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Optimization of Polymerase Chain Reaction Conditions of Denaturing Gradient Gel Electrophoresis for North Black Soil Microbes

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ABSTRACT

This paper studies the effects of concentrations of Mg^{2+} and dNTP, the annealing temperature, extension and cycling times in PCR of north black soil microbes using an orthogonal experiment. Results showed that the feasible PCR reaction system for soil microbes should be carried in a volume of 50 µl, composed of 50 ng soil microbial DNA template, 15 pm primer, 5 u Pfu enzyme, 0.2 mmol.L^{-1} Mg_2SO_4, and 0.3 mmol.L^{-1} dNTPs. The PCR reaction procedures were set up under two conditions. At first, the soil microbial DNA was denatured at 94°C for 5 min followed by 20 cycles of each 94°C for 1 min, 65-55°C for 1 min (descending at 0.5°C per cycle), and finally 72°C for 1 min. The second reaction conditions also included 20 cycles, each of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and a final extension temperature of 72°C for 7 min. The work was repeated several times on the same model using the same primers and PCR conditions to acquire clear and pure bands.

Keywords: PCR reaction system, agarose gels, DNA extraction

INTRODUCTION

Soil is a complex and heterogeneous habitat where a vast diversity of microorganisms exists. The majority of studies on soil are based on culturing organisms, which can characterize only a small fraction of all bacteria living in soil due to the fact that a large proportion of soil bacteria can not be cultured (Torsvik *et al*. 1996).

Species identification and population enumeration are critical in studying microbial communities. Traditionally, microbial species are cultured and then characterized by their respective physiological and biochemical properties. This method, however, has a serious drawback because most of the bacteria cannot be readily isolated and cultured. Amann (1995) estimated that only less than 1% of bacteria in the natural environment can be cultured. Researchers have applied various molecular methods to the study of microbial communities, including polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*. 1993).

Numerous researchers are using the method combining PCR and DGGE to study microbial communities in various environments (Fournier *et al*. 1998), including biofilms (Devereux *et al*. 1996) and activated sludge (Curtis and Caine 1998). Since PCR is the basis of the study, the optimization of PCR conditions is important for subsequent applications.

The aim of the present study was to analyze the feasibility of a PCR system including effects of concentrations of Mg^{2+} and dNTP, annealing temperature, extension time and cycling times of north black soil bacteria microbes using an orthogonal experiment.

MATERIALS AND METHODS

Soil samples

To confirm the feasibility of a PCR system, two soil samples (open field-soil and greenhouse-soil) were selected to ensure stability and consistency of the PCR system. Soil samples were collected from the surface layer (10-20 cm) nearby the roots in a horticultural experiment base of Northeast Agricultural University. The physicochemical characteristics of selected soil samples are listed in **Table 1**.

DNA extraction

DNA was extracted from soil samples by a slight modification of the method described by Xia *et al*. (1998). Briefly, 10 g of soil samples were ground with liquid nitrogen using a mortar and pestle for about 2 min until forming a fine powder. The soil powder was added into 15 ml of extraction buffer (0.1 mol.L^{-1} phosphate buffered saline, 0.1 mol.L^{-1} EDTA, 0.1 mol.L^{-1} Tris, 1.5 mol.L^{-1} NaCl, 1% CTAB, pH = 6.5) with 100 µl protease K in a 50 ml centrifuge tube. Following vibration (225 rpm) for 30 min at 37°C, 1.5 ml of 20% SDS was added and samples were incubated in a water bath at 65°C for 2 h with gentle shaking every 15-20 min.

After centrifugation at 6000 rpm for 10 min, the supernatant was transferred into another 50 ml tube. Five ml of DNA extraction buffer was added to the primary centrifuge tube and 0.5 ml of 20% SDS, incubated at 65°C for 10 min, and centrifuged again as above. Both supernatants were mixed together and added with an equal volume of chloroform: isoamyl alcohol (24:1). Then the mixture was centrifuged at 6000 rpm for 15 min and precipitated with 0.6 volumes of isopropanol by keeping at room temperature

Table 1 Physico-chemical characteristics of selected soil samples.

Soil type	O.M. (%)	Total N (%)	Alkaline N (mg/kg)	Total P (%)	Readily available P (mg/kg)	Slowly available K (mg/kg)	Readily available K (mg/kg)	EC ms/cm	pH
Open field soil (A)	3.48	0.449	178.5	0.100	255.4	864.9	468.1	0.34	7.44
Green House Soil (B)	6.14	1.276	275.0	0.272	345.2	865.9	588.2	0.67	7.66

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for 24 h. The crude nucleic acid pellets were obtained by centrifugation at 1000 rpm for 45 min at room temperature, and washed with cold 75% ethanol. The pellets were suspended in 200 ml of sterile deionized water, and stored at -20°C.

DNA purification

DNA was purified using Promega Wizard Extraction Kit according to the manufacturer's recommendations. Integrity of DNA was checked on 1% agarose gels. DNA concentrations were between 50-100 ng μl^{-1} (**Fig. 1**).

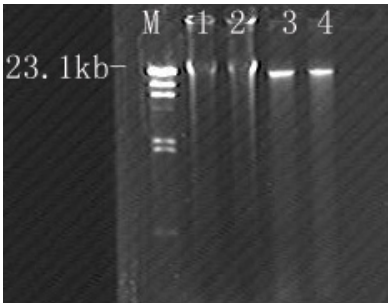

Fig. 1 Electrophoretogram of DNA purification.
Bands 1, 2: crude DNA;
Bands 3, 4: pure DNA;
Bands 1, 3: DNA of soil type A;
Bands 2, 4: DNA of soil type B;
M: λ-*Hind* III

Optimization of PCR conditions

The effects of concentrations of Mg^{2+} and dNTP, the annealing temperature, the extension time and the cycling times are important for optimization of PCR. PCR amplification was conducted following the method described by van Hannen *et al*. (1999). The pri-mers for PCR were specific for conserved bacterial 16S rRNA se-quences (Muyzer *et al*. 1993). A bacterial 16S rRNA fragment of about 230 bp was amplified with primers F_{341}GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and R_{518} (5′-ATT ACC GCG GCT GCT GG-3′).

Effects of concentrations of Mg^{2+} and dNTP

By using an orthogonal experiment, it was possible to establish the feasible concentrations of Mg^{2+} and dNTP, as described earlier (Zheng *et al*. 1998) (**Tables 2, 3**).

Table 2 Feasible concentrations of dNTP and Mg^{2+}.

Component	Concentration ($mmol.L^{-1}$)			
dNTP	0.15	0.20	0.25	0.30
Mg^{2+}	1.5	2.0	2.5	3.0

Table 3 Orthogonal design of PCR with different concentrations of dNTP and Mg^{2+}.

Experiment number	dNTP	Mg^{2+}
1	0.15	1.5
2	0.20	2.0
3	0.25	2.5
4	0.30	3.0
5	0.15	2.0
6	0.20	2.5
7	0.25	3.0
8	0.30	1.5
9	0.15	2.5
10	0.20	3.0
11	0.25	1.5
12	0.30	2.0
13	0.15	3.0
14	0.20	1.5
15	0.25	2.0
16	0.30	2.5

Assessment of annealing temperature, the extending time and the cycle times on PCR amplification

To study the effects of the annealing temperature, 55, 56 and 57°C were used. To study the effects of extension time, 30, 45 and 60 seconds were used. To study the effects of cycle times, 30, 35 and 40 cycles were used in PCR amplification.

RESULTS

Effect of Mg^{2+} and dNTP concentrations on PCR amplification

Mg^{2+} significantly affected the amplification by indirectly affecting the energy of the Pfu enzyme. A very high or very low concentration of Mg^{2+} induced failure of amplification.

dNTP was directly affected the PCR amplification. Great errors could be seen at high concentrations of dNTP. Furthermore, high concentrations of dNTP decreased the amount of Mg^{2+} and affected the Pfu enzyme (**Fig. 2**).

Fig. 2 shows that different concentrations of Mg^{2+} and dNTP resulted in different amplification results. The PCR products of 12 and 15 were better, and 8 and 11 also showed marked feasibility. All others were not feasible with polymer. The PCR products of both 12 and 15 consisted of Mg^{2+} at 0.2 $mmol.L^{-1}$, whereas the concentration of dNTP was 0.3 $mmol.L^{-1}$ and 0.25 $mmol.L^{-1}$, respectively. The PCR product of 12 was more significant than that of 15. So we got feasible production when the concentration of Mg^{2+} was 0.2 $mmol.L^{-1}$ and that of dNTP was 0.3 $mmol.L^{-1}$.

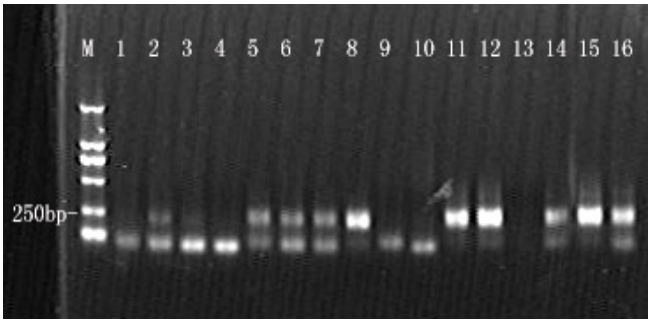

Fig. 2 Orthogonal design electrophoretogram. Bands 1-16: Orthogonal design electrophoretogram of experiment numbers 1~16 in **Table 3**.

Effect of different annealing temperatures on PCR amplification

Different annealing temperatures have different effects on amplification. Broadly, the higher the temperature, the stronger the specificness. The ideal amplification was acquired at an annealing temperature of either 55°C or 56°C (**Fig. 3**), the latter being more significant than that at 57°C.

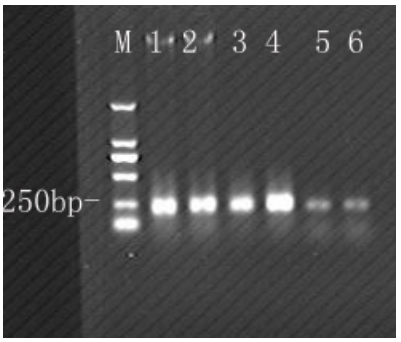

Fig. 3 Effect of different annealing temperatures on PCR amplification.
Bands 1, 2: 55°C;
Bands 3, 4: 56°C;
Bands 5, 6: 57°C;
Bands 1, 3, 5: DNA of soil type A;
Bands 2, 4, 6: DNA of soil type B.
M: Marker 2000

Effect of different extension times on PCR amplification

A feasible extension time has significant effects on the PCR product. Pfu enzyme extends very quickly and adequate product results in a minute at 70-72°C. Since the bands of extension time for 30 s were not clear, an extension time for 45 s or 60 s resulted in clearer banding and thus an extension time for 60 s was adopted to get adequate products (**Fig. 4**).

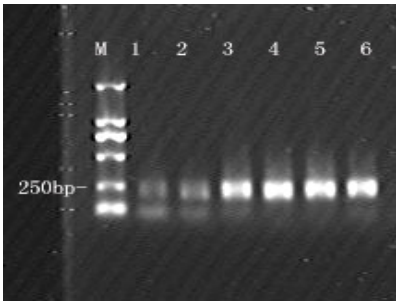

Fig. 4 Effect of extending time on PCR amplification.
Bands 1, 2: 30 s;
Bands 3, 4: 45 s;
Bands 5, 6: 60 s;
Bands 1, 3, 5: DNA of soil type A;
Bands 2, 4, 6: DNA of soil type B.
M: Marker 2000

Effect of cycle times on PCR amplification

Cycle times also have an effect on the amplification. According to a previous study, the theoretical products of amplification were 2N (N = cycle times) (Zheng *et al*. 1998).

Generally, PCR products of fewer cycles cannot be detected and too many cycles will saturate the products, while only wasting time. Forty cycles resulted in more accurate and stronger bands in both soil samples than that of other cycle times, and this may be suitable for amplification (**Fig. 5**).

