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Efficient DNA Extraction and Amplification of Samples from the Archeological Site of Pompei (Italy)

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Summary: Due to their growth and metabolism, microorganisms can cause severe damage to Cultural Heritage, through mechanical and chemical processes. In this paper we used molecular approaches to study bacterial communities on samples from the archeological site of Pompei (Italy). In particular four DNA extraction methods without prior cultivation of the microflora were compared. In terms of efficiency, the DNA extraction method based on the Tri-Reagent™ kit gave the best results. All extraction protocols coupled with 16S rDNA fragments PCR amplification were satisfactory; but the best results were obtained by Klentaq DNA LA Polymerase™; this enzyme can be considered an effective tool for amplification of DNA from Cultural Heritage and possibly other environmental samples.

Keywords: biodegradation; DNA extraction; electron microscopy; morphology; PCR

Introduction

It is established knowledge that microorganisms cause deterioration of materials.[1]

The study of microbial communities on monuments is usually accomplished by using standard culture methods. Culture-based approaches, while extremely useful for understanding the physiological potential of isolated organisms, do not necessarily provide a comprehensive information on the composition of microbial communities. Several studies have employed culture-indipendent techniques to show that cultivated microorganisms from diverse environments often may represent very minor components of the microbial community as a whole.[2] It is believed that only a small percentage of extant microorganisms have been discovered as cultivation methods recover less than 1% of the total microorganisms present in environmental samples,[3,4] therefore, microbial investigations based only on cultivation strategies can not be regarded as reliable in terms of reflecting the microbial diversity present in art samples. In addition, extensive cultivation strategies require more sample material than the quantity generally obtained from art objects and are generally time-consuming.

The application of culture-indipendent techniques on art objects based on molecular biological methods (especially the PCR amplification of 16S rRNA gene) avoids problems of conventional cultivation methods.[4–6] The application of molecular methods on art objects revealed the presence of microorganisms that had never been identified in these environments before. By applying these methods, the potential of such methods to investigate biodeterioration processes was demonstrated and it was suggested that such techniques should be integrated as a part of restoration strategies.[7–10]

However, the applications of molecular investigation strategies have to undergo permanent improvement to overcome any inherent limitations. One of the most important areas of improvement is the extraction of

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DNA from little sample material. DNA extraction methods have to be efficient for all types of microbial cells to allow subsequent DNA amplification and analysis. [1] In addition, samples from works of art often contain PCR inhibitors such as mineral salts, pigments, exopolysaccharides, humic acids and other unknown substances.[11,12] In addition to the above mentioned advantages, molecular investigation methods can be useful to analyse the recurrence of microorganisms that may alter artworks in different environments.

Finally, molecular methods can be used in the detection/selection of new microorganisms of potential industrial interest, due to the ecological features of stoneworks exposed to open air, such as dryness, wide temperature ranges, etc., which stimulate the selection of particular genotypes and phenotypes.[13]

The objective of the research discussed in the present paper was the selection of the most efficient DNA extraction method and 16S rRNA gene fragments amplification methods to study the bacterial communities on cultural heritage samples. Four approaches have been used to extract genomic DNA, including commercially available kits which have provided rapid and simplified procedures for genomic DNA extraction directly from Cultural Heritage samples.

Materials and Methods

Site Description and Sampling

Investigations were carried out on three samples of lateritious stone from two different areas located in the archaeological site of Pompei (Italy) (area A: ''*Tiepidarum*'', Terme del Foro, see Figure 1; area B: Casa del Principe). The sampling was carried out where there was a rosy discoloration. The samples were taken by scraping off surface material and plaster (1 cm^2, to a depth of 3 mm), using a sterile glass fiber pencil. Fragments were then ground in a fine powder by sterile mortar and immediately analyzed. For each sample, 50 mg were used for DNA extraction.

DNA Extraction

From natural samples, DNA was extracted using three commercial kits, Plant GenElute™, Tri-Reagent™ and Plant Extract-N-Amp™ kits (Sigma-Aldrich, Milano, Italy), according to the manufacture's recommendations and using a laboratory procedure based on chemical lysis. All the four DNA extraction protocols were

