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*Original Article*

# Comparison of five methods for extraction of genomic DNA from a marine Archaea, *Pyrococcus furiosus*

*Hamid Mirmohammadsadeghi[1], Daryoush Abedi[2], Hamid Reza Mohmoudpour[3], Vajihe Akbari[4]*

**ABSTRACT**

***Objective:*** Archaea are one of the interesting forms of life due to their unique structural, physiological, biochemical and genetic features. For molecular analysis, high quality DNA should be extracted from these species.

***Methodology*:** In the present study, five extraction methods were evaluated for the isolation of DNA from *Pyrococcus furiosus* and the results compared. The DNA extracts were analyzed using gel electrophoresis and UV-spectophotometry.

***Results*:** Our results showed that a combination of enzymatic lysis (proteinase K) and chemical lysis (SDS) is the most suitable procedure for cell disruption of *P. furiosus*. This study showed that using freeze/thaw alone is ineffective in cell disruption. Recovering nucleic acids from cell lysates using silica-based DNA binding and solvent extractions was not suitable for DNA recovery and purification.

***Conclusions*:** The method proposed by Ramakrishnan and his coworker for isolation of genomic DNA from elemental sulfur-reducing hyper- thermophilic Archaea was successful for recovering DNA with good quantity and quality.

**KEY WORDS**: DNA extraction, *Pyrococcus furiosus*, Cell disruption, Enzymatic lysis, Chemical lysis.

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## INTRODUCTION

Archaea are one of the interesting domains of life due to their unique structural, physiological, biochemical and genetic features. Many archaea can survive under harsh conditions, including high/low temperatures, high/low pH, high salinity, anoxic environments and high pressure.[1] Scientific communities are focusing on extremophile archaea to evaluate molecular, physiological and evolutionary mechanisms of their adaptation. The study of archaea also helps in understanding eukaryotic cell biology, since compared to eukaryotic systems, they can provide a suitable system with higher stability and lower complexity. For example, obtaining the crystal structure of RNA polymerase from archaea helps to understand the structure and function of RNA polymerase II.[2] Due to their unusual features, extremophile archaea are considered as valuable resources in biotechnological and industrial processes. They can be used for bioremediation of toxic compounds. Their enzymes are important for industrial applications. Other metabolites of extremophile archaea, such as lipids, are suitable for the formation of liposomes as thermostabe drug delivery systems.[3] Their chaperones and chaperonins can be used for refolding, stabilization and solubilization of recombinant proteins.[4,5]

Considering the importance of archaea, there are different molecular techniques regarding their

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applications in research and biotechnology. Before any molecular analysis, such as gene probing, PCR, cloning, sequencing and expression, it is required to extract high quality DNA that can be used for these analyses. To purify nucleic acids, many protocols have been proposed. These protocols are different in the means of cellular lysis and isolation of DNA. However, in some cases, DNA extraction may be more difficult than expected, as some bacteria and archaea are resistant to cell disruption.[6] Different methods have been developed for cell disruption, including chemical, enzymatic or physical treatment or a combination thereof.[7]

The objective of the present study was to compare the effectiveness of five extraction methods for the isolation of DNA from a thermophile archaea, *Pyrococcus furiosus.*

## METHODOLOGY

***Organism, media and growth conditions:*** *Pyrococcus furiosus* (DSM 3638) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the medium of ATCC 1915 was used with some modifications. Approximately 0.3 g of powdered sulfur was added to each test tube. The 1915 Pyrococcus medium for cultivation of *P. furiosus* was composed of the following components (per liter), which were dissolved in seawater: $KH_2PO_4$, 0.5 g; $NiCl_2.6H_2O$, 2 mg; yeast extract, 1 g; tryptone, 0.5 g; and trace element solution (see below), 10 ml. The trace element solution contained the following ingredients (per liter): $MgSO_4.7H_2O$, 3 g; $MnSO_4.H_2O$, 1 g; NaCl, 1 g; $FeSO_4.7H_2O$, 0.1 g; $CoCl_2.6H_2O$, 0.1 g; $ZnSO_4.7H_2O$, 0.1 g; $CuSO_4.5H_2O$, 0.01 g; $AlK(SO_4)_2.12H_2O$, 0.01 g; $H_3BO_3$, 0.01 g; and $Na_2MoO_4.2H_2O$, 0.01 g. Ten ml of this medium was added to each test tube and prior to inoculation the reducing agent sodium sulfide was added to a final concentration of 0.5 g/L. The tubes were placed in anaerobic jars and the cultures were kept up to 48 h at 96°C.

