogram et al. (1987) for recovering DNA from sediments. Some methodologies such as using physical disruption without separating the cells from the environmental matrix followed by alkaline extraction of the DNA, and purification of the extracted DNA was performed, cesium chloride density gradient centrifugation, and hydroxyapatite column chromatography (Steffan et al., 1988).

DNA purification

Although direct lysis of bacterial cells and subsequent purification of the released nucleic acid typically yields higher amounts of DNA than the cell fractionation method, it also extracts much more humic compounds than the original cell fractionation method (Torsvik, 1980).

Humic substances are naturally occurring, hetergeneous organic substances that are yellow to black in colour and resistant to degradation (Aiken et al., 1985). They contain anionic functional groups (e.g., partially deprotonated phenolic and carboxylic groups), as well as hydrophobic components (aromatic and aliphatic moieties) (Stumm and Morgan, 1996). It has been previously reported that trace amounts of humic substances can inhibit the amount of enzyme-dependent manipulation of nucleic acid such as endonuclease digestion of extracted DNA by the restriction enzymes or PCR (Steffan et al., 1988; Tsai et al., 1992a), are hampered by the present of inhibitory natural substancy in environmental samples. The main impediment when using PCR on environmental soil sample is the presence of humic substances or other components such as iron that can inhibit the polymerase activities or binding of primers and reduce the sensitivity of detection (Tsai et al., 1992a) and also cause false-negative results from contaminated DNA template. Due to the impurity in DNA was extracted, which may not be removed by standard DNA purification techniques, therefore, many of the modified protocols have been developed to minimize humic contamination as defined in Table 1.1.

Table 1.1 Summary of the methodologies for elimination of humic substances during extraction procedure

Group of researcher	Applied methodologies	Soil type
Tsai et al., 1991	Elutip-d column	Sediment
Selenska and Klingmuller, 1991	CsCl-EtBr density gradient centrifugation at refraction index 1.386, 180,000 x g for 16 h and dialysed overnight against TE buffer	Sandy loam
Jacobsen et al., 1992	Gene-Clean glass beads	Sandy loam
Tsai et al., 1992b	2 types of spun columns: a.) Bio-Gel P-6 and P-30 polyacrylamide gel columns b.) Sephadex G-50 and G-200	Sediment
Smalla et al., 1993	CsCl and KAc precipitation followed by spermine-HCl or glass milk purification	Sandy loam, Silt loam
Zhou et al., 1996	a.) single minicolumn (Wizard minicolumn) b.) double minicolumn c.) gel plus minicolumn d.) gel plus centrifugal concentrator	Loams, Sandy loams, Sandy clay loam (clay content ranging from 5 to 31%)
Jackson et al., 1997	Sepharose 4B, Sephadex G-200, and G-50	Woodland soil, Hydrocarbon-contaminated, soil, aquatic sediment, aquatic biofilm
Miller et al., 1999	SpinBind column and Sephadex G-200	Silt loam, Sediment
Edgcomb et al., 1999	Sephacryl S-300 microspin column	Sediment

Recent developments in nucleic acid extraction methods for environmental samples have focused on purification strategies to remove humic substances (Herrick et al., 1993; Moran et al., 1993; Tsai et al., 1992a; Young et al., 1993). A variety of purification methods to remove humates have been described including selective precipitations and extractions (Guo et al., 1997), various chromatographic matrices (including ion exchange, size exclusion, and hydrophobic

interaction matrices) (Ogram, 1998), captured by agarose gels (Young et al., 1993), and magnetic beads (Jacobsen, 1995). Times required for complete processing typically range from 4 to 12 hrs.

One of the most widespread techniques is the use of spin columns packed with various matrices. One commonly used packing gel is Sephadex G-200, which usually removes humic substances sufficiently to yield DNA that is pure enough for amplification, although further techniques may be required (Tsai et al., 1992b). In 1997, Jackson and colleagues also reported about the comparison of efficiency of three different matrixes (Sepharose 4B, Sephadex G-200, and Sephadex G-50) as a means of remaining humic contaminants from DNA extracted of environmental samples. It was demonstrated that Sepharose 4B gave superior separation of DNA from humics, and DNA in this way showed consistently greater amplification than DNA purified by the other materials.

A few companies (e.g. MoBio, www.mobio.com; Bio101, www.Bio101.com) now market commercial kits for purification of soil DNA which have been running successfully for many applications, and provide very rapid purification (less than 30 min) (Ogram, 2000).

Even though significant advances have been made in the efficiency and speed of DNA purification from soils, this is still the limiting step in many cases. It is likely that the primary reason so many procedures for soil DNA purification have been published is that most procedures are optimized for a specific soil. This implies that any given procedure may not be universally applicable to all soil (Zhou et al., 1996). Soils are highly variable with regard to physical and chemical properties, particularly with regard to the structure of organic carbon. Co-purification of organic carbon, particularly humic and fulvic acids, is the primary obstacle to efficient recovery of DNA suitable for amplification by PCR. A procedure that efficiently separates humic compounds from one soil may not do so from another soil (Ogram, 2000). Operator variability is also likely to be a factor in the variability of DNA recovery between laboratories, prompting the development of new procedures by individual laboratories. Even within laboratories, considerable variability between quality and amount of isolated DNA is only sometimes observed.

Development of an acceptable strategy for evaluating lysis and purification efficiency would assist in developing a universal purification procedure. A variety of approaches have been used, including those based on recovery of exogenous labeled nucleic acids (Lee et al., 1996; Ogram et al., 1995) or cells (Steffan et al., 1988) added to a sample, and those based on estimations of purification from crude DNA extracts from a given sample (Sandaa et al., 1998; Zhou et al., 1996). None of these approaches is without drawbacks, and the discipline would be well served if a benchmark method for assessing purification efficiency was developed.

The detection system

The study of prokaryotic biodiversity has been hampered for many years by the difficulty of characterizing microorganisms prior to their isolation in pure culture. This methodological limitation has forcefully restricted and based our view of microbial diversity, very often in an unpredictable way. The lack of strongly informative morphology found in complex microorganisms, has precluded the understanding of the community structure at the microbial level in most ecosystems. Nevertheless, the situation has been changed in the last 10 years. PCR has allowed the study of microbial genes directly amplified from samples without any need of cultivation. In some ways PCR amplification is reminiscent of a liquid culture or enrichment (and could have many of its limitations and biases). However, since whole cell growth is not required, the methodology in principle is much more universal and direct. In addition, even if the biodiversity studies based on PCR are biased (a controversial issue) they are so in a different way from those based on culture, and therefore provide very useful information. Naturally, one critical step in these studies is the choice of gene(s) to be amplified (Table 1.2) (Garcia-Martinez et al., 1999).

Table 1.2 Examples of the PCR techniques demonstrated in environmental system

Microorgainsm	Gene probe	Habitat	Detection limit (cells per g soil or per 100 ml water)	Reference
E. coli	16S ribosomal gene	Sediment Soil	Less than 10 Less than 3	Tsai et al., 1992a
E. coli	16S ribosomal gene	Sediment	Less than 1	Tsai et al., 1992b
Rhizobium leguminosarum	*npt II*	Soil	1-10	Pillai et al., 1991
Pseudomonas fluorescens R2f	*pat*	Soil	0.602	Van Elsas et al., 1991
Frankia spp.	16S ribosomal gene	Soil	0.2×10^5 genomes	Picard et al., 1992
P. putida VNM43	*mer A*	Soil	4.3×10^4	Tsai et al., 1991

PCR is a very powerful, specific, and sensitive analytical technique with application in many diverse fields, including molecular biology, clinical diagnosis, forensic analysis, and population genetics. One approach to studying complex ecosystems is to study subsets of the taxa present in hopes of better understanding the roles of individual groups within the large community. If specific gene are amplified from community DNA, it is desirable that such target sequences be widely abundant within capable of amplifying such genes exist. Perhaps the greatest advance in microbial ecology methods during the second half of the twentieth century came when Norman Pace (Hugenholtz et al.,1998) translated Carl Woese's concepts of rRNA phylogeny (Woese, 1987) to the analysis of natural microbial communities. This greatly expanded our knowledge of the diversity of soil bacteria and provided the basis for most current methods of diversity analysis. Most of these methods are based on the diversity of 16S rDNA sequences present in a sample, and include cloning and sequence analysis (Liesack et al., 1992), amplified rDNA restriction analysis (Moyer et al., 1994), denaturing gradient gel electrophoresis (Muyzer et al., 1993) and terminal-restriction fragment length polymorphism analysis (Liu et al., 1997). These approaches are useful for analyzing the richness (i.e. numbers of species) of small

segments of a soil community through the use of PCR primers specific for a given phylogenetic subset of the total community, such as the α-Proteobacteria**.**

In particular, sequence variation in rRNA has been exploited for inferring phylogenetic relationships among microorganisms and for designing specific nucleotide probes for the detection of individual microbial taxa in natural habitats. These techniques have also been applied to determining the genetic diversity of microbial communities and to identifying several uncultured microorganisms in complexes in environmental sample. They constitute the cloning of ribosomal copy DNA or PCR-amplified ribosomal DNA (rDNA) followed by sequence analysis of the resulting clones (Dunbar et al., 1999; McCaig et al., 1999). For bacterial diversity assessments the most widely specific gene, 16S rRNA genes, have been used predominantly in several studies by Wang, GC.-Y., and Wang, Y. in 1997 and Munson et al. in 1997. Many PCR-based techniques such as polymerase chain reaction fragment length polymorphism (PCR-RFLP), reverse transcription PCR (RT-PCR), competitive PCR (cPCR) were used to monitor the relative distribution in environmental variation (Barns et al., 1999; Felske et al., 1998; and Johnsen et al., 1999, respectively).

The gene for the 16S rRNA is the most widely used. First the nature of this sequence with a mosaic of highly conserved regions interspersed with variable and hypervariable stretches makes it extremely convenient for PCR primer design (Stackebrandt and Rainey, 1995; Gurtler and Stanisich, 1996). In addition the vast database of sequences available for this gene makes finding cultivated close relatives feasible, or even if that is not the case, the sequence can be placed in a frame of relationships with other microorganisms that are often assumed to have phylogenetic value with implications on probable physiological behavior (Amann et al., 1995).

However, there are some pitfalls in the use of 16S rDNA for studies of biodiversity. One is that in terms of size the genes for the 16S molecule are extremely constant (with a total variation of about 200 bp for a mean length of 1550 pb [Linton et al., 1994a; Linton et al., 1994b; Rainey et al., 1996]) and therefore different genes cannot be easily separated by size. For sequencing, the total amplicon generated has to be cloned and the individual clones are sequenced

separately. This is a methodological limitation for large scale screening where many samples need to be compared, and in any case requires the tedious and expensive sequencing of many clones often revealing identical (or nearly so) sequences. Additionally, the 16S sequence, in spite of having hypervariable and extremely informative regions for close relationships, is often not divergent enough to give good separation in close relationships, e.g. species of the same genus (Normand et al., 1996). Both problems are highly simplified if the spacer region located between the 16S and 23S rRNA genes is included in the PCR amplification. This region is extremely variable in size and sequence even within closely related taxonomic groups (Gurtler et al., 1996) they are more suitable for this purpose. Size patterns can be used to characterize different communities of Bacteria or Archaea. The most common gene arrangement for the different subunits within the operon, with very few exceptions, follows the order 16S-23S-5S (Gurtler et al. 1996; Pisabarro et al., 1998; Roth et al., 1998). This kind of structure finds most of its deviations associated with small-genome bacteria, often parasitic (Andersson and Krland, 1998). This means that between 16S and 23S genes, and between the 23S and 5S, lie intergenic spacer regions of variable length. The size of the spacer may vary considerably for the different species, and even among the different operons (Condon et al., 1995). This variation in length is mainly due to the presence of several functional units within them such as tRNA genes, present in most of the microorganisms studied in a number of one or two per spacer (Gurtler et al., 1996; Normand et al., 1996). Stretches of the 16S molecule can be also amplified (by simply using a primer located within the 16S gene) to the extent of including the whole 16S rDNA in the amplicon. Due to its larger size and the fact that there are more sequence data available for 16S genes than for 5S, analyses of 23S-5S spacers are less common (Yoon et al., 1997).

