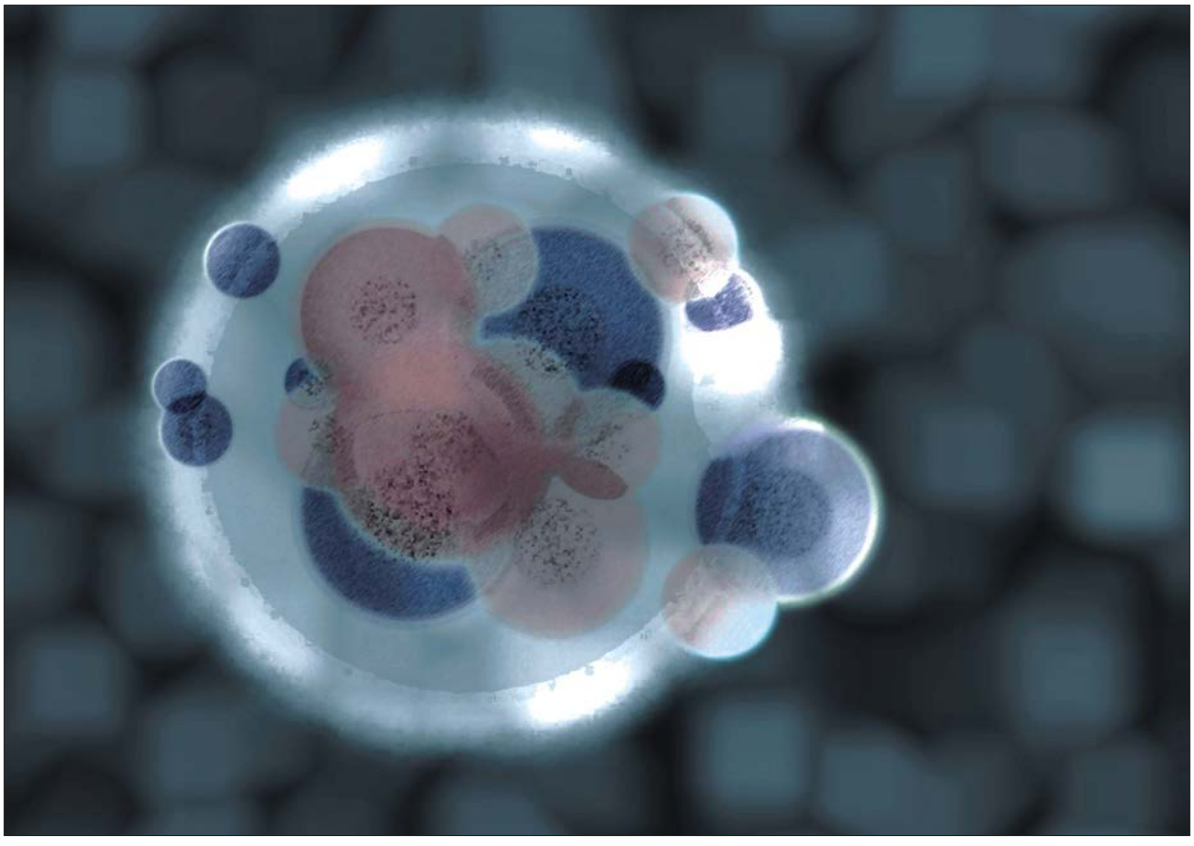

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Identification of an Isolate of *Pseudomonas aeroginosa* Deposited in PTCC as a PHA Producer Strain: Comparison of Three Different Bacterial Genomic DNA Extraction Methods

[1,2]Hamid Mir Mohammad Sadeghi, [1,3]Ali Jahanian Najafabadi,
[1]Daryoush Abedi and [1]Abbas Jafarian Dehkordi
[1]Department of Pharmaceutical Biotechnology and Isfahan Pharmaceutical Research Center,
School of Pharmacy, Isfahan University of Medical Sciences and Health Services, Isfahan, Iran
[2]Applied Physiology Research Center,
Isfahan University of Medical Sciences and Health Services, Isfahan, Iran
[3]Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran

Abstract: In this study, we used a nucleic acid based method to identify the capability of Polyhydroxy alkanoate (PHA) production of two isolates of *Pseudomonas aeroginosa* deposited in Persian Type Culture Collection (i.e., *Pseudomonas aeroginosa* PTCC 1310 and 1740) and present results showed this capability for the PTCC1310 but not for the other isolate. This identified isolate could be used for massive production of the mentioned polymers or its *pha* locus could be cloned and used for the functional expression of the PHA biopolymers. Here we also compared three bacterial genomic DNA extraction methods with respect to their difficulty, cost and the time of procedure. These methods included simple boiling method, phenol-chloroform method and commercial DNA extraction kit. All three methods gave reasonable amount of DNA that could be used as templates for the detection of *pha* gene. In conclusion we have identified a *Pseudomonas aeroginosa* strain present in Iran containing the necessary genes for PHA production and suggest that the simple boiling method can be utilized for further screening of bacterial samples for the presence of these genes.

Key words: PHA, *Pseudomonas aeroginosa* PTCC 1310 and 1740, genomic DNA extraction

INTRODUCTION

The unlimited use of petroleum based plastics for different applications and purposes have created many problems related to the disposal of solid wastes (Kessler and Witholt, 1999; Salehizade and Van Loosdrecht, 2004). Therefore, worldwide programs have been started to solve the problems caused by the accumulation and non-decompository properties of these materials (Braunegg *et al*., 1998; Lee and Chang, 1995). One of the solutions to solve the problem is the use of biodegradable polymers, which will decompose after their disposal (Shimao, 2001). Among the candidates for biodegradable biopolymers, polyhydroxyalkanoates (PHAs) have received much attention because of their similar properties to synthetic plastics and their potential use as biodegradable and biocompatible thermoplastics (Aldor and Keasling, 2003; Salehizade and Van Loosdrecht, 2004) used for different industrial, marine, agricultural and also medical applications (Vidal-Mas *et al*., 2001; Agus *et al*., 2006a). Absorbable surgical suture, prosthesis or tissue engineering material and controlled release of drugs are some applications of these biopolymers in medicine (Zinn *et al*., 2001; Wang *et al*., 2003, Rossi *et al*., 2004).

PHAs are natural storage compounds which are mostly produced by a great number of microorganisms when in the presence of excessive carbon sources they encounter nutrient limitation. Some microorganisms, however, accumulate these polymers from the beginning of the exponential phase (Vidal-Mas *et al*., 2001; Agus *et al*., 2006b; Lemos *et al*., 2006). These biodegradable biopolymers are classified into three groups including short chain length (scl) PHAs containing 3 to 5 carbon atoms in monomer units, medium chain length (mcl) PHAs containing 6 to 14 carbon atoms in monomer units and scl-co-mcl containing 3 to 14 carbon atoms in monomer units (Solaiman and Ashby, 2005a).

PHA synthases are enzymes responsible for the polymerization and production of PHA. On the basis of structure, substrate specificity and organization of the

Corresponding Author: Hamid Mir Mohammad Sadeghi, Department of Pharmaceutical Biotechnology,
School of Pharmacy and Pharmaceutical Sciences,
Isfahan University of Medical Sciences and Health Services,
P.O. Box 81746-73471, Isfahan, Iran Tel: +98 311 792 2616 Fax: +98 311 668 0011