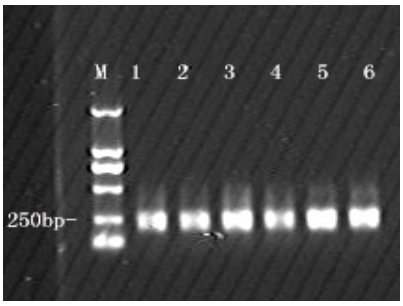

Fig. 5 Effect of number of cycles on PCR amplification.
Bands 1, 2: 30 cycles;
Bands 3, 4: 35 cycles;
Bands 5, 6: 40 cycles;
Bands 1, 3, 5: DNA of soil type A;
Bands 2, 4, 6: DNA of soil type B.
M: Marker 2000

DISCUSSION

Nowadays, quantitative molecular techniques – such as T-RFLP, SSCP, DGGE, etc. – have been developed for the rapid analysis of microbial community diversity in soil environments. T-RFLP or Terminal Restriction Fragment Length Polymorphisms, for example, employs PCR in which one of the two primers used is fluorescently labeled at the 5′ end and is used to amplify a selected region of bacterial genes encoding 16S rRNA from total community DNA. The PCR product is digested with restriction enzymes, and the fluorescently labeled terminal restriction fragment is precisely measured by using an automated DNA sequencer. Liu *et al*. (1997) used T-RFLP to rapidly assess sewage sludge bacterial communities. Single-strand-conformation polymorphism (SSCP) of DNA, a method widely used in mutation analysis, was adapted to the analysis and differenttiation of cultivated pure-culture soil microorganisms and noncultivated rhizosphere microbial communities. SSCP is an electrophoretic technique which has been developed, like Denaturing gradient gel electrophoresis, or DGGE (Fischer *et al*. 1979), for the detection of mutations. Under nondenaturing conditions, single-stranded DNAs will fold into secondary structures (conformations) according to their nucleotide sequences and their physicochemical environment (e.g., temperature and ion strength). Due to different electrophoretic mobilities, different conformations can be separated by nondenaturing polyacrylamide gel electrophoresis (Orita *et al*. 1989). Since no GC clamp primers, gradient gels, or specific apparatus is required, SSCP is potentially more simple and straightforward than DGGE or TGGE by using SSCP in combination with an automated DNA sequencer.

DGGE (Fischer *et al*. 1979) is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically. This method has been applied to the analysis of 16S rRNA genes from environmental samples (Muyzer *et al*. 1993) and allows the separation of a heterogeneous mixture of PCR amplified genes on a polyacrylamide gel. Individual bands may be excised, reamplified and sequenced (Ferris *et al*. 1996), or challenged with a range of oligonucleotide probes (Muyzer *et al*. 1993) to give an indication of the composition and diversity of the microbial community. DGGE is relatively rapid to perform, and many samples can be run simultaneously. The method is, therefore, particularly useful when examining time series and population dynamics.

Many molecular techniques, such as the ones discussed above, must be combined with Polymerase Chain Reaction (PCR). PCR, as an *in vitro*- rather than *in vivo*-based DNA amplification procedure has so far become a popular and very important tool for gene cloning in molecular biology research. A large amount of alternative forms of PCRs for different purposes and applications have been developed based on a deep understanding of the basic principle of PCR. Despite the fact that PCR is characterized by its three Ss: selectivity, sensitivity, and speed (Arnheim *et al*. 1992), workers in practice often feel that the common traditional three-step PCR including denaturation at higher, annealing at lower, and elongation at middle temperature cannot satisfy requirements for the frequently appeared nonspecific products in PCR.

Touch down PCR was originally designed to circumvent spurious priming that frequently appeared in PCR (Don *et al*. 1992). This approach took advantage of the exponential nature of PCR reactions according to the inference that the specific annealing between a piece of prime and its corresponding complementary sequence on the single stranded template always occurs at a higher temperature than a nonspecific priming for the presence of a partial sequence mismatched between the primer and nonspecific single-stranded templates. In the touchdown PCR, first carried out by Don *et al*. (1992), the annealing temperature of the reaction is decreased 1°C every second cycle from 65°C to a touchdown at 55°C, followed by 30 cycles of reactions annealing at around 50°C. Some authors (Zhang *et al*. 2002) have compared the specificity of touchdown and common PCRs by use of the most frequently used *Taq* DNA polymerase and the *Pfu* DNA polymerase of higher fidelity. Results with both DNA polymerases clearly demonstrated that touchdown PCR had higher specificity than the common PCR.

Several authors have reported that the efficiency of PCR amplification of target soil DNA depends on the relationship between the level of target DNA and that of contaminants in the crude extract, which in turn is affected by the efficacy of the purification procedure (Saano *et al*. 1995). Because of the presence of high organic matter in greenhouse soil, it is very difficult to amplify the DNA of north black soil. The organic matter of soil cannot be removed easily, and has a significant effect on the *Pfu* enzyme activity.

With an orthogonal study, we can get a feasible PCR reaction system for north black soil bacteria microbes and ideal products for use in further experiments. In this study, the feasible concentrations of Mg^{2+} and dNTP were 0.2 $mmol.L^{-1}$ and 0.3 $mmol.L^{-1}$, respectively. The feasible PCR reaction system included: 56ºC annealing temperature, 60 s extension time, 40 cycles and a final extension temperature of 72ºC. The work was repeated several times on the same model using the same primers and PCR conditions to acquire clear and pure bands. Thus, our results showed that the DNA extraction method we used was fit for north black soil and organic matter removal using the kit.

Since there are many factors involved in PCR amplification, the changes or effects of any unfeasible factor would significantly affect PCR amplification. Thus we must seek an optimal reaction system that can be carried out with different apparatuses, different reagents, and different objectives. The present study gave optimal factors including temperature, extension times and cycle times for PCR amplification of north black soil microbes, and the effort taken in this study will help for future works.

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REFERENCES

Amann RI (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**, 143-169

Arnheim N, Erlich H (1992) Polymerase chain reaction 5 strategy. *Annual Review of Biochemistry* **61**, 131-156

Curtis TP, Caine NG (1998) The comparison of the diversity of activated sludge plants. *Water Science and Technology* **37**, 71-78

Devereux R, Hines ME, Stahl DA (1996) S cycling: characterization of natural communities of sulfate reducing bacteria by 16S rRNA sequence comparisons. *Microbiological Ecology* **32**, 283-292

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19**, 4008

Ferris MJ, Muyzer G, Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial community. *Applied and Environmental Microbiology* **62**, 340-346

Fischer SG, Lerman LS (1979) Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell* **16**, 191-200

Fournier D, Lemieux R, Couiland D (1998) Genetic evidence for the diversified bacteria populations in wastewater sludge during biological leaching of metals. *Biotechnology Letters* **20**, 27-31

He ZW, Liu YS (1998) Optimization of PCR condition with orthogonal design intuitive analysis. *Hu Nan Medical University Journal* **23**, 403-404

Muyzer G, de Waal EC, Uitterrlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction – amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**, 695-700

Muyzer G, Waal EC, Uitterlinden AG (1993) Profiling of complex microbial population by DGGE analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. *Applied and Environmental Microbiology* **62**, 2676-2680

Muyzer G, Ellen CW, Andre GU (1993) Profiling of complex microbial populations by denaturing gradient gel Electrophoresis analysis of polymerase chain reaction genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**, 695-700

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences USA* **86**, 2766-2770

Saano A, Tas E, Pippola S, Lindstrom K, van Elsas JD (1995) Extraction and analysis of microbial DNA from soil. In: Trevors JT, van Elsas JD (Eds) *Nucleic Acids in the Environment: Methods and Applications*, Springer-Verlag, Heidelberg, pp 49-67

Torsvik V, Sorheim R, Goksoyr J (1996) Total bacterial diversity in soil and sediment communities – a review. *Journal of Industrial Microbiology* **17**, 170-178

van Hannen EJ, Zwart G, van Agterveld MP, Gons HJ, Ebert J, Laanbroek HJ (1999) Changes in Bacterial and Eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. *Applied and Environmental Microbiology* **65**, 795-801

Wen TL, Terence LM (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63**, 4516-4522

Xia BC, Zhou J, Tiedje Z (1998) Application of molecular biology method in microbial ecology. *Zhong Shan University Journal* **37**, 97-101

Zhang GX, Yuan BM, Xu PR, Wang JM, Xue LX (2002) Modified Touchdown PCR as a high efficient approach to improve the specificity of PCR. *Life Science Research* **6**, 314-317