The genetic structure of bacterial community was investigated using a culture independent technique, the rRNA intergenic spacer analysis (RISA), that relies on the length polymorphism of the intergenic spacer between the small (16S) and large (23S) subunit rRNA genes (Borneman and Triplett, 1997). These so-called fingerprinting methods provide band profiles that are representative of the genetic structure of the community as a whole or of a section of it, as defined by selected primers (Muyzer, 1998). These methods are valuable tools for characterizing complex bacterial communities and detecting shifts following environmental

perturbations and are less time-consuming and labor-intensive that strategies such as small-subunit rRNA gene clone library construction (Ranjard et al., 2000). Fingerprinting methods based on DNA or rRNA sequence analysis such as the sequence-dependent separation of fragments in a denaturing or temperature-gradient gel electrophoresis (DGGE and TGGE), the amplified ribosomal DNA restriction analysis (ARDRA), and the rRNA intergenic spacer analysis (RISA) were demonstrated to be relevant for the studies of complex bacterial communities such as those indigenous to soil (Engelen et al., 1998; Ovreas and Torsvik, 1999; Smit et al., 1997). Among all these methods, the RISA fingerprinting was used because it is easy to perform, allows the rapid examination of the composition of complex bacterial communities, and can also be performed without the use of specific and expensive equipment (Acinas et al., 1999; Borneman et al., 1997; Robleto et al., 1998).

In 1997, Borneman and colleagues reported that RISA was used to show significant microbial population differences between a mature forest soil and an adjacent pasture soil from eastern amazonia. Each soil type showed numerous bands that were unique to that environment. These differences were presumably the result of the different soil properties associated with the conversion to pasture. And in 1998, Robleto and colleagues also reported that RISA was used to estimate effects of disturbance on diversity, such as the introduction of an antibiotic-producing bacterium into soil.

Moreover RISA has also been used to distinguish bacterial community structures associated with different soil microenvironments (Ranjard et al., 2000). They found that the fingerprinting approach used in they study may not be appropriate for an estimation of diversity in terms of richness and evenness since (i) only the most abundant populations were represented in a community profile, (ii) several sequences are certainly contained in one RISA band (Fisher and Triplett, 1999), and (iii) a single organism could be represented by several RISA bands (Jensen et al., 1993). Such observations led them to limit they study to a comparative analysis of the genetic structure of the whole soil community and the microenvironment pools.

As one attempt to obtain an overview of the structural diversity of microbial communities, denaturating gradient gel electrophoresis and temperature gradient gel electrophoresis analysis (DGGE/TGGE) has been introduced into microbial ecology (Muyzer, 1999; Muyzer et al., 1993). Separation in DGGE is applied on the basis of decreasing electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (DGGE) or a temperature gradient (TGGE), which is decreased compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Molecules with different sequences may have different melting behaviors and thus migrate to different positions in a gel. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different position in the denaturing gradient and hence can be separated effectively by DGGE. This method is particularly useful when temporal and spatial dynamics of the population structure are analyzed, since it avoids the laborious aspects of cloning and sequencing.

This technique has been successfully applied to identifying sequence variations in a number of genes from several different organisms. DGGE can be used for direct analysis of genomic DNA from organisms with genomes of millions of base pairs. This is carried out by transferring separation patterns to hybridization membranes using capillary blotting with modified gel media or by electroblotting followed by analysis with DNA probes (Heuer et al., 1999; Kowalchuk et al., 1999). Alterantively, PCR can be used to selectively amplify the sequence of interest before DGGE is used (El Fantroussi et al., 1999). It has been reported that the combination of PCR-RFLP with DGGE can be used to identify their products by hybridization with a hierarchical set of oligonucleotide probes designed to detect ammonia oxidizer-like sequence clusters in the genera *Nitrosospira* and *Nitrosomonas* (Kowalchuk et al. 1999).

Another useful method for consortium analysis is terminal restriction fragment length polymorphism (T-RFLP) analysis of PCR-amplified 16S rDNA fragments (Liu et al., 1997; Marsh; 1999). This method identifies 16S rDNA fragments based on the restriction endonuclease digestion patterns rather than the sequences. It appears to be advantageous over D/TGGE due to its resolution and simplicity of identification.

Khan and colleagues (1998) reported about the successful PCR amplification of DNA from GEMs in soils and sediments requires a sequence of events, including cell lysis, removal of the DNA from soil or sediment, removal of humic acids and phenolic substances, prior to PCR amplification itself. Clearly, impairment of any step in this complex chain will diminish yields. This new method eliminates several lengthy steps and the detection limit was higher than with conventional plating procedures.

Thus, selection of an appropriate DNA extraction and purification procedure form among the procedures that have been described to date remains a major problem in the application of molecular techniques to studies of soil and sediment microbial communities.

OBJECTIVE

The aims of this study were to develop the promising method for direct extraction of bacterial DNA from various type of soils with regard to percentage of DNA recovery, an efficient purification of extracted DNA procedure, and the detection limit of this protocol.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Soil samples

Various types of soils were collected as followed: from rice field (Khon Kaen Province), cassava field (Nakhon Ratchasima Province), undisturbed forest (Prachin Buri Province) and sediment (Nakhon Ratchasima Province). Characterization of the soil samples were performed according to the standard method of Black (1965).

2.1.2 Chemicals and reagents

Cell lysis

- skim milk powder solution

milk powder	0.1	g
H_2O	25	ml

- SDS extraction buffer

SDS	3	g
NaCl	8.18	g
Sodium acetate	4.10	g
H_2O	1,000	ml

Direct extraction

- lysis buffer

NaCl	2.92	g
Na_2EDTA	3.72	g
Tris-HCl	7.88	g
SDS	10	g
H_2O	1,000	ml

- proteinase-K (Boehringer, Mannheim, Germany)
  : final concentration = 0.28 mg/ml in water
- lysozyme (Fulka, USA)
  : final concentration = 20 mg/ml in water
- phenol:chloroform:*iso*-amylalcohol
  : 25:24:1, vol/vol/vol
- 10% SDS in water
- 0.2 N NaOH in water

DNA precipitation

- 70% EtOH in water
- 99.7% Isopropanol
- 3 M Sodium acetate in water
- Rnase A (BioLab, USA)

Gel electrophoresis

- Tris-borate EDTA (TBE)
  : 0.04 M Tris-acetate, 0.001 M EDTA
- 1% agarose gel in TBE
- 10% acrylamide gel
- Ethidium bromide (<0.5 mg/ml)
- Silver sequence DNA staining reagent
  fix/stop solution: 10% glacial acetic acid
  staining solution : 0.1% silver nitrate, 0.056% formaldehyde
  developing solution: 0.3% sodium carbonate, 0.056% formaldehyde, 0.0002% sodium thiosulfate

DNA purification

- MicroSpin Sephacryl S-300 columns (Pharmacia Biotech, USA)
- Quantum Prep Freeze N Squeeze Spin Column (Bio-Rad, USA)

PCR

- *Taq* polymerase Kit (Promega, USA): the concentrations of reagent are depending on type of primer

Bacteria cultivation

- Plate count agar (PCA)

: 5% tryptone, 2.5% Yeast extract, 1% Dextrose and 1.5% agar

Bacterial strains

- *Bradyrhizobium japonicum* USDA 110
- *Pseudomonas aeruginosa*

2.2 Methods

2.2.1 DNA isolation by cell extraction method

As indicated in Figure 2.1, each 0.25 g of soil samples was ground in liquid nitrogen by using a mortar and pestle for 5 min or until a fine powder remains. The powdered soil was suspended in 0.5 ml of skim milk powder solution by vigorous vortexing. The soil debris was removed by centrifugation at 9,000 rpm for 10 min at 4^{o}C and the supernatant was thoroughly mixed with 2 ml of SDS extraction buffer by vortexing. Then an equal volume of phenol was added, the phases were mixed by intermittent vortexing for 2 min at room temperature and then separated by centrifugation at 9,000 rpm for 10 min. The nucleic acid in the aqueous phase was precipitated with 2 volumes of 100% isopropanol for at least 30 min at -20^{o}C. The precipitate was collected by centrifugation at 13,000 rpm for 10 min at 4^{o}C, and the pellet was washed twice with 70% ethanol by centrifugation at the same speed and time. The dried pellet was dissolved in 250 μl of sterilized deionized water and stored at -20^{o}C until used (Volossiouk et al., 1995).

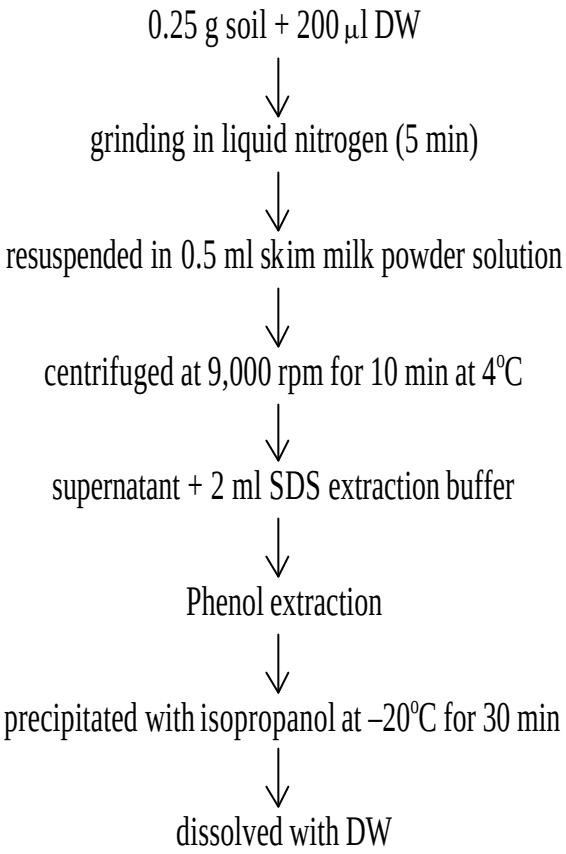

Figure 2.1 Outline of DNA isolation by cell extraction methodology

2.2.2 DNA direct extraction methods

There were three procedures were developed in this study. For the first trial methodology, the bacterial DNA from soil was extracted as previously described (Schwieger et al., 1998). Ten grams of soil sample was added with 0.85% NaCl solution. The suspension was pelleted in 15 ml test tubes by centrifugation at 9,000 rpm for 15 min. The supernatant was discarded and the remaining soil samples were resuspended in 5 ml of lysis buffer. The suspensions were subjected to five cycles of freeze-thawing. Each cycle consisted of 5 min of freezing in liquid nitrogen, 5 min of thawing in 65°C water bath with gentle agitation, and vortexing for 10 sec at the highest speed setting. Proteinase-K (final concentration = 0.28 mg/ml in water) was added to each sample, and the tubes were incubated at 65°C for 1 h in a water bath with reciprocal shaking at speed 150 rpm. Samples were placed on ice and mixed with 1 volume of phenol:chloroform:*iso*-amylalcohol (25:24:1, vol/vol/vol). The suspensions were then

centrifuged for 10 min at 9,000 rpm at 4°C. The upper phase was carefully transferred to 1.5 ml tubes. DNA solution was then precipitated with 2 volume of isopropanol at -20°C for 30 min. Precipitated DNA was collected by centrifugation at 13,000 rpm for 10 min at 4°C. Pellets were washed with cold 70% ethanol, dried at room temperature, and resuspended in sterilized deionized water. As showed in Figure 2.2.