gene locus these enzymes are classified into four classes. Various types of PHA producer microorganisms harbor different types of *pha* gene loci (Rehm, 2003). The class II *pha* operon consists of two PHA synthase genes (*phaC1* and *phaC2*) flanking a PHA depolymerase gene (*phaZ*) and is found in mcl-PHA producing pseudomonas (Rehm, 2003; Solaiman and Ashby, 2005b). Because of the importance of the PHAs mentioned above, many groups developed various methods including lipophilic dye staining and nucleic acid based methods to identify the PHA producing microorganisms (Solaiman and Ashby, 2005b; Tyo *et al*., 2006). Among the nucleic acid based methods such as Southern blot hybridization based method developed by Timm *et al*. (1994) and PCR based methods developed by Lopez *et al*. (1997) or Solaiman *et al*. (2000), the later is highly specific for the identification of microorganisms harboring class II PHA polymerase. Since it has been shown that some *Pseudomonas* strains can produce these polymers, in the present study we focused on the identification of the PHA genes in *Pseudomonas aeroginosa* available in Persian Type Culture Collection (i.e., *Pseudomonas aeroginosa* PTCC 1310 and PTCC 1740).

To date various genomic DNA extraction methods developed and reported by different groups. The most important differences between these methods are the used reagents, the cost, time and the difficulty of procedure. In case of screening a large amount of samples a less expensive and easier method is preferred. In order to find such an optimal method for our later studies on the screening of other bacteria for their PHA production capability, here we also compared three different genomic DNA extraction methods including phenol-chloroform method, simple boiling method and extraction of genomic DNA using a commercial kit to clarify the advantages and disadvantages of each method against the others.

MATERIALS AND METHODS

Bacterial strains: *Pseudomonas aeroginosa* PTCC 1310 and PTCC 1740 were obtained from Persian Type Culture Collection (Tehran, Iran). As positive control *Pseudomonas putida* DSMZ 1693 and *Pseudomonas oleovorance* DSMZ 1049 were obtained from DSMZ, Germany. All the used bacteria were cultivated on nutrient agar or in nutrient broth before the extraction of genomic DNA.

Genomic DNA extraction: In this study three different methods of genomic DNA extraction were compared:

Simple boiling: In this method bacterial colonies were suspended in 1 mL TE buffer (1 mM EDTA, 10 mM Tris, pH = 8) and boiled for 10 min. Subsequently, the samples were transferred to -70°C and kept for 5 min. Finally the samples were placed at 4°C without any centrifugation for later use.

Phenol-chloroform method: The bacteria pelleted by centrifugation at 3500 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in 200 μL of digestion buffer (100 mM Tric-Cl (pH 8.0), 5 mM EDTA (pH 8.0), 1% SDS) containing freshly thawed proteinase K (500 μg mL^{-1}). The sample was then incubated at 55°C in a water bath overnight. After the incubation, the tube content was mixed with an equal volume of phenol-chloroform-isoamyl alcohol solution (25:24:1), then centrifuged for 5 min at 13000 rpm. The supernatant was transferred into another clean tube carefully and without touching the pellet. Then, to remove the remained amount of phenol it was washed with the chloroform-isoamyl alcohol (24:1) solution. In the next step, 3 M sodium acetate was added to the mixture at a final concentration of 0.3 M. Two fold volume of ice-cold absolute ethanol was added and the content was centrifuged at 0°C and 13000 rpm for 10 min resulting in a white precipitate. The upper liquid was discarded and 70 μL ethanol 70°C was added to the tube and centrifuged for 5 min. The upper liquid was discarded and the precipitated DNA was dissolved in 200 μL double distilled water and kept at 4°C for later use.

Commercial kit: Genomic DNA of the used strains was extracted using High Pure PCR template preparation kit (Roche, Germany) following the manufacturer's instruction.

PCR: In order to screen the capability of PHA production, a PCR based method developed by Solaiman *et al.* (2000) was applied and the oligonucleotides 5′-ACA GAT CAA CAA GTT CTA CAT CTT CGAC-3′ and 5′-GGT GTT GTC GTT GTT CCA GTA GAG GAT GTC-3′ were used as suggested by that study. PCR condition included a primary denaturation time at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C and a final extension time of 5 min at 72°C. PCR reaction contained: 1 unit Taq DNA polymerase, 3 μL of 25 pM of each primer, 1 μL template DNA, 5 μL of 10 X PCR buffer and 1 μL dNTP (100 mM) at a final volume of 50 μL. The PCR products were examined on a 0.7% agarose gel containing a concentration of 0.5 μg mL^{-1} ethidium bromide to visualize the bands.