***DNA extraction:*** Residual sulfur present in the cultures was removed by decantation. After centrifugation for 5 min at 3000 × g, the cell pellet was washed with 200 µl of PBS buffer. For the comparative study, genomic DNA of these samples was extracted using five different methods.

***Procedure 1:*** DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) as recommended by the manufacturer. Briefly, to each sample 5 µl of lysozyme buffer (10 mg/ml in 10 mM Tris-HCl, pH 8.0) was added and incubated 15 min at 37°C, then 200 µl of binding buffer (6 M guaninidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4) and 40 µl of proteinase K was added and incubated for 10 min at 70°C. After adding 100 µl of isopropanol, this mixture was pipetted into the upper buffer reservoir of the High Filter Tube and centrifuged for 1 min at 8,000 × g. The flow through liquid was discarded and 500 µl of inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6) was added to the filter tube and centrifuged for 1 min at 8,000 × g. Again, the flowthrough liquid was discarded and 500 µl of wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) was added to the filter tube and centrifuged for 1 min at 8,000 × g. The washing step was repeated twice, and to elute the DNA, 200 µl of prewarmed (70°C) elution buffer (10 mM Tris-HCl, pH 8.5) was added to the filter tube and centrifuged for 1 min at 8,000 × g.

Ethanol precipitation was used to concentrate DNA. To each sample, 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of absolute ethanol were added and incubated for 5 min at -70°C. This mixture was centrifuged at 12,000 × g for 10 min at 4˚C and the pellet was resuspended in 200 µl of 70% ethanol. The sample was centrifuged at 12,000 × g for 5 min at 4˚C, the pellet was dried at room temperature and then was dissolved in 20 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

***Procedure 2:*** Extraction of genomic DNA was performed using a freeze/thaw method as elsewhere reported with some modifications.[8,9] Briefly, the bacterial pellet was dispersed in 1 ml of suspending buffer (50 mM Tris-HCl, 200 mM NaC1, 100 mM EDTA and 1 % SDS; pH 8). For better lysis, this mixture was alternatively frozen (at -70°C) thawed (at 60°C). The lysate was extracted twice with phenol, then was subjected to three ether extractions to remove residual phenol. The DNA was concentrated by ethanol precipitation. The dried DNA pellet was dissolved in 50 mM Tris-HCl, pH 8; 1 mM EDTA and subjected to RNase treatment (0.1 mg/ml, 30 min at 37°C). Phenol and ether extractions were repeated and the DNA pellet was washed with ethanol, dried and dissolved in 50 µl of water.

***Procedure 3:*** The genomic DNA was isolated using a Genomic DNA Prep Kit (A&A Biotechnology, Poland) as described by the manufacturer. Briefly, the bacterial pellet was suspended in 100 µl of TB buffer (10 mM Tris-HCl, pH 8.5) then 200 µl of Total Lysis buffer and 20 µl of proteinase K was added and incubated at 37°C for 20 minutes. After incubation at 70°C for 5 min, this sample was centrifuged for two minute at 10,000 rpm. The supernatant was

Table-I: Different concentrations of SDS and EDTA were used to neutralize the positive charge of DNA in lysates of procedure 3.

| *Sample* | *DNA extract* | *SDS (0.1%)* | *EDTA (500 mM)* | *SDS final concentration* | *EDTA Final concentration of* |
|---|---|---|---|---|---|
| A | 6 µl | 3 µl | 1 µl | 0.03% | 50 mM |
| B | 5 µl | 4 µl | 1 µl | 0.04% | 50 mM |
| C | 4 µl | 5 µl | 1 µl | 0.05% | 50 mM |
| D | 3 µl | 6µl | 1 µl | 0.06% | 50 mM |
| E | 2 µl | 7 µl | 1 µl | 0.07% | 50 mM |
| F | 3 µl | 7 µl | - | 0.07% | - |

transferred to the spin column and centrifuged for one minute at 10,000 rpm. Five hundred microliters of wash solution A1 was added and centrifuged for one minute at 10,000 rpm. The washing step was repeated and to elute DNA, 100 µl of TB buffer (preheated to 75°C) was added and incubated for 5 min at room temperature. The spin column was then centrifuged for one minute at 10,000 rpm and flow through liquid was collected for gel electrophoresis.

***Procedure 4:*** DNA extraction with phenol/chloroform/isoamyl alcohol was used as described by Barbier et al.[10] Briefly, the bacterial pellet was dispersed in 1 ml of suspending buffer (100 mM Tris-HCl, 100 mM NaC1, 0.5 mM EDTA, pH 8). For better lysis, SDS (1% w/v) and proteinase K (0.4 mg/L) were added and incubated for three hour at 40°C. The lysate was subjected to three phenol-chloroform-isoamyl alcohol DNA extractions. Phenol extraction was repeated and the DNA pellet was washed with ethanol, dried and dissolved in 50 µl of TE buffer.

***Procedure 5:*** This procedure was performed as previously reported by Ramakrishnan and his coworker, but with some modifications.[11] Briefly, the cell pellet was dispersed in cell suspension solution (500 mM Tris-HCl, 200 mM EDTA, 460 mM NaOH; pH 8). Then 50 µl of RNase and 100 µl of cell lysis/denaturing solution (15% SDS; pH 6.6) was added to the sample and incubated at 55°C for 15 minutes.

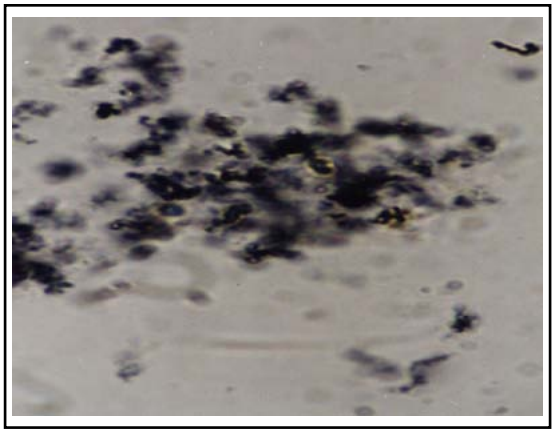

Fig.1: Light microscopy of P. furiosus.

After adding 25 µl of proteinase K and incubation at 55°C for 60 minutes, 500 µl of NaCl (5 M) was added and incubated at 4°C for 10 minutes. The sample was centrifuged and the supernatant was collected. To the supernatant, 2 ml of TE buffer and 8 ml of ethanol was added and incubated for two minutes at room temperature. After centrifugation for 15 min at 5,000 rpm, the dried DNA pellet was dissolved in 100 µl of TE buffer.

***Gel electrophoresis and spectrophotometry:*** DNA extracts (5 µl) were electrophoresed using a 0.7% agarose gel at 100 V and then stained with ethidium bromide visualized under UV illumination. The amount of DNA was evaluated spectrometricaly (Shimadzu, 2100, Japan) based on absorbance at 260 nm. The quality of the extracted samples was evaluated by the ratio of the absorbance at 260 and 280 nm. For some samples, before analyzing with gel electrophoresis, different ratios (see Table-I) of SDS and EDTA were added.