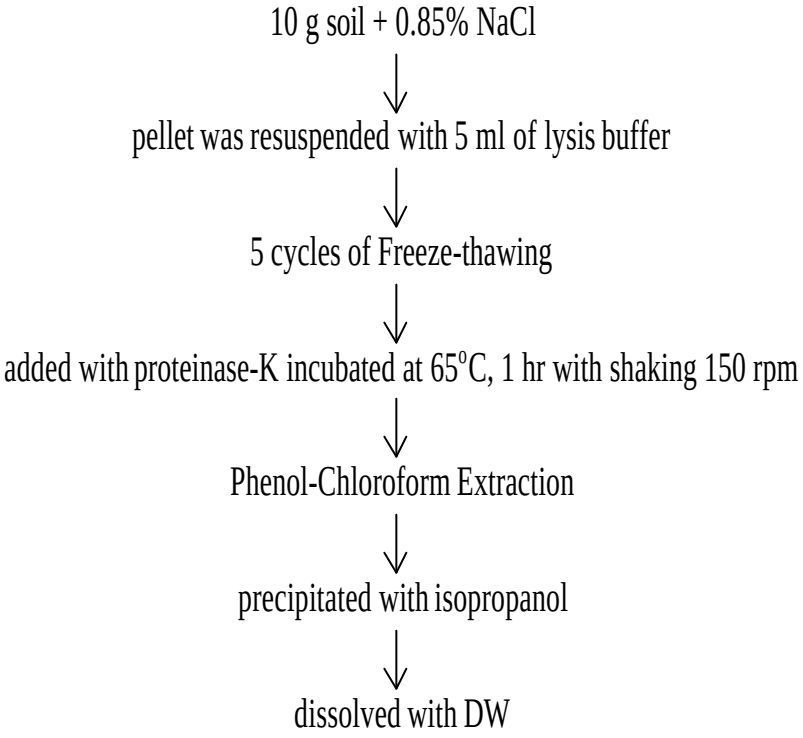

Figure 2.2 Outline of the first direct extraction methodology

The second protocol was also developed and partially modified from the first protocol. The wash step was used as described in the 1st protocol. The washed sample was ground in liquid nitrogen then resuspended with 5 ml of lysis buffer. The suspensions were applied to the step of 5 cycles of freeze-thawing followed by additional of proteinase-K (at the same concentration of the 1st protocol) and incubated at 65°C for 1 hr. these 2 techniques were repeated 3 times. After the final round of freeze-thawing and proteinase-K treatment the protocol was performed by following the 1st method (Figure 2.3).

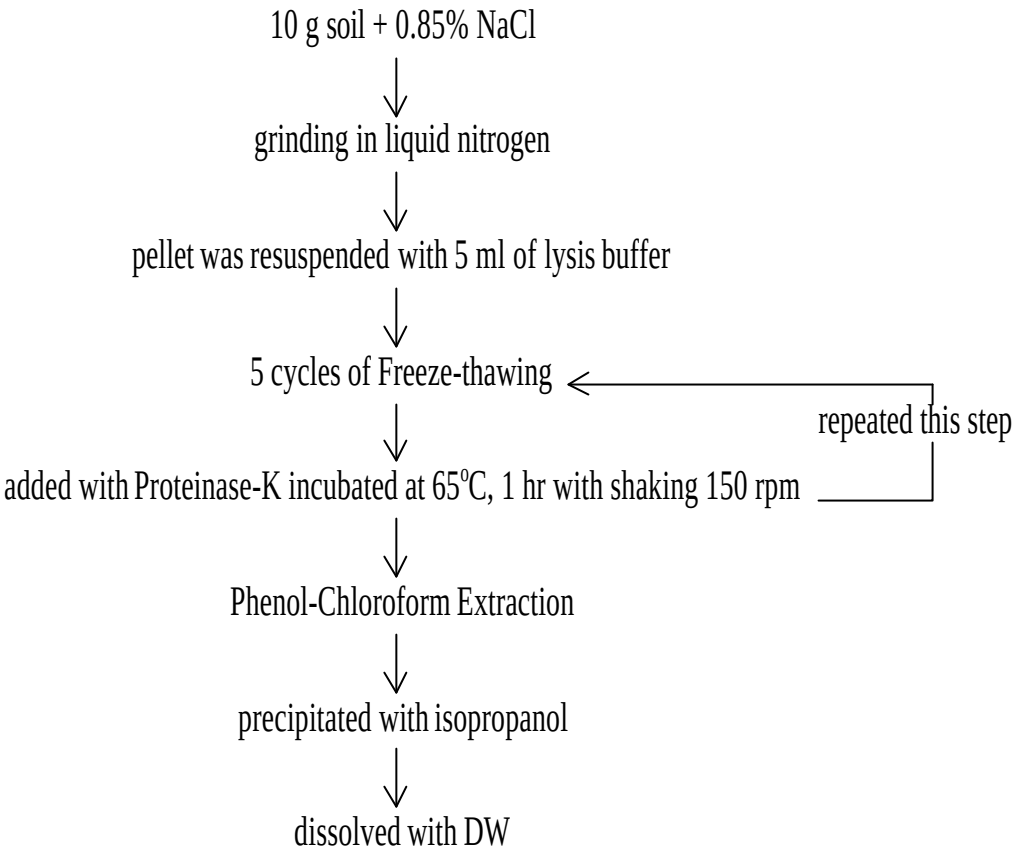

Figure 2.3 Outline of the second direct extraction methodology

Thirdly, the addition of lysozyme and NaOH were used to enhance cell disintegration efficiency (Figure 2.4). One gram fresh weight of soil sample was washed in 0.1 M phosphate buffer (pH 7). After centrifugation, the pellet was resuspended with lysozyme solution (20 mg/ml at final concentration) and incubated at 37°C for 30 min. The soil sample was then added with 1 ml of 10% SDS and incubated at 50°C for 30 min. The soil slurry was further applied with proteinase-K solution before incubation at 65°C for 1 hr. The mixture after added with 1 ml of 0.2 N NaOH was shaken at room temperature for 15 min. Three cycles of freezing in liquid nitrogen and thawing at 70°C were carried out. An equal volume of phenol was added, the phases were mixed by intermittent vortexing and then separated by centrifugation at 13,000 rpm 10 min. The nucleic acid in aqueous phase was precipitated with 2 volumes of isopropanol and kept at -20°C for 30 min. or overnight. The DNA pellet after centrifugation was washed with 70% ethanol and dried. The dried pellet was dissolved in sterilized deionized water.

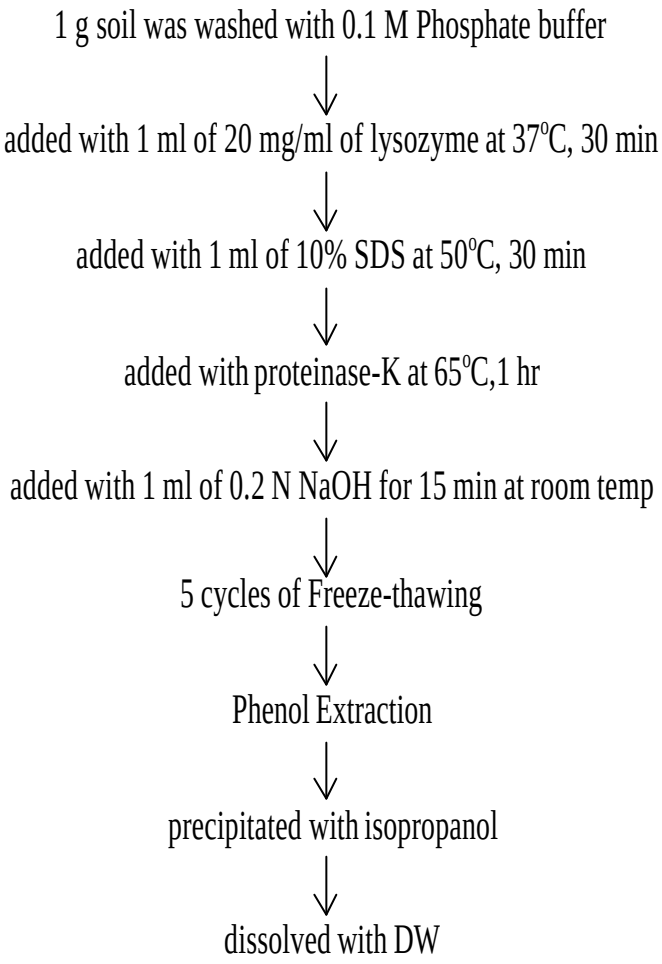

Figure 2.4 Outline of the third direct extraction methodology

2.2.3 Gel electrophoresis

The DNA extracts may be contained RNA contaminants, introduction of RNase during extraction procedure was necessary. Tsai and colleagues (1991) reported that impurities such as humic materials in the extracts did not affect RNase or DNase digestion, but may affect DNA hybridization efficiency. Then the presence and size of the extracted DNA from soil were determined after treated with RNase A (1 mg/ml at final concentration) and incubated at 55°C for 30 min by 1% agarose gel electrophoresis at 80 V for 30 min.

2.2.4 Determination of bacterial extraction efficiency

Enumeration of the culturable bacteria in soil samples at each steps of DNA extraction was determined by serial dilution of samples and followed by plating suspensions on plate count agar (PCA) to investigate the remained bacterial population and lysis efficiency. The culture was incubated at 30^{o}C for 3-5 days before examined.

2.2.5 DNA purification and quantification

As illustrate in Figure 2.5, crude DNA from extraction protocols was treated with RNase then further purified by applying through MicroSpin Sephacryl S-300 columns (Pharmacia Biotech, Piscataway, NJ) (Edgcomb et al., 1999) twice before applied onto agarose gel electrophoresis. The purified DNA was extracted from agarose gel by using Quantum Prep Freeze N Squeeze Spin Column and applied through Sephacryl S-300 microspin column (Bio-Rad, Hercules. CA) again. Both of crude and purified DNAs were quantified by comparing the fluorescence intensities of ethidium bromide stained DNA sample bands to the fluorescence intensities of standards on agarose gel.

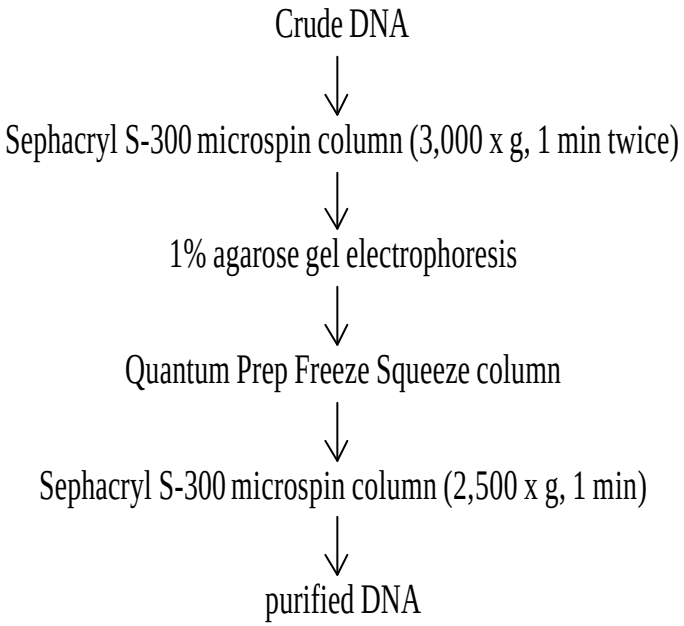

Figure 2.5 Outline of the purification methodology

2.2.6 PCR inhibition assay by using rRNA intergenic spacer analysis (RISA) as target sequence

To determine whether PCR inhibitors presented in the purified soil DNA solution, the intergenic spacer region between the SSU and large-subunit rRNA genes which is extremely variable in size and sequence even within closely related taxonomic groups (Borneman et al., 1997) were used for amplification in 25 μl PCR mixtures at the following final concentrations or total amounts: 500 ng of soil DNA, 50 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 400 nM (each) primer, and 2.5 U of *Taq* polymerase. The PCR primers were 1406F (5'-TGYACACA CCGCCCGT-3') (universal rRNA small subunit) and 23SR (5'-GGGTTBCCCCATTCRG-3') (bacterial 23S rRNA large subunit) (B = G, C or T; R = A or G; Y = C or T). The varied volume amount of extracted bacterial DNA from soil added with 50 ng of extracted DNA from pure culture were used as template. All reagents were combined and heated at 94°C for 3 min. Thirty-five cycles of PCR were performed with a Perkin-Elmer-ABI 9700 thermocycler system (Perkin-Elmer, Singapore) at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 90 sec, followed by elongation at 72°C for 10 min. Whole amount of PCR product in each reaction was loaded in one lane of 1% gel electrophoresis.

2.2.7 Investigation of detection limit

Pseudomonas aeruginosa was inoculated into each of autoclaved five soil samples at 10^5, 10^4, 10^3, 10^2, 10 and 1 cells per gram soil. Inoculated soil were extracted by the third direct extraction procedure and then purified as previously described. Purified DNA was used as template by using RISA primer. The quantity of RISA-PCR products were determined by applied whole amount of PCR product in each reaction into 10% acrylamide gel electrophoresis by using a minislab gel (gel size, 90 by 80 by, 0.75 mm.; Hoefer Pharmacia Biotech, San Francisco, Calif.). Gels were run at 80 V for 3 hrs or until the bromophenol blue migrated to the end of the gel at room temperature. Gels were silver stained with SILVER SEQUENCE DNA staining reagents (Promega, Madison, USA) (Borneman et al., 1997; Robelto et al., 1998) or by the protocol described by Bassam and colleagues (1991).

2.2.8 Southern hybridization analysis

RISA-PCR products generated from DNA extracted from pure culture of *P. aeruginosa* were used as a probe to verify the PCR products generated from soil DNA. Southern blotting was performed by separated PCR products on 1% agarose gel and the DNA was capillary transferred to positively charged nylon membrane. Hybridization was performed at 58°C. Anti-Digoxygenin-AP, Fab fragments (Boehringer Mannheim, Germany) was used to detect probe DNA-target DNA hybrids according to the manufacturer's instructions (DIG DNA Labeling and Detection Nonradioactive, Boehringer Mannheim, Germany). The probes were produced by DIG-High Prime according to the manufacturer's instructions (DIG-High Prime; Boehringer Mannheim, Germany).

2.2.9 The test of other primer amplification

Other sets of DNA primer were used. The sequences of *nifD* derived from *Azotobacter vinelandii* M. 20568 were 5'-TARTCCCANGAGTGCATYTGNCGGA-3' and 5'-ATSGARTW CAACTTCTTCGG-3'. The reaction condition with 10 ng of template DNA was 3 min at 72°C and 0.5 min at 94°C for the third 30 cycles 3 min at 72°C and ∞ at 4°C. Short tendemly repeated repetitive sequence (STRR) primer was 5'-CCARTCCCCARTCCCC-3'. The 25μl reaction mixture contained the following final concentration or total amounts: 500 ng of DNA 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.9670 pM each primer, and 2.5 U of *Taq* polymerase. The reagents were mixed and then heated to 95°C for 6 min. Thirty cycles of PCR were then run at 94°C for 1 min, 56°C for 1 min, and 65°C for 5 min followed by 65°C for 16 min (Rasmussen and Svening, 1998).

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Soil characterization

The development of DNA extraction procedures were examined by using various types of soil sample collected from rice field (clay), undisturbed forest (sandy clay loam), and cassava cultivation area (sand). The results of soil characteristics were summarized in Table 3.1. Among the soil samples, sediment (loamy sand) contained the lowest amount of organic matter and viable bacterial cells (0.73% and 6.5 x 10^5 CFU/g soil, respectively). Sample collected from undisturbed forest contained the highest organic matter (4.25%) and rice field had a maximum number of viable bacterial cells (9.7 x 10^5 CFU/g soil). The pH range varied from 4.9 to 7.3. Whereas, other two samples (sediment and peat), which had a substantially high difference in the term of organic matter were represented to determine DNA impurity assay.

Table 3.1 Soil characteristics

Soil sample	pH	Particle distribution			Humic content (%)	Organic content (%)	Total viable bacterial count (CFU/g soil)
		Sand (%)	Silt (%)	Clay (%)			
1. Rice field	7.3	20.87	28.67	50.46	0.20	1.07	9.7 x 10^5
2. Undisturbed forest	4.9	62.71	17.43	19.87	0.55	4.25	7.2 x 10^5
3. Cassava cultivation area	7.0	92.39	9.41	1.81	0.42	3.38	8.3 x 10^5
4. Sediment	6.3	79.3	9.5	11.2	0.08	0.73	6.5 x 10^5
5. Peat	7.0	ND			ND	62.77	5 x 10^4

Notes ND = Non Determined

3.2 Development of DNA extraction methodology

The first DNA direct extraction protocol was conducted on the basis of using lysis buffer, freeze-thawing, proteinase-K treatment, and phenol:chloroform:*iso*-amylalcohol extraction. The results showed that DNA could be obtained from rice field soil sample about 7 ng DNA per gram of soil (dry weight) (Figure 3.1a), invisible DNA in agarose gel from undisturbed forest soil sample and large amount of DNA but in shearing form from cassava cultivation area (Figure 3.1b). As shown in lane 4-7 of Figure 3.1b, the size distribution of the DNA fragments (more than 9.4 kb to less than 2.0 kb) obtained from cassava cultivation soil sample by gel electrophoresis revealed that some limited shearing took place during extraction regardless of the physical disruption method employed. It has been reported that physical treatments can shear DNA to sizes of 5 to 10 kb or less (Holben et al., 1988; Ogram et al., 1987), and in at least one study, the average fragment size was 100 to 500 bp as indicated by agarose gel electrophoresis (Simonet et al., 1991). Such DNA may not be suitable for community analysis based on *Taq* DNA PCR, because of the risk of forming chimeric products with smaller template DNA (Liesack et al., 1991). Furthermore, the humic substances also still showed up in the agarose gel electrophoresis as a bright blue green fluorescent band under UV-light migrating ahead of the DNA (as shown in Figure 3.1a-b).

The efficiency of the soil bacterial fraction was determined by comparing the amount of culturable bacteria recovered with the number of bacteria remaining in each step of the DNA extraction procedure. Enumeration of bacteria was performed on the 0.1 ml of the extracted aliquot from each step suspension was spread on PCA medium. The recovery efficiencies were summarized in Table 3.2. The efficiency in each step of extraction from these 3 soil samples was varying. For the soil samples from cassava cultivation and undisturbed forest which was contained less amount of clay than rice cultivation area showed that freezing-thawing step was able to disturb the soil bacteria greater than other steps, while the result of rice field soil sample, the proteinase-K treatment presented the high efficiency. Thus, it seemed that soil texture would be one of the factors which involved in cell disintegration efficiency. This protocol also suggested that even using extreme physical treatments together with enzymatic treatments, the number of

survival bacteria were still remained in the range of 10 - 4.48 x 10^2 cells per gram of soil (dry weight) and it took 3 hrs and 30 min for completion (Table 3.3). However, this protocol was not reproducible when it was repeated.

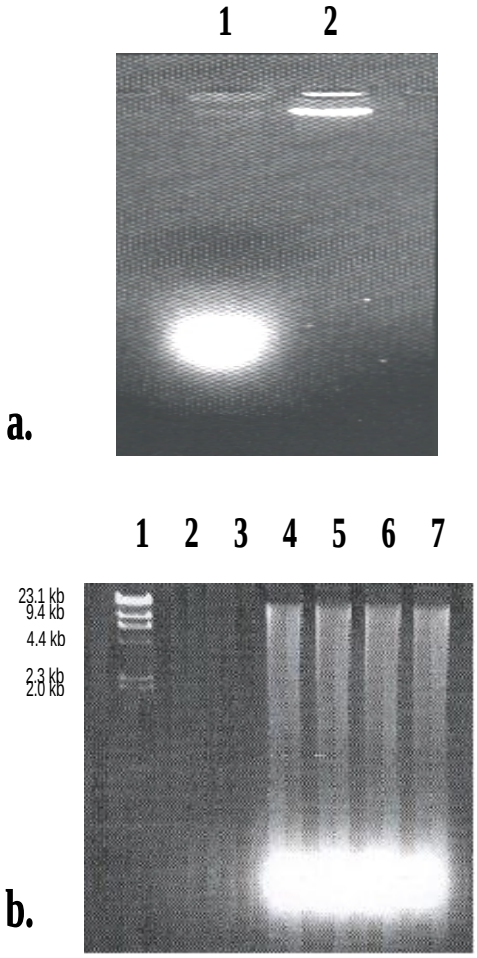

Figure 3.1 (a.) Extracted DNA from rice field soil sample by 1st direct extraction procedure. Lane 1: crude DNA (humic contaminant represent in bright blue green fluorescent band migrating ahead of the DNA), Lane 2: λ DNA marker
(b.) Extracted DNA from undisturbed forest and cassava cultivation soil sample by 1st direct extraction procedure. Lane 1: λ DNA marker digested with the Hind III, Lane 2-3: crude DNA from undisturbed forest soil sample, Lane 4-7: crude DNA from cassava cultivation soil sample

Table 3.2 The recovery of soil bacterial cells at each step of the 1st direct DNA extraction procedure

Step of DNA extraction*	CFU/g soil			Bacterial survival (%)			Recovery efficiency (%)		
	Rice field	Cassava cultivation area	Undisturbed forest	Rice field	Cassava cultivation area	Undisturbed forest	Rice field	Cassava cultivation area	Undisturbed forest
1	9.7×10^5	8.3×10^5	7.2×10^5	100	100	100	0	0	0
2	6.41×10^5	2.33×10^5	1.09×10^4	66.08	28.07	1.51	33.93	71.98	98.49
3	1.2×10^4	5.73×10^3	7.2×10^3	1.24	0.69	1.00	65.06	27.33	0.51
4	1.64×10^2	4.48×10^2	3.6×10^1	0.017	0.054	0.005	1.23	0.636	0.995

(*) Numbers in the column are 1 = Initial total viable count, 2 = After 5 cycles of freeze- thawing, 3 = After proteinase-K treatment and incubated at 65°C, 1 hr, and 4 = After phenol-chloroform extraction.

Table 3.3 Time consumption in each step of the 1st direct extraction DNA procedure

Step	Time consumption
Washing	15 min
Freeze-thawing	50 min
Proteinase K treatment	60 min
Phenol-chloroform treatment	10 min
DNA precipitation and RNase treatment	75 min
Total	3 hrs 30 min

Therefore, an attempt was further made to develop more efficient methodology (2nd extraction procedure), the grinding of soil samples in liquid nitrogen together with repeating of the freeze-thawing step followed by proteinase-K treatment twice at 65°C for an hour was developed. From this protocol, amount of obtained DNA was increased into 3 folds when compared with the previous approach (Figure 3.2). This is possibly because the 2nd protocol requires more steps of extraction than the previous one. The recovery of viable cell and efficiency of each step in this study were presented in Table 3.4. The grinding step showed the highest efficiency among all soil samples and the number of bacterial survival from each soil samples were still remained but lower in number than those applied with previous protocol (in the range of 3.32- 7.92 x 10^2 cells per gram soil). However, to implement this protocol, it was found that this method performed rather laborious such as the step that soil sample was ground in liquid nitrogen. Moreover, to achieve good yield of DNA from this protocol it too, considerable amount of time especially in 3 rounds of 5 cycles freeze-thawing and Proteinase-K treated that took at least 7 hrs 30 min (Table 3.5). The estimated recovery efficiency of DNA by the direct lysis method was also reported to be as high as 90% from sediments containing 19 to 44% clay and 3 to 16.5% organic carbon (Steffan et al., 1988). The indigenous microorganisms in the soil and sediments were lysed by using lysozyme and freeze-thaw procedure by Tsai and others in 1991. The lysate was extracted with SDS and phenol-chloroform. In addition to a high recovery efficiency (>90%), the yield of DNA were as high as 38 and 12 μg/g (wet weight) sediments and soil, respectively.

This method also generated minimal shearing of the extracted DNA. Thus, to avoid these obstacles another approach was developed on the basis of physical, chemical and enzymatic disruption.