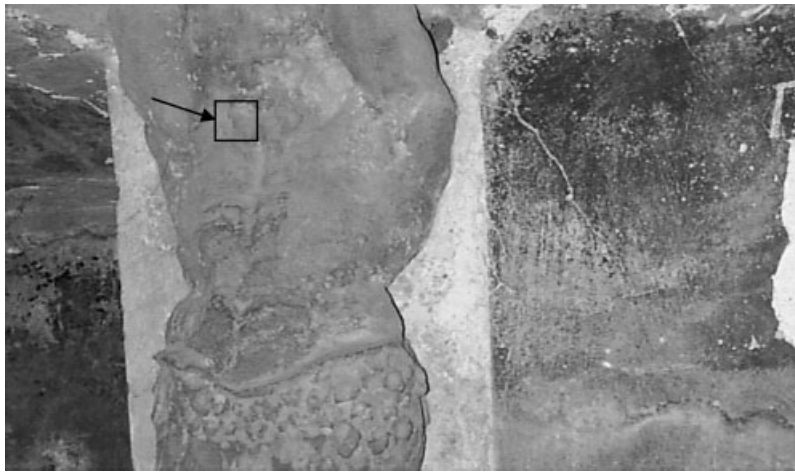

Figure 1.
''*Tiepidarum*'', Terme del Foro, archaeological site of Pompei (Italy). The arrow indicates where the samples were taken (area A).

carried out in triplicate. The laboratory method based on chemical lysis was here reported. Briefly, 1 mL of a solution containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 1% (w/vol) sodium dodecyl sulphate (SDS) was added to 50 mg of sample material. Cells were lysed enzymatically with proteinase K at 37 °C overnight, after which the samples were centrifuged at 14,000× *g* for 10 min.

The collected supernatants were extracted with phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and incubated for 10 min on ice with 1/10 volume of 3M sodium acetate and centrifuged at 14,000× *g* for 10 min. After precipitation with 1 volume of ice-cold isopropanol, the nucleic acids were washed with 70% ethanol.

Finally, genomic DNA was purified by elution through silica gel membranes to eliminate completely the DNA inhibitors. Table 1 reports the main characteristics of the adopted methods to evaluate DNA quantity and effectiveness.

Purity and Yield of DNA

The purity of DNA was assessed spectrophotometrically by calculating A_{260}/A_{230} and A_{260}/A_{280} ratios for humic acid contamination and protein impurities respectively. In general, A_{260}/A_{230} ratios < 2.0 indicate the presence of humic contaminants, which might inhibit the PCR amplification, while A_{260}/A_{280} ratios < 1.7 indicate protein contaminants.[14,15]

To check the quality and size of DNA, the crude DNA extracts were tested by electrophoresis on 1% agarose gel in TBE buffer, stained with 0.5 μg ml^{-1} ethidium bromide and compared with 1-kb DNA Ladder (Sigma-Aldrich, Milano, Italy).

PCR Amplification

All extracted DNAs were amplified using specific primers targeting the 16S rRNA gene.[16] Two different Taqs, RedTaq Readymix™ and Klentaq DNA LA Polymerase™ (Sigma-Aldrich, Milano, Italy) were used for amplifications, according to the manufacturer instructions provided by Sigma-Aldrich. RedTaq Readymix™ is a ready-to use mixture of Taq DNA Polymerase and an inert red dye that allows visual recognition. KlenTaq LA DNA Polymerase Mix is a mixture of KlenTaq-1 with a proofreading enzyme.

All reactions were carried out in 30 μl volumes, containing 0,2 μM of each primer, 15 μl of Red Taq buffer, 2,5 μM $MgCl_2$, 200 μM dNTPs and 10 ng of template, made up to 30 μl with sterile water. PCR was performed in a PCR Mastercycler (Eppendorf, Hamburg, Germany) with one cycle denaturing (4 min, 94 °C), followed by 30 cycles denaturing (20 s, 92 °C), annealing (30 s, 50 °C) and extension (45 s, 72 °C) and a final cycle of extension (5 min, 72 °C). All reactions included a negative (sterile water) control. All PCR products (10 μl) were analysed by electrophoresis in 1.5% (w/v) agarose gel, stained with ethidium bromide (0.5 μg mL^{-1}) and visualized under UV-light. The yield of the 16S rRNA gene PCR reactions was measured in order to estimate the impact of the extraction method and of the matrix on PCR efficiency. The 16S

Table 1.
– Beneficial features of the four investigated DNA extraction methods

Methods	Description	
	Lysis	DNA purification
GenElute™Plant Genomic DNA Miniprep kit (I)	Detergent, chaotrope	Silica gel membranes
TRI Reagent™ kit (II)	Acid guanidine thiocyanate	Phenol/chloroform extraction
Extract-N-Amp™ PCR Plant kit* (III)	Extraction solution	Silica gel membranes
Laboratory method (IV)	SDS- proteinase K	Phenol/chloroform plus silica membranes

* Kit includes a PCR ReadyMix but it was not used in these trials.

rRNA gene amplification efficiency was estimated by image analysis.