## RESULTS

***Microorganism culture:*** The black color of medium and $H_2S$ odor indicated the growth of the microorganism, it was also observed under light microscope (Nicon, HFX-DX, Japan). (Fig.1)

**DNA extraction:** Five different DNA extraction methods were tested to evaluate their efficacy in

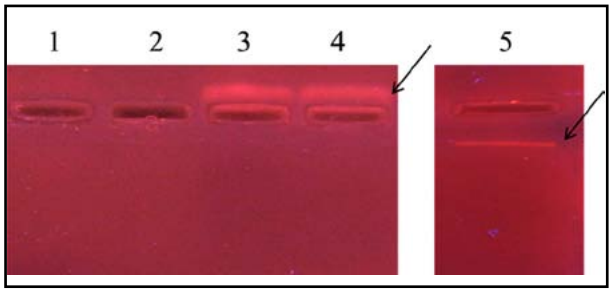


Fig.2: Agarose gel electrophores of the products obtained using different methods of DNA extraction. Lane 1: procedure 1; lane 2: procedure 2; lane 3: procedure 3; lane 4: procedure 4 and lane 5: procedure 5. DNA extracts were electrophoresed on 0.7% agarose gel at 100 V, stained with ethidium bromide and visualized under UV illumination.

isolation of *P. furiosus* genomic DNA. Procedures 1 and 2 were not successful and were even not able to disrupt the cell wall (lysis efficiency was checked by microscope). Procedures 3 and 4 were able to lyse bacterial cells. But after electrophoresis, the DNA extracted by these methods migrated towards cathode. (Fig.2) To resolve this problem, different concentrations of SDS and EDTA were used to neutralize the positive charge of DNA in lysates. As Fig.3 shows, 50 mM EDTA and 0.07% SDS can efficiently neutralize the positive charge and DNA extracts migrate towards the anode. After treatment of cell lysates with the optimum concentration, DNA was purified using phenol/chloroform and silica membrane. The yield of DNA recovery was very low and no visible bands were observed on an agarose gel.

Finally, procedure 5 resulted in a purified DNA, which after electrophoresis, migrates towards the anode. The UV spectrophotometry of DNA extracts showed the higher efficiency of procedure 5 for DNA isolation. DNA quality is basically marked by the ratio of $A_{260}$ to $A_{280}$ being higher than 1.8 and this ratio for the extracted DNA by procedure 5 was 2.

## DISCUSSION

*P. furiosus* is a marine organism and, as reported previously, the process of DNA purification from these organisms are challenging due to the presence of extracellular materials such as glycoproteins that can interfere with DNA isolation by absorbing the DNA.[12] *P. furiosus* is also a member of archaea and has different cell wall and cytoplasmic membranes. The cell wall of most archaea (except for two strains) is composed of glycoproteins called the surface layer or S-layer.[1] The most critical and first step of DNA isolation is cell disruption. In this study, five different cell lysis strategies were evaluated. Procedure 1 was not successful in efficient cell disruption. The basis of cell lysis with the High Pure PCR Template Preparation Kit is using lysozyme, which hydrolyzes the β-1,4-glycosidic linkage between the N-acetylmuramic acid-N-acetylglucosamine of the peptidoglycan layer existing in the cell wall of most bacteria. However, Archaea can tolerate lysozyme activity as their cell walls don't contain peptidoglycan.[13] However, Radax et al reported effective lysis of halophilic archaea using a combination of lysozyme, SDS, bead beating and thermal shock.[14] For procedure 2, lysis of cells was better than for procedure 1 but cell disruption was still not efficient. Cell disruption in procedure 2 was performed using physical treatment. Alternative freezing and thawing can change the fluidity of cell membranes, which causes them to be more susceptible to enzymatic and detergent treatment.

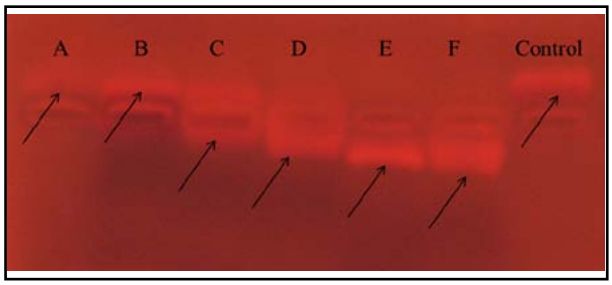


Fig.3: Agarose gel electrophores of samples treated with different concentrations of SDS and EDTA. A: 50 mM EDTA and 0.03% SDS; B: 50 mM EDTA and 0.04% SDS; C: 50 mM EDTA and 0.05% SDS; D: 50 mM EDTA and 0.06% SDS; E: 50 mM EDTA and 0.07% SDS; F: 0.07% SDS without EDTA and Control: without SDS and EDTA.

However, due to their thermostable membrane, this method for thermophile archaea may not be successful.[15] Boiling and freeze/thaw methods have been previously used for cell lysis of pure cultures of two archaea strains, which proved to be ineffective in cell disruption.[16] Using the Genomic DNA Prep Kit in procedure 3, a clear lysate was obtained, and microscopic evaluation of lysates showed efficient disruption of cells. In this method, a combination of lysis buffer and proteinase K was used. Proteinase K is a serine protease isolated from the fungus *Tritirachium album*.[17] This protease can lyse the glycoprotein cell wall of different species, but certain species may be more or less resistant to this process. In procedure 4, cell disruption was carried out successfully using SDS (1% w/v) and proteinase K. Finally, a high concentration of SDS and proteinase K in procedure 5 led to efficient cell lysis. SDS is an ionic detergent that binds to and denatures proteins and helps cell lysis. Our results showed that a combination of enzymatic lysis (proteinase K) and chemical lysis (SDS) is the most suitable procedure for cell disruption of *P. furiosus*.

Recovering nucleic acids from cell lysates is the next step in the isolation of genomic DNA. In the present study, silica-based DNA binding and solvent extractions were used for DNA recovery and purification. Presence of DNA in the cell lysate of procedures 3 and 4 was confirmed using gel electrophoresis (Fig.2) but recovery of DNA from these lysate was not successful. Using silica-based membrane in procedure 3 failed to purify DNA with high quantity and quality. Eluates from silica columns had only little amounts of DNA (shown by UV-spectrophotometry) and did not produce visible bands on an agarose gel. The basis of DNA

purification in this kit is interaction between the negative charge of DNA and silica gel. In the case of archaea, the negative charge of DNA may be neutralized by organic and inorganic cations.[18] The chromatin of archaea is associated with positively charged proteins including histone-like proteins and Alba proteins. Also, in thermophile archaea, mono and divalent cations at high concentrations protect DNA against cleavage of phosphodiester bonds.[19] For example, intracellular concentrations of potassium ions in thermophile strains is higher than other strains.[20]

After cell lysis with procedures 3 and 4, the DNA remained associated with positive components and migrated towards the cathode (Fig.2). Different concentrations of SDS and EDTA were used to neutralize the positive charge of DNA in lysates. After treatment of cell lysates with the optimum concentration, DNA was purified using phenol/ chloroform and silica membrane. The yield of DNA recovery was very low and no visible bands were observed on an agarose gel. The presence of SDS and EDTA may be interfering with DNA purification processes.

In addition, extensive phenol/chloroform extractions may lead to a low yield of DNA extraction. Procedure 5 was able to recover DNA with good quantity and quality. This method was proposed by Ramakrishnan and his coworker for isolation of genomic DNA from elemental sulfur-reducing hyper-thermophilic archaea.[11] In this method, proteinase K and high concentrations of SDS were used to disrupt the cell wall and a high concentration of NaCl was used to precipitate cell debris. Isolation of DNA was performed using ethanol precipitation. In summary, our observations suggest that the extraction method needs to be carefully chosen, especially when dealing with archaea or other similar organisms.

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### *Author Contribution:*

Hamid Mir Mohammad Sadeghi: Design, interview, analysis and writing the manuscript. Daryoush Abedi: Design, interview and writing the manuscript. Hamid Reza Mohmoud Pour: Interview, analysis and collection of data. Vajihe Akbari: Interview, analysis, drafting and writing the manuscript.