RESULTS

PCR amplification in order to identify the capability of PHA production: Genomic DNA of *P. putida* (DSMZ 1693) and *P. oleovorans* as positive controls and also genomic DNA of *P. aeroginosa* PTCC 1310 and PTCC 1740 were extracted using commercial kit and examined by agarose gel electrophoresis (figure not shown). All the extracted genomes were used as templates for PCR reaction.

In order to screen the existence of type II *pha* gene locus a primer pair (see Materials and Methods) was used for PCR analysis. A band at about 540 bp was expected if this locus was present. As shown in Fig. 1, gel electrophoresis of the PCR products for *P. putida* (DSMZ 1693), *P. oleovorans* (DSMZ 1049) and *P. aeroginosa* PTCC1310 but not *P. aeroginosa* 1740 gave the expected band and these results were reproducible. For more examination, the PCR reaction for *P. aeroginosa* PTCC 1740 was performed using different concentration of $MgCl_2$ ranging from 2 to 4 mM or under different annealing temperature ranging from 52 to 60°C but the desired band was not observed (data not shown).

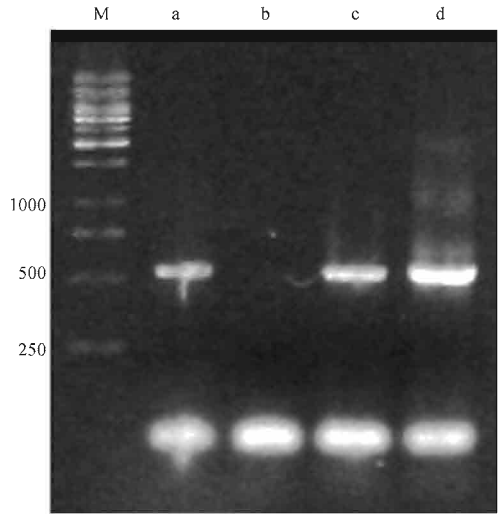

Fig. 1: Gel electrophoresis of PCR products using genomic DNA extracted by commercial kit as PCR template. As it shows the isolate *P. aeroginosa* 1310 and the two positive controls including *P. putida* DSMZ 1693 and *P. Oleovorans* DSMZ 1049 revealed the expected band at about 540 bp region (lanes a, c and d respectively). But the PCR amplification reaction for the isolate *P. aeroginosa* PTCC1740 resulted in no product. M represents molecular size marker. Two microliter of each sample was run

Comparison of different genomic DNA extraction methods: Having proved the existence of *pha* locus in genomic DNA of *P. aeroginosa* PTCC 1310, different genomic DNA extraction methods were performed to extract its genomic DNA. In fact, commercial kit, simple boiling method and phenol-chloroform method were performed to extract genomic DNA (see materials and methods) and the extracted products were gel electrophoresed (Fig. 2).

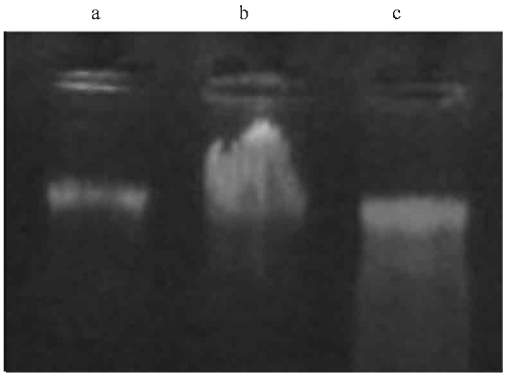

Fig. 2: Genomic DNA extraction of *P. aeroginosa* PTCC1310 by using different methods including (a) commercial kit, (b) simple boiling and (c) phenol-chloroform method. Two microliter of each sample was electrophoresed