SEM Observations

The samples for scanning electron microscopy (SEM) observations were treated overnight in a solution of 2% glutaraldehyde (0.01 mol L^{-1} phoshate buffer), and then immersed in 1% osmium tetroxide. A microscope operating at 10 kV (Zeiss DSM 940A; LEO Elektronenmikroscopie GmbH)[17] with EDAX X-ray analyzer was used (Oxford Link INCA).

Results and Discussion

DNA Extraction

The analysis of 1% agarose gel (data not shown) revealed fragments of size range of 1–12 kb. There appeared to be no differences in DNA fragment size between commercial kits and the laboratory method used. Extraction protocols II and IV, although were more time consuming than extraction protocols I and III, yielded more DNA than the other extraction protocols tested in this study. The A_{260}/A_{280} and A_{260}/A_{230} purity ratios of DNA samples obtained were low, $A_{260}/A_{280} < 1.7$ and $A_{260}/A_{230} < 2.0$. We have carried out the PCR protocols reaction although these results.

However, DNA extracted with protocols I and III contained less humic acid contamination (A_{260}/A_{230}) than other methods. This is perhaps due to the fact that I and III protocols contain a purifying step in which silica gel membranes are used, more efficient than other purifying steps. On the other hand the purifying reduces the yield of DNA extraction.

PCR Amplification

Various factors can affect the application of the PCR reaction; in fact initial sample composition is important for the performance of PCR. DNA isolated from site A seemed to contain less inhibitors than DNA from site B resulting in a more efficient PCR (Figure 2 lanes 2, 4). PCR reaction of DNA extracted from site A by Extract-N-Amp™ procedure yielded an opalescent precipitate probably due to inhibitors (Figure 2, top of the lane 5). Lab Meth. IV protocol provided a positive performance of PCR in both samples (Figure 2, lanes 7 and 8).

The use of a more efficient and processive DNA polymerase than other native Taq polymerase such as Klentaq DNA LA Polymerase™ has overcome this problem (Figure 3).

Anywhere differences in PCR performance among samples were observed; such differences may be attributed to co-extracted inhibitors or shearing DNA (Fig. 2 lane 5,6), caused by DNA degradation during the extraction.

SEM Observations

Figure 4a and Figure 4b show the scanning electron microscopy (SEM) observation of sample A from the *Terme del Foro,* (Pompei , Italy) and its EDAX profile respectively.

SEM observations, supported by the EDAX profiles, could provide important information on the presence of cells with different shapes, microlayers, mineral salts and exopolysaccharides and their degree of adhesion to the surface of altered materials.

Table 2.
– Performance of the investigated DNA extraction methods

Methods	Time	Beneficial features
GenElute™Plant Genomic DNA Miniprep kit (I)	40 min	Rapid extraction and purification of genomic DNA
TRI Reagent™ kit (II)	90 min	Efficient isolation of DNA from a variety of starting materials
Extract-N-Amp™ PCR Plant kit (III)	15 min	Rapid extraction and amplification of genomic DNA
Laboratory method (IV)	12 h	Efficient isolation of genomic DNA from environmental samples

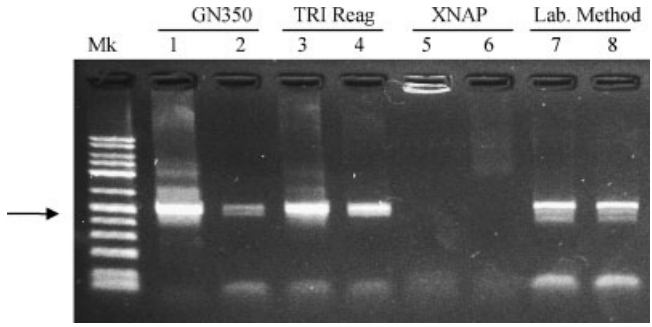

Figure 2.
Amplification of 500bp fragment of rRNA gene by RedTaq™ ReadyMix™. Mk: Euroladder M, arrow 525 bp. Lanes 1, 3, 5, 7 archeological site A. Lanes 2, 4, 6, 8 archeological site B.

SEM observations of the samples from the area A revealed the presence of numerous bacteria and fungi. This microbial colonization might result in severe biodeterioration, including aesthetic, chemical and mechanical degradation.

Conclusions

Commercial kits allowed rapid and simplified procedures for genomic DNA extraction directly from cultural heritage samples. Some of them resulted to have peculiar features in terms of rapidity, yield or purity of DNA. The results demonstrate that the method based on Tri-Reagent™ kit gave the best results for our objective; in fact this method yielded an amount of DNA, that was significantly higher than those obtained with other kits and laboratory method (data not shown) perhaps due to a more efficient lysis step. All kits we tested coupled with RedTaq Readymix™ have provided an effective tool of investigation in heritage bacterial community. Otherwise, when more complex samples, must be analysed, Klentaq DNA LA Polymerase™ should be considered as an effective tool for amplification of DNA. The results of this study showed that the DNA extraction protocol II was to be preferred as it facilitated the successful extraction of PCR-amplifiable DNA from very little sample material containing PCR inhibitors. The combination of different analytical techniques (SEM and biomolecular tools) provided a good overwiew of the bacterial community on the artworks studied. The set up method could be applied in a future work for the

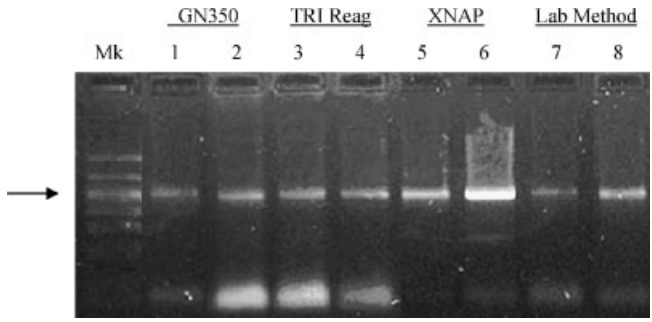

Figure 3.
Amplification of 500 bp fragment of rRNA gene by Klentaq DNA LA Polymerase™. Mk: Euroladder M, arrow 525 bp. Lanes 1, 3, 5, 7 archeological site A. Lanes 2, 4, 6, 8 archeological site B.

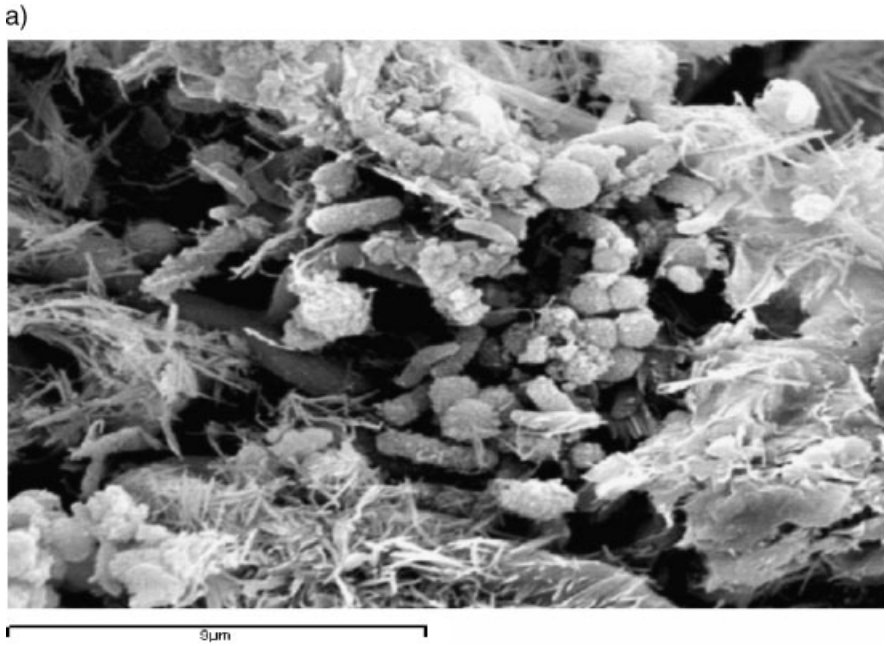

Figure 4a and 4b.
SEM observation of bacteria and EDAX profile on fragment A from Terme del Foro, archaeological site of Pompei (Italy).

bacterial communities characterisation of Cultural Heritage samples.

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[1] C. Schabereiter-Gurtner, G. Piñar, W. Lubitz, S. Rölleke, *J. Microbiol. Meth.* **2001**, 45, 77.
[2] G. Piñar, C. Schabereiter-Gurtner, W. Lubitz, in: "*Molecular Biology and Cultural Heritage, Proceedings of International Congress on Molecular Biology and Cultural Heritage*" Sevilla, C. Saiz-Jimenez, Eds., Balkema Publishers, Lisse, **2003**, 35.
[3] D.M. Ward, R. Weller, M.M. Bateson, *Nature* **1990**, *345*, 63.
[4] R.I. Amann, W. Ludwig, K.H. Schleifer, *Microbiol. Rev.* