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 µl Pfu enzyme, 0.2 mmol.L$^{-1}$ Mg$_2$SO$_4$, 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.

Table 1 Physico-chemical characteristics of selected soil samples.

<table>
<thead>
<tr>
<th>Soil type</th>
<th>O.M. (%)</th>
<th>Total N (%)</th>
<th>Alkaline N (mg/kg)</th>
<th>Total P (%)</th>
<th>Readily available P (mg/kg)</th>
<th>Slowly available K (mg/kg)</th>
<th>Readily available K (mg/kg)</th>
<th>EC ms/cm</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open field soil (A)</td>
<td>3.48</td>
<td>0.449</td>
<td>178.5</td>
<td>0.100</td>
<td>255.4</td>
<td>864.9</td>
<td>468.1</td>
<td>0.34</td>
<td>7.44</td>
</tr>
<tr>
<td>Green House Soil (B)</td>
<td>6.14</td>
<td>1.276</td>
<td>270.0</td>
<td>0.272</td>
<td>345.2</td>
<td>865.9</td>
<td>588.2</td>
<td>0.67</td>
<td>7.66</td>
</tr>
</tbody>
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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 (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](image)

**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 primers for PCR were specific for conserved bacterial 16S rRNA sequences (Muyzer et al. 1993). A bacterial 16S rRNA fragment of about 230 bp was amplified with primers F341GC (5’-CGC CGG GCC GGC GGG GCG GGG GCA CGG GCC TAC GGG AGG CAG CAG-3’) and R518 (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>
<thead>
<tr>
<th>Component</th>
<th>dNTP (mmol.L$^{-1}$)</th>
<th>Mg$^{2+}$ (mmol.L$^{-1}$)</th>
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<tr>
<td></td>
<td>0.15</td>
<td>1.5</td>
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<td>0.20</td>
<td>2.0</td>
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<td>0.25</td>
<td>2.5</td>
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</table>

**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 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 specificity. 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.

**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).
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).

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 differentiation 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 non-denaturing 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, re-amplified 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 non-specific 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 (Saanol et al. 1995). Because of the presence of high organic matter in 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 Mg²⁺ and dNTP were 0.2 mmol.L⁻¹ and 0.3 mmol.L⁻³, respectively. The feasible PCR reaction system included: 56°C annealing temperature, 60 s extension time, 40 cycles and a final extension temperature of 72°C. 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.
ACKNOWLEDGEMENTS

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REFERENCES