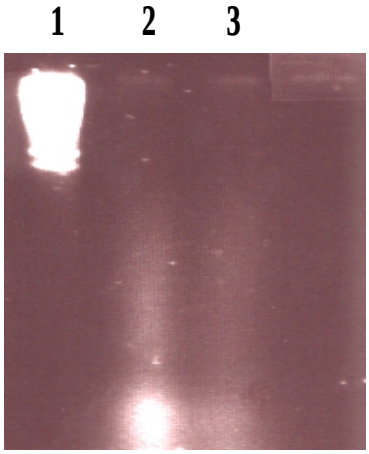

Figure 3.2 Extracted DNA from rice field soil sample by 2nd direct extraction procedure. Lane 1: λ DNA marker digested with the Hind III, Lane 2-3: extracted crude DNA

Table 3.4 The recovery of soil bacterial cells at each step of the 2nd direct DNA extraction procedure

Step of DNA extraction*	CFU/g soil			Bacterial survival (%)			Recovery efficiency (%)		
	Rice field	Cassava cultivation area	Undisturbed forest	Rice field	Cassava cultivation area	Undisturbed forest	Rice field	Cassava cultivation area	Undisturbed forest
1	9.7×10^5	8.3×10^5	7.2×10^5	100	100	100	0	0	0
2	2.65×10^4	6.86×10^4	8.64×10^4	2.73	8.26	12	97.25	91.71	87.98
3	1.6×10^4	3.97×10^4	3.47×10^4	1.64	4.78	4.8	1.1	3.49	7.22
4	1.12×10^4	1.44×10^4	3.37×10^4	1.15	1.74	4.68	0.49	3.05	0.12
5	4.85×10^3	5.73×10^3	3.89×10^3	0.5	0.69	0.54	0.66	1.06	4.14
6	4.17×10^3	2.32×10^3	3.6×10^3	0.43	0.28	0.5	0.06	0.41	0.04
7	3.78×10^3	1.83×10^3	2.6×10^3	0.39	0.22	0.36	0.05	0.07	0.14
8	3.59×10^3	1.58×10^3	1.22×10^3	0.37	0.19	0.17	0.02	0.02	0.19
9	6.79×10^2	3.32×10^2	7.92×10^2	0.07	0.04	0.11	0.3	0.15	0.06

(*) Numbers in the column are 1 = Initial total viable count, 2 = After grinding in liquid nitrogen, 3 = After 1st freeze-thawing, 4 = After 1st treated with proteinase K, 5 = After 2nd freeze-thawing, 6 = After 2nd treated with proteinase K, 7 = After 3rd freeze-thawing, 8 = After 3rd treated with proteinase K, and 9 = After phenol-chloroform extraction

Table 3.5 Time consumption in each step of the 2nd direct extraction DNA procedure

Step	Time consumption
Washing	15 min
Grinding in liquid nitrogen	15 min
1st round of Freeze-thawing	50 min
1st round of Proteinase K treatment	60 min
2nd round of Freeze-thawing	50 min
2nd round of Proteinase K treatment	60 min
3rd round of Freeze-thawing	50 min
3rd round of Proteinase K treatment	60 min
Phenol-chloroform treatment	10 min
DNA precipitation and RNase treatment	75 min
Total	7 hrs 25 min

As described above, the clay particles can be the bacterial protector from stress condition (Frostegard et al., 1999), therefore, soil from rice field was chosen as the most difficult to extract bacterial DNA. In 1999, Miller and colleagues reported that procedures which chloroform or phenol was included in the lysis mixture yielded more DNA than comparable procedures in which these organic solvents were not used. Furthermore, this study was developed from the 2nd protocol into the 3rd method due to the laborious and time consuming. In this study, it was found that the third methodology which treated the soil sample with lysozyme, 10% SDS, proteinase-K, NaOH, freeze-thawing, and phenol, respectively, gave a highest amount of DNA yield when compared with previous protocols. Besides bacterial DNA from soil, extracted products also procured the humic contaminants. As demonstrated in Figure 3.3, lane 3-8 were the amount of extracted DNA from rice field. The DNA migration was hampered by an enormous contaminant, which represented in bright blue green band ahead of the DNA. And lane 9-14 were extracted DNA from rice field that were purified by using sephacryl S-300 microspin column. DNA intensities were increased. This result suggested that humic substances were not only obstructed the

migration but also retarded the visualization of extracted DNA from rice field soil sample. However, the intensity of DNA from agarose gel electrophoresis could be estimated about 50 ng, which higher than the 1st and 2nd protocol. The recovery of viable cells and efficiency of each step in the 3rd protocol was summarized in Table 3.6. In this protocol the step that treated with 10% SDS performed the highest efficiency.

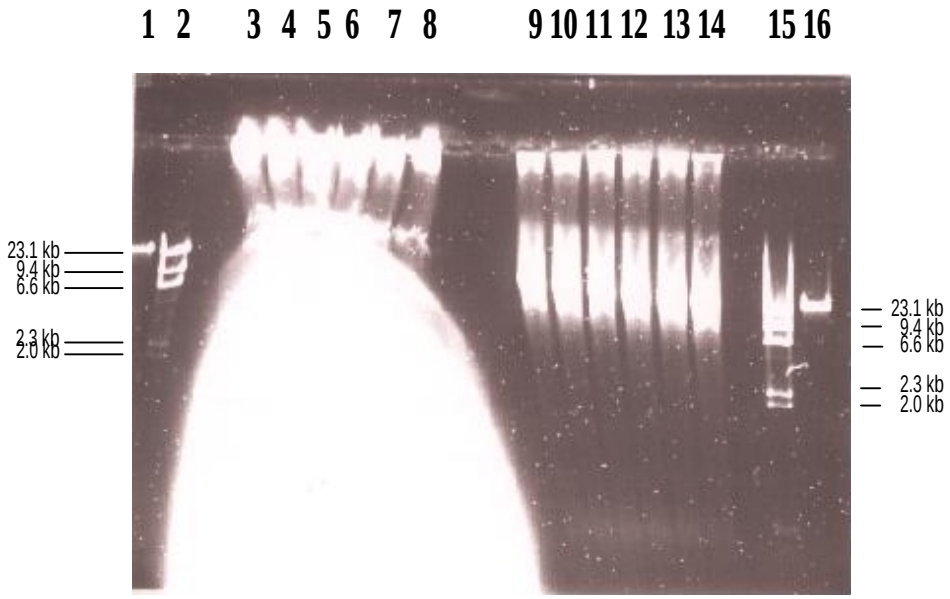

Figure 3.3 Extracted DNA from rice field soil sample by 3rd direct extraction procedure. Lane 1 and 16: λ DNA marker, Lane 2 and 15: λ DNA marker digested with the Hind III, Lane 3-8: extracted crude DNA, Lane 9-14: Purified DNA with Sephacryl S-300 microspin column twice

Table 3.6 The recovery of soil bacterial cells from rice field soil sample at each step of the 3rd direct DNA extraction procedure

Step of DNA extraction	CFU/g soil	Bacterial survival (%)	Recovery efficiency (%)
1	9.7×10^5	100	0
2	8.38×10^5	86.44	13.56
3	3.98×10^4	4.1	82.34
4	3.62×10^4	3.73	0.37
5	3.29×10^4	3.39	0.35
6	2.96×10^4	3.05	0.33
7	1.26×10^3	0.13	2.92

(*) Numbers in the column are 1 = Total viable count,
2 = After treated with lysozyme, 3 = After treated with proteinase-K,
4 = After treated with 10%SDS, 5 = After treated with NaOH,
6 = After freeze-thawing, and 7 = After phenol extraction

The percent recoveries of viable soil bacteria in final step, which was treated with the phenol reagent of the 1st, 2nd, and 3rd direct extraction procedures were compared. It was found that the efficiency of treatment with pure phenol twice followed by chloroform in 3rd protocol were higher than phenol-chloroform treatment due to the higher acid stress condition (Miller et al., 1999). The colour developed from different soil samples treated with phenol was also different (Figure 3.4).

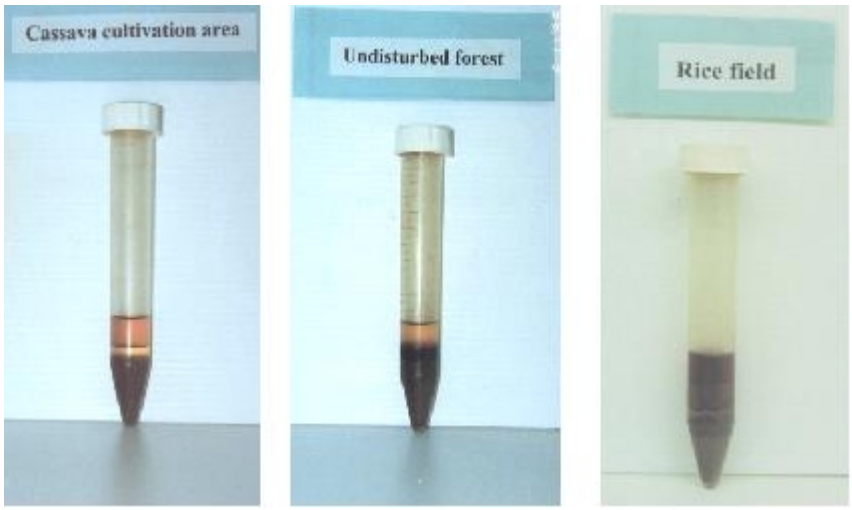

Figure 3.4 The colour developed from different soil samples that treated with phenol

The variation in cell lysis apparently reflects differences in soil characteristics and bacterial community composition i.e., soil exhibiting low cell lysis may have contained higher proportions of gram-positive cells (Zhou. et al., 1996). When investigated the survival bacteria in final step of DNA extraction procedure, the result showed that almost all of remaining bacterial population were endospore-forming cells (Figure 3.5), which were difficult to disrupt due to their rigid wall (Frostegard et al., 1999). The results indicated that this approach was rather suitable for investigation of gram negative bacterial population than gram positive bacteria in each soil sample. This study should be further determined by inoculating gram positive bacteria into soil contain among of vegetative bacterial cell. The 3rd protocol might be disturbed gram positive

vegetative cell but only ineffective for endospore-forming cells. However, the value of percent of bacterial survival in final step of each protocol for rice field sample were compared. Percentage of bacterial survival were 0.017, 0.07, and 0.13%, respectively, it was found that the 3rd protocol gave the highest survival cell, which contrast to the results of the quantity of obtained DNA among of these 3 procedures. It could be suggested that the 3rd method was efficient for extraction the unculturable cell or free-DNA adsorbed to soil particles than the other two protocols. To implement this protocol, it required 5 hrs (Table 3.7). The results supported that the methodology developed here was very rapid detection when compared with the most rapid detection method.

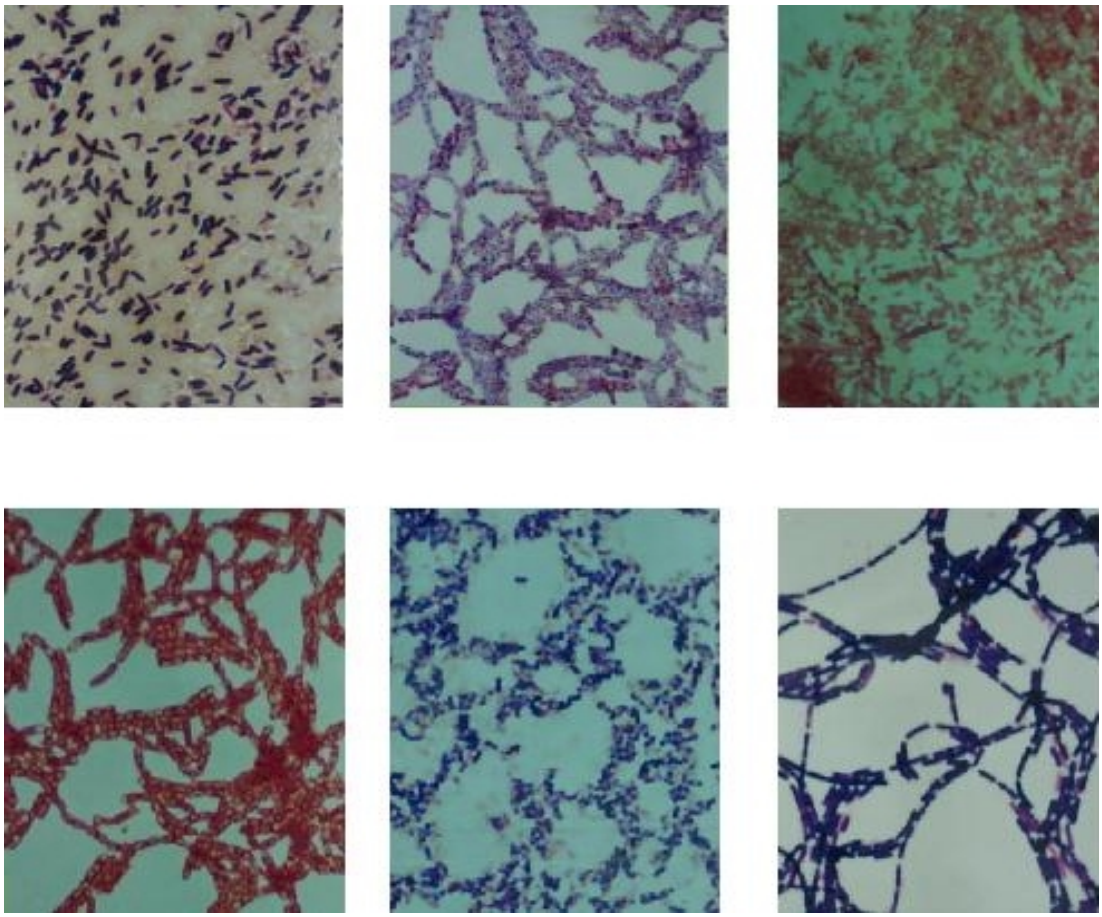

Figure 3.5 Some remaining bacteria after final step of DNA extraction

Table 3.7 Time consumption in each step of the 3rd direct extraction DNA procedure

Step	Time consumption
Washing	15 min
Lysozyme treatment	30 min
10% SDS treatment	30 min
Proteinase K treatment	60 min
NaOH treatment	15 min
Freeze-thawing	30 min
Phenol followed by chloroform extraction	30 min
DNA precipitation and RNase treatment	75 min
Total	4 hrs 45 min

Not only direct extraction procedure, cell extraction methodology was also developed following Volossiouk et al. (1995) in this study. The protocol was combined the application of skim milk to be effective as a carrier in order to reduce background and humic contaminant, it was found that DNA could not be recovered even when increase amount of soil samples to 10 grams or applied the step of 2 cycles of freeze-thawing before grinding in liquid nitrogen. This was confirmed and supported by several experiments included this study that to recover the bacterial cell from soil particles was somewhat insufficient.

According to earlier general procedures, this was time consuming; the original procedure required sample size of 60-90 g and at least 3 days for completion (Trosvik, 1980). Cell extraction involves separation of the bacterial fraction from soil which was accomplished by using modification of the fractionated-centrifugation technique (first described by Faegri et al., 1977). Fractionated centrifugation involves homogenization of soil sample in a buffer salt solution, followed by a low-speed centrifugation step which pellets the soil debris and fungal mycelia while leaving the bacterial cells in suspension. Cells were lysed with lysozyme and hot SDS, and the DNA was purified by using hydroxyapatite column (Trosvik, 1980). Another drawback was the spreading of organisms by aerosolization and other spills were practically unavoidable during

the repeated blending and centrifugation steps (Jacobsen et al., 1992). In 1988, Holben and co-workers improved the lysis method and inoculated polyvinylpolypyrollindone (PVPP) to reduce humic contamination of the extracted DNA. This study showed the recovery about 33% of the bacterial cells from soil. However, the disadvantage of this methodology was inefficient of separation of bound cells or DNA from soil particle.

A difficulty to differentiate in cell properties and in the extent to which soil structures protect cell from lysis treatments (Forstegard et al., 1999). Different bacterial groups adhere more or less strongly to soil particles (Prieme et al., 1996), which might bias the picture of the composition of the microbial community in the sample. Therefore, the extraction method was changed from cell extraction protocol to direct extraction protocol (as described above), which has been reported to have potential to circumvent problems of representative provided that the cell from all groups of microorganisms are lysed in equal proportions (Briglia et al., 1996).

3.3 Development of DNA purification protocol

DNA extracted from environmental samples was frequently contaminated with coextracted organic substances. A major consideration in the isolation of bacterial DNA from soil was to purify the DNA from soil contaminants, which make DNA refractory to complete restriction endonuclease digestion (Zhou et al., 1996), hybridization analysis (Alm, Zheng, and Raskin, 2000), and PCR amplification (Jackson et al., 1997). The organic matter can be coextracted with DNA from soil. Hence the DNA purification methods was an important step for detection bacterial community on the basis of DNA technique. The most methodologies for separations of DNA from organic matters are depended either differential levels of binding of organic matter and nucleic acids to a polymeric matrix or differential size fractionation. These methods varied in their rapidity and ease of use, as well as in their effectiveness.

In 1996, Zhou and others demonstrated that the single- or double-minicolumn purification appeared to give DNA which was incompletely digested with some restriction endonuclease and less suitable for PCR amplification, as well as lower recovery efficiency. However, they were very rapid and less expensive. They reported that gel plus column methods gave very pure DNA. Jackson et al., (1997) also found that among of three different gels (Sepharose 4B, Sephadex G-200, and Sephadex G-50), Sepharose 4B gave superior separation of DNA from humics, while Sephadex G-200 was less effective that further dilution may be required. Whereas Sephadex G-50 was ineffective at purifying DNA from environmental samples. Later, Miller and colleagues (1999) reported that humic acid in their sample were retained slightly longer on the Sepharose 4B column. This was suggested that none of purification methods tested completely removed all of contaminants in different type of samples. Thus an achievement of purification for all soil types should be regarded.

From all instructions the combination of Sephacryl S-300 microspin column gel electrophoresis and Freeze N Squeeze microspin column was chosen as DNA purification protocol in this study based on their rapidity and purity of DNA recovery. A dark brown colour DNA was applied into the column. It was found that DNA eluent from a spin column can not completely decolourized, and the resulting purified extracts retained a yellowish colour, indicative of residual humic acid contamination. Thus, a partial purified DNA was further purified by applied into the 2nd Sephacryl column.

To ensure to obtain DNA purity, DNA eluent from spin column was loaded into agarose gel electrophoresis and then extracted from gel by using Quantum Prep Freeze N Squeeze Spin Column. Gel electrophoresis of the purified DNA solutions resulting from the column was shown in Figure 3.6. Visual comparison under UV-light intensities of stained DNA allowed a semi-quantitative estimation of the efficiencies of the purification protocol. The intensity of DNA was increased due to reduction of humic substances with DNA yield of 4 ug per gram soil (dry weight).

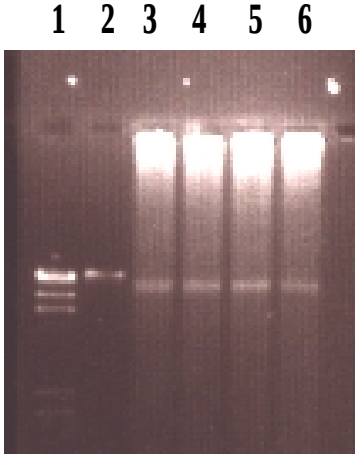

Figure 3.6 Purified DNA from rice field soil sample by 3rd direct extraction procedure. Lane 1: λ DNA marker digested with the Hind III, Lane 2: λ DNA marker, Lane 3-6: Purified DNA (obtained from 0.25 g of soil per lane)

The levels of DNA recovered from the rice field soil sample based on an assumed average value of 9 fg of DNA per cell (Ingraham et al., 1983). Assuming that this holds true for average bacteria, the density of bacteria from rice field soil sample would represent 8.62 ng of DNA per gram soil, which lower than total extracted DNA for 3.992 ug. This is possibly due to the amount of obtained DNA extracted from other microorganisms in soil, which cannot be detected by culturing on PCA medium. This result was corresponded to observations made by other investigators, who reported that only a very small portion of the natural bacterial community was culturable (Amann et al., 1994-5; Ferguson et al., 1984; Rondon et al., 1999; Roszak et al., 1987). The 'viable but nonculturable' (VBNC) hypothesis holds that certain normal culturable bacteria can enter a state in which they are still viable but cannot be cultured by standard microbiological methods (Barer et al., 1993; Colwell, 1993). Microgram quantities of DNA can be recovered from 10 g of soil in such purity that the isolated material can be fragmented with restriction enzymes, but one cannot distinguish between DNA from soil organisms and free DNA. It is estimated that 99% of the recovered DNA comes from microorganisms, which exist in rich soil at a density of 10^9-10^{10} cells per 100 g of soil. However,

less than 10% of the microbes from the environment can be cultivated under artificial laboratory condition. DNA either existing as extracellular DNA or stored in microorganisms may persist in soil or water for prolonged periods of time (Doblhoff-Dier et al., 2000).

Moreover, an excess recovered DNA may result from a consequence of cell death, DNA and proteins are degraded by hydrolytic enzymes. DNA fragments, however, may escape complete depolymerization and exist as oligomers of up to 10^4 bp. Within a biological system, for example, in bacteria or cells from higher organisms, DNA is always associated with many ligands, preferentially with proteins. Inside cells, no naked or free DNA exists. Whenever DNA exists outside a cell, it is normally bound to substances, for example, to proteins or to soil constituents such as clay. Thus, DNA outside the cell is unlikely to be free or naked. The expression 'extracellular DNA' or 'foreign DNA' will be used when it is necessary to distinguish it from 'intracellular DNA' (Doblhoff-Dier et al., 2000).

Eventhough, this protocol can be applied for various types of soil but further development should be emphasized due to the Sephacryl S-300 microspin column is costly. An entire this purification step took approximately 750 Bahts per sample at present time.

3.4 Inhibitory effect of contaminant from extracted DNA to PCR reaction

Since DNA extracted from environmental samples was frequently contaminated with co-extracted organic substances (Moran et al., 1993; Ogram et al., 1995) it was necessary to evaluate the effects of the presence of these contaminants on quantitive PCR amplification. To ensure the quality of extracted DNA obtained from soil, purity was then estimated by using a PCR inhibitory assay. Purified DNA was used as a template to determine whether humic contaminants were still remained and were able to inhibit the PCR reaction.

The experiment was designed by using extracted DNA from pure culture of *Bradyrhizobium japonicum* USDA 110 mixed with varied amount of extracted bacterial DNA from soil as templates in PCR. RISA primer was performed on DNA extracted from rice field, undisturbed forest, cassava cultivation area, sediment, and peat samples. Analysis by gel electrophoresis showed that the decreased of a major immerging band intensity of the PCR products, corresponding to different amounts of template DNA from soil. It was suggested that the PCR inhibitors had still remaining in the purified DNA from soil template. The PCR products were generated from only the reaction that lowest volume of DNA from rice field soil sample supplemented with DNA from pure culture (Figure 3.7, lane 10-13). From this result, the extracted DNA from gel was further purified by applied through Sephacryl S-300 microspin column again in order to remove amount of other contaminant obtained from gel electrophoresis system such as loading dye, EtBr, and salinity of buffer. From this result, the RISA PCR products were obtained from this purification protocol in both of DNA from soil template and DNA from soil supplemented with DNA from pure culture (Figure 3.7, lane 14-17).

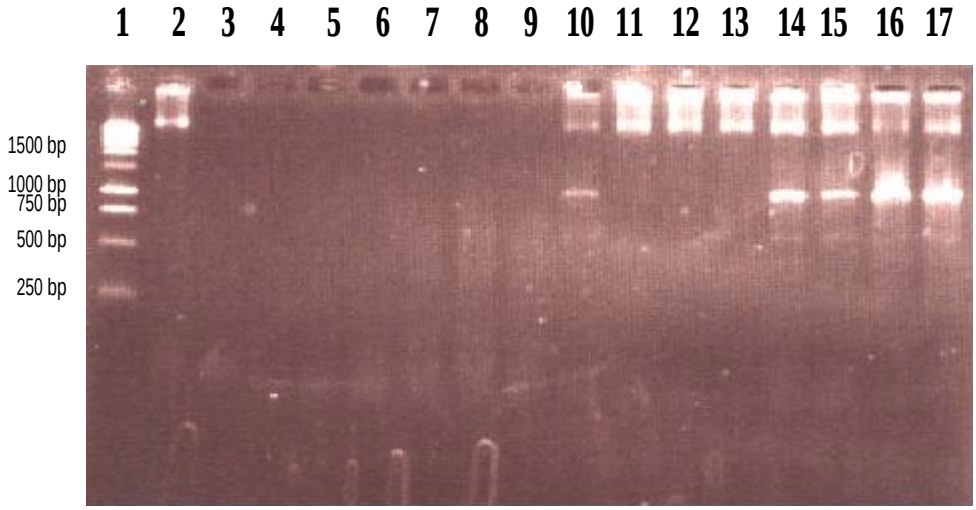

Figure 3.7 RISA-PCR products generated from DNA directly extracted from rice field soil sample supplemented with 50 ng DNA extracted from pure culture of *Bradyrhizobium japonicum* USDA 110. Lane 1: 1 kb marker, Lane 2 and 17: positive control, Lane 3-5: PCR products from soil DNA as a template (1, 3, 5 μl, respectively), Lane 6-9: PCR products from soil DNA through out the 3rd Sephacry column (1, 3, 5, 7 μl, respectively), Lane 10-13: PCR products from soil DNA supplemented with DNA from pure culture as template (1, 3, 5, 7 μl, respectively), Lane 14-16: PCR products from soil DNA through out the 3rd Sephacry column supplemented with DNA from pure culture as template (1, 3, 5 μl, respectively)

PCR amplification of DNA from varied amount of bacterial soil DNA template showed that the RISA-PCR products were detected in lowest volume tested. The signal strengths could be increased significantly by further dilution of the extract presumably because concentrations of inhibitory substances were reduced. In terms of soil sample type, amount of humic contaminants remaining in extracted DNA from undisturbed forest was higher than another samples suggested that it could be from the higher organic acid. While the PCR products from sediment sample revealed that it was not inhibited as much, resulting from more efficient amplification at higher DNA volume (Figure 3.8).

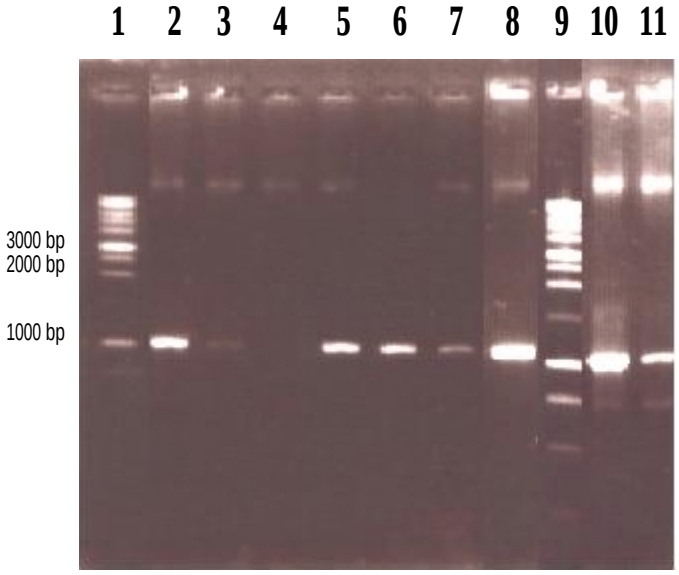

Figure 3.8 RISA-PCR products generated from DNA directly extracted from several soil samples supplemented with 50 ng DNA extracted from pure culture of *Bradyrhizobium japonicum* USDA 110. Lane 1 and 9: 1 kb marker, Lane 2-4: DNA from undisturbed forest soil sample was used as template (1, 3, 5 μl, respectively), Lane 5-7: DNA from cassava cultivation area soil sample was used as template (1, 3, 5 μl, respectively), Lane 8,10, and 11: DNA from sediment sample was used as template (1, 3, 5 μl, respectively)

Similar to this assay, amount of the soil bacteria *Pseudomonas aeruginosa* was represented. For all soil samples, the sephacryl S-300 microspin column plus electrophoresis purification methods allowed highest quality for PCR amplification at the lowest sample volume and the achievement can be decreased when the higher extracted DNA volume was amended (Figure 3.9a). Whereas the peat sample needed to be diluted 100-fold (Figure 3.9b). Thus, this purification protocol was an effective method for removing PCR inhibiting substances. It also was found that these results were in good agreement with the amount of organic matter (Table 3.1) in all soil samples. DNA obtained from peat sample, which has a highest organic matter, was purified and used as a template. The result of PCR showed that it needed more dilutions to be successful. While purified DNA obtained from the lowest organic matter (sediment) sample can be used directly in PCR without dilution. This result was also similar to those 3 other kinds of soils (undisturbed forest, rice field, and cassava cultivation area), which were in range of 1.07-4.25% organic matter.

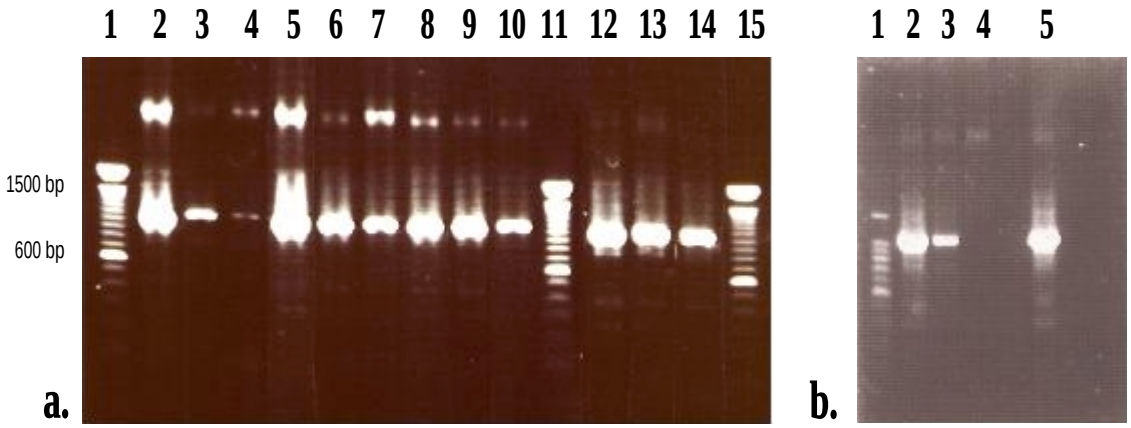

Figure 3.9 (a.) RISA-PCR products generated from DNA directly extracted from several soil samples supplemented with 50 ng DNA extracted from pure culture of *Pseudomonas aeruginosa*. Lane 1, 11, and 15: 100 bp marker, Lane 2-4: DNA from undisturbed forest soil sample was used as template (1, 3, 5 μl, respectively), Lane 5-7: DNA from cassava cultivation area soil sample was used as template (1, 3, 5 μl, respectively), Lane 8-10: DNA from rice field soil sample was used as template (1, 3, 5 μl, respectively), Lane 12-1: DNA from sediment sample was used as template (1, 3, 5 μl, respectively)

(b.) RISA-PCR products generated from 100-fold dilution DNA directly extracted from peat samples supplemented with 50 ng DNA extracted from pure culture of *Pseudomonas aeruginosa*. Lane 1: 100 bp marker, Lane 2-4: DNA from peat sample was used as template (1, 3, 5 μl, respectively), Lane 5: only DNA extracted from pure culture of *Pseudomonas aeruginosa* was used as template

3.5 Demonstration of microbial community diversity analysis

The differences between the microbial communities inhabiting the rice field, undisturbed forest, cassava cultivation area soil sample, sediment sample, and Peat were illustrated by different RISA banding patterns obtained from each sample (Figure 3.10). Each soil type presented numerous bands that are unique to each environment. These differences were suggested that each soil contain unique microbial populations, as well as the clear distinction between the bacterial communities.

The main limitation for this technique derives from the random patterns of variation of the spacer size and their unpredictability. Some closely related species can have very similar sizes and the reverse. However, the sequence information used in addition to the size pattern solves most of the problems, with only a small investment of time and resources. Community fingerprinting, the diversity which can be described, is limited by the amount of length variation of the spacer region. This turn to be a limitation for the analysis of complex microbial systems on a high hierarchical level, for example the whole bacterial domain (Garcia-Martinez et al., 1999).

To solve some problems such as similar size, further analysis by phylogenetic identification of the organisms represented by the RISA band can be investigated by excising the bands, extracting and cloning the DNA, and then sequencing the SSU rRNA of the RISA molecule. Alternatively, a larger portion of the SSU rDNA molecule could be obtained to provide more phylogenitic information. This could be accomplished by PCR amplification of soil DNA using a primer designed to hybridize the sequence of interest and a universal rRNA primer (Borneman et al., 1997).

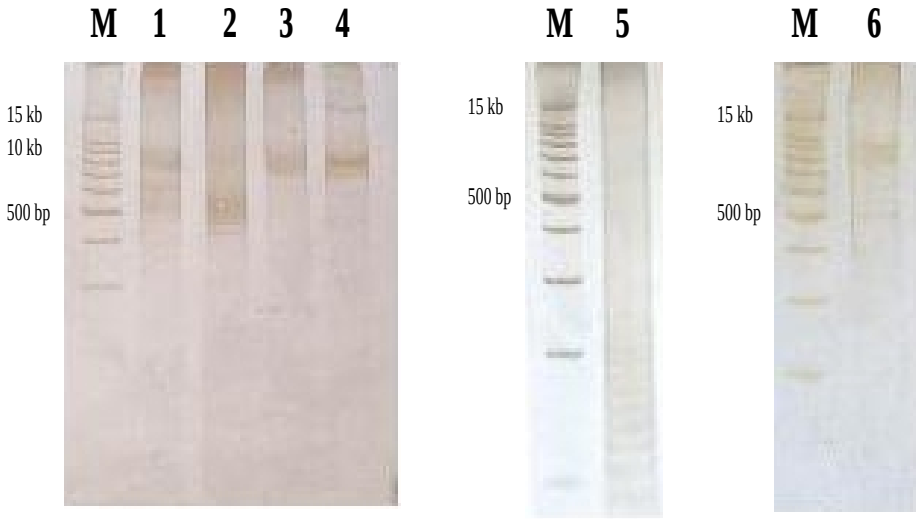

Figure 3.10 Silver staining of RISA-PCR products generated from
Lane 1: extracted DNA from rice field
Lane 2: extracted DNA from undisturbed forest
Lane 3: extracted DNA from cassava cultivation area
Lane 4: extracted DNA from pure culture of *Pseudomonas aeruginosa*
Lane 5: extracted DNA from sediment
Lane 6: extracted DNA from peat
Lane M: 100 bp DNA ladder

In addition, an attempt to obtain an overview of the structural diversity of microbial communities, denaturating gradient gel electrophoresis and temperature gradient gel electrophoresis analysis (DGGE/TGGE) has been introduced into microbial ecology (Muyzer et al., 1993). To investigate the bacterial diversity in each soil sample, the system was developed. The methodology was simplified on the basis of DNA denaturation. DNA material is denatured by denaturation agent such as urea to single-stranded DNA, which is subjected to polyacrylamide gel electrophoresis. The mobility of single-stranded DNA in the gel is dependent on its secondary structure, as determined by the nucleotide sequence. And the change in electrophoretic mobility may have been due to gel matrix interaction with single-stranded DNAs (Kumeda and Asao, 1996). Both techniques had been reported to be interchangeable, giving comparable fingerprints of microbial communities (Heuer and Smalla, 1997). Thus, these two strategies can also be used to diversification comparison. As an alternative to DGGE and TGGE, Peters and colleagues (2000) has been developed a protocol which allows the application of single-strand-conformation polymorphism (SSCP) (Hayashi, 1991; Orita et al., 1989) for the cultivation-independent assessment of microbial-community diversity (Schwieger et al., 1998) In contrast to DGGE and TGGE, no GC clamp or construction of gradient gels is required, and thus the SSCP method has the potential to be more easily applied (Lee et al., 1996).

One of the most common community fingerprinting methodologies is the separation of the PCR products by DGGE. This technique has been extremely useful in molecular microbial ecology (Muyzer et al., 1993; Teske et al., 1996; Kowalchuk et al., 1997). However, it requires the use of special polyacrylamide gels and the use of specific electrophoresis apparatus is convenient. Generated fragments ranging in size from approximately 300 to 1,500 bp. This size range is adequate for conventional agarose gels although the use of high resolution agaroses such as NuSieve® or Methaphor® is recommended. Acrylamide gels or even automatic sequencers can be used to obtain finer patterns in which many bands can be analyzed and compared and a very precise identification of them can be obtained (Hain et al., 1997).

From the RISA fingerprinting can be further analyzed, pairwise comparisons of RISA profiles were manually performed using the negatives and matrices (presence-absence and relative intensity of each band) constructed. In this analysis, whether two bands are present or absent as well as changes in band intensity are equally informative. Euclidian distances between pools were computed and subjected to hierarchical cluster analysis using the agglomerative second-order moment algorithm known as Ward's method (Ward, 1963) to produce the dendograms.

3.6 Determination of detection limit

To determine detection limit of this methodology, *P. aeruginosa* cells were inoculated at different amounts into 1 gram autoclaved soil sample. Then extraction and purification along with this protocol were performed in parallel with 3rd protocol. Figure 3.11 demonstrated the detection limit of target DNA in sediment, rice field, cassava cultivation area, undisturbed forest, and peat were 1, 10, 10^3, 10^4, and 10^5 cells per grams soil (dry weight) respectively. Results showed the correlation of PCR detection limit with the amount of organic matter content (in range of 0.73-62.77%) of each sample. These suggested that PCR was detectable as low as 1 cell per gram of soil in the presence of organic matter particularly in sediment sample.

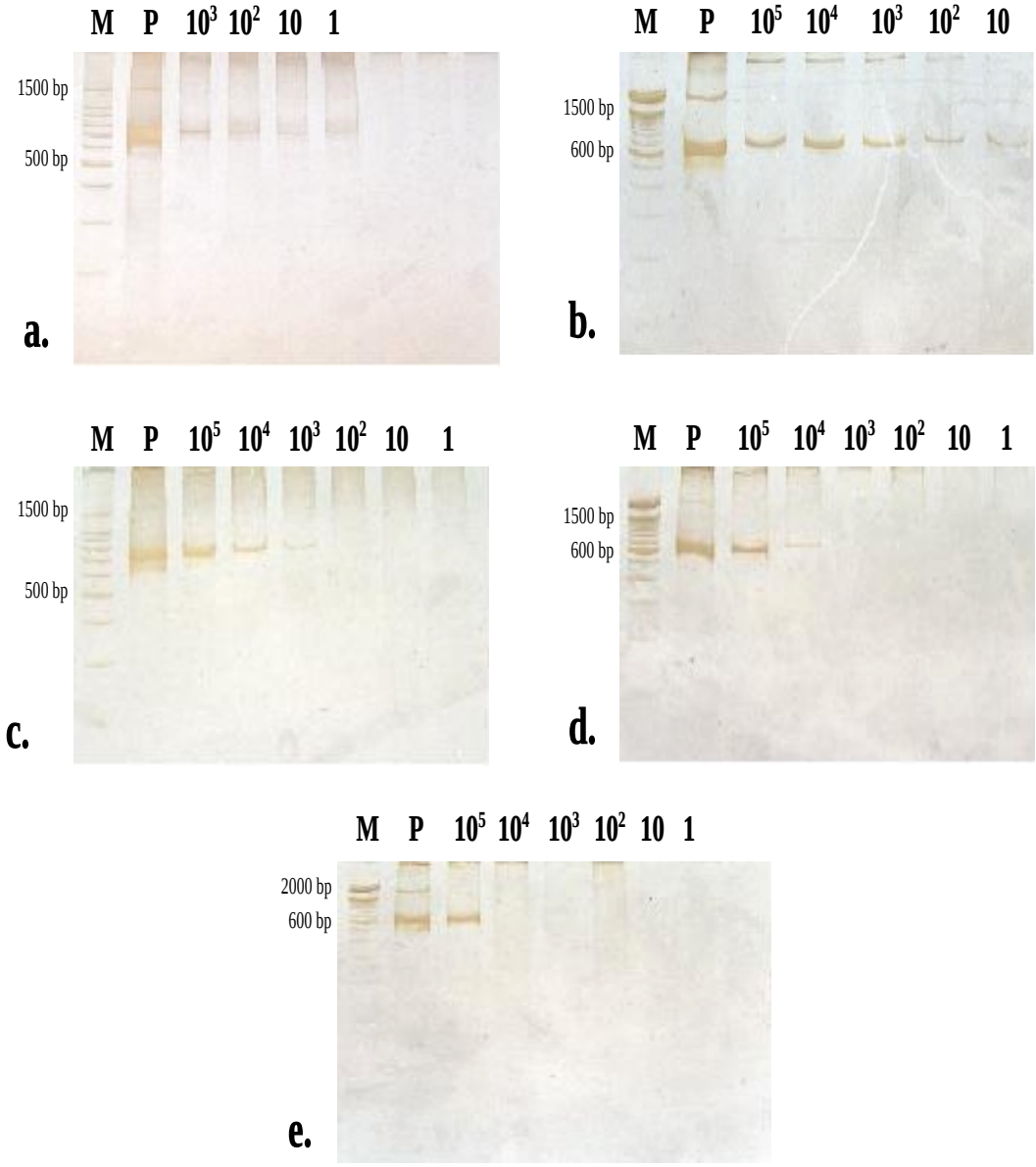

Figure 3.11 Silver staining of RISA-PCR products generated from DNA extracted from autoclaved soil samples inoculated with *Pseudomonas aeruginosa* (a.) sediment, (b.) rice field, (c.) cassava cultivation area, (d.) undisturbed forest, and (e.) Peat sample. Lane M: 100 bp DNA ladder and Lane P: RISA-PCR products generated from extracted DNA from pure culture of *Pseudomonas aeruginosa* Lane 10^5, 10^4, 10^3, 10^2, 10 and 1: amount of *Pseudomonas aeruginosa* inoculated

Southern blot hybridization technique was also employed to confirm the generated RISA-PCR products not artifact from rice field soil sample instead of sequencing. RISA-PCR products generated from DNA extracted from pure culture of *P. aeruginosa* was used as a probe. This trial was done to verify the reveal banding from detection limit experiment (especially at low concentrate inoculation) were not generated from others impurities or nonspecific target. The hybridization signals decreased when decreasing amounts of inoculated cells as well as the result of detection limit determination.

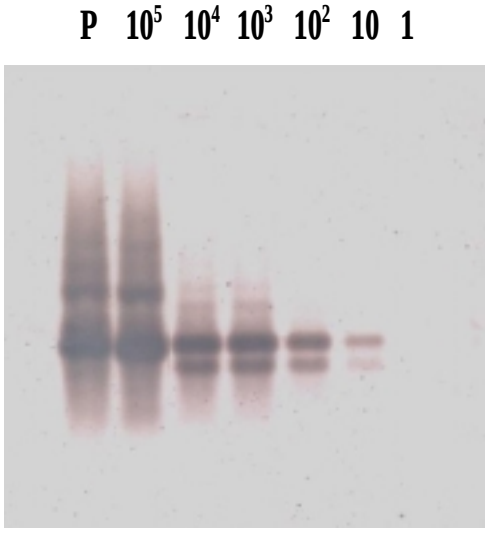

Figure 3.12 Southern blot hybridization of RISA-PCR products generated from DNA extracted from autoclaved soil from rice field samples, which inoculated with *Pseudomonas aeruginosa* by using RISA-PCR products generated from extracted DNA from pure culture of *Pseudomonas aeruginosa* as a probe Lane P: RISA-PCR products generated from extracted DNA from pure culture of *Pseudomonas aeruginosa* Lane 10^5, 10^4, 10^3, 10^2, 10 and 1: amount of *Pseudomonas aeruginosa* inoculated

3.7 The test of other primer amplification capability

Extracted DNA from various types of soil were tested with other primers without known species inoculation. For detection a group of interested from extracted DNA, *nifD* primer derived from conserved sequence of free-living N_2-fixing *Azotobacter vinelandii* was applied. The results of *nifD*-PCR products can be used to identify the phylogenetic diversity among nitrogen fixer (Zehr et al., 1998). The other primer was short tendemly repeated repetitive sequence (STRR) derived from repeated repetitive sequence of heterocytous cyanobacteria, this was applied to identify in a number of cyanobacteria genera and species (Mazel et al., 1990). Figure 3.13 and 3.14 showed *nifD*-PCR products and STRR-PCR products, which generated from DNA directly extracted from cassava cultivation area and rice field, respectively. This was suggested that not only RISA primer could be attained to identify bacterial DNA, another primers can also be used to study the complexity in ecosystems. To investigate whether interested bacteria present in soil sample is depending on an appropriate primer to specific gene exist. This rapid method can be applied in many diverse fields, including molecular biology, clinical diagnosis (Geldreich, 1995; Volossiouk et al., 1995), forensic analysis, population gentics (Ferris et al., 1996; Kumeda et al., 1996), and GMOs detection (Khan et al., 1998).

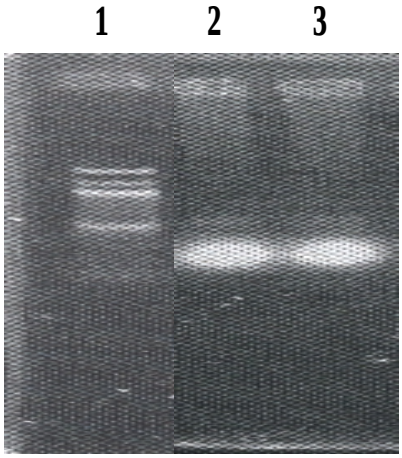

Figure 3.13 *nifD*-PCR products generated from DNA directly extracted from cassava cultivation soil sample. Lane 1: 100 bp marker, Lane 2-3: PCR Products

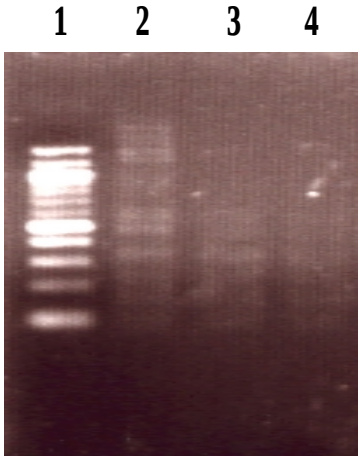

Figure 3.14 STRR-PCR products generated from DNA directly extracted from rice field soil sample. Lane 1: 100 bp marker, Lane 2: PCR products (template was DNA from pure culture of Cyanobacteria), Lane 3-4: PCR products (template was extracted bacterial DNA from soil)

CHAPTER 4
CONCLUSIONS

Methods using *in situ* cell lysis followed by DNA extraction and quantification offer new ways of studying microorganisms in the environment that make it possible to avoid biased related to cultivation. However, other problems still prevent the detection of a large part of the soil microbial community. For instance, the lysis treatment is ineffective with some organisms, either because they are resistant to the treatments or because they are protected by soil structures. An appropriate methodology for DNA direct extraction from various types of soil was created in this study. The methodology was developed by using lysozyme and proteinase-K followed by alkali treatment and physical disruption. This procedure required approximately 5 hrs for completion. The high amount of purified DNA approximately 4 μg per gram soil (dry weight) was obtained from this direct extraction procedure.

Application of Sephacryl S-300 microspin column twice, agarose gel electrophoresis followed by Freeze N Squeeze column, and applied the DNA through the Sephacryl S-300 microspin column again was the most efficient purification protocol. However, methods of extract purification need to be improved, especially for the study of soils with a high organic matter content. From the test of inhibitory effect of contaminants from extracted DNA to PCR reaction, DNA obtained from peat sample was found to be the most inhibited extract in the detection assay. Comparison with all others extracted DNA, peat required at least one additional 100-folds dilution to successfully PCR amplifying DNA.

The purified DNA could be used as DNA templates for PCR by using RISA primer when every soil samples were tested. The generated RISA PCR patterns in 10% acrylamide electrophoresis could be used to distinguish the microbial community in the environmental samples.

In this study the sensitivity was developed and detection limit was at least 1 cell per gram soil when inoculating with *P. aeruginosa*. This approach was a promising method for direct extraction of bacterial DNA from soil with regard to extraction efficiency and reduction of humic contamination, as well as high sensitivity for the target bacterial sequence. Since both methods, those for direct extraction and purification of crude DNA, are relatively fast, they can be implemented by investigators who study *in situ* gene occurrence. These methods might also facilitate the great benefit to scientists tracking genetically engineered microorganisms released into natural environment.

CHAPTER 5
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