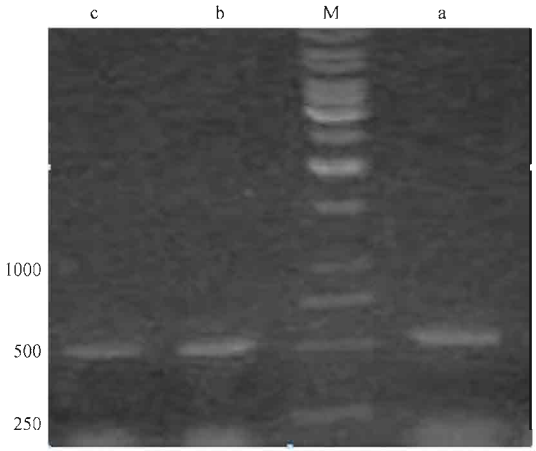

Fig. 3: Gel electrophoresis of PCR products using genomic DNA extracted by (a) commercial kit, (b) phenol-chloroform and (c) simple boiling methods. As it shows when DNA template obtained from these methods were used, the obtained PCR products were similar in size with almost equal intensity and sharpness. M represents DNA size marker. Two microliter of each sample was electrophoresed

In order to examine the efficiency of extraction methods, extracted DNA was used as template for PCR amplification using the mentioned primers. Gel electrophoresis of the PCR products revealed same bands at about 540 bp for all three DNA samples (Fig. 3).

DISCUSSION

To date different methods have been used to screen the existence of *pha* locus in various microorganisms and also the capability of PHA production by them. Lipophilic dyes such as sudan black B, Nile blue A and Nile red have been traditionally used for the screening of PHA containing bacteria (Spiekermann *et al*., 1999; Gorenflo *et al*., 1999; Vidal-Mas *et al*., 2001). However, because of their non specific lipid binding property, these dyes could also stain other lipid materials. Because of this non specific staining property and some other problems such as being time consuming and the possibility of false negative results which would occur when a bacterium harbor the genetic organization for PHA production but the condition is not suitable to produce it, several nucleic acid based methods were developed by different groups like Timm *et al*. (1994), Lopez *et al*. (1997) and Solaiman *et al*. (2000); the latest group developed the first PCR procedure that specifically detects class II *pha* genes. They used a pair of primers designed on the basis of a highly conserved region of class II *pha* loci of various *Pseudomonas*. In this study, we used the primer pair suggested by Solaiman *et al*. (2000) as we examined the PHA production of two *Pseudomonas aeroginosa* isolates. Gel electrophoresis of the PCR products revealed the expected bands for the positive controls (*P. putida* DSMZ 1693 And *P. oleovorans* DSMZ 1049) and also for *P. aeroginosa* PTCC 1310 but not for *P. aeroginosa* 1740 confirming the capability of *P. aeroginosa* PTCC 1310 to produce medium chain length polyhydroxyalaknoates.

Here we also compared three different genomic DNA extraction methods. The comparison was based on different criteria like accuracy, difficulties, time and cost of the experiment and reproducibility of results. Commercial kits, despite their fast and accurate procedure are expensive. In comparison phenol-chloroform method is less expensive and also accurate, but it is time consuming and the procedure should be performed under specific condition to prevent phenol oxidation and also chloroform evaporation. Simple boiling method revealed reasonable results. This method is fast, inexpensive, repeatable and also does not need any specific reagents. But the most important problem of this method is limited time of keeping samples for later use. In this method DNA is not separated from cell lysates and other cytoplasmic and membrane components which could destruct the genomic DNA. Therefore, beside the easy, inexpensive and fast properties of this method, further modifications are required.

In conclusion, in this study we identified the *Pseudomonas aeroginosa* PTCC1310 as a PHA producer microorganism which could be used in later studies for the production of the precious PHA materials, or for the cloning and molecular study of its *pha* gene locus in the expression host for the over production of PHA. Here we also found the simple boiling method as a reliable, reproducible, inexpensive and fast method for the extraction of bacterial genomic DNA in order to screen a large amount of bacteria to identify their PHA production capability on the basis of the mentioned PCR based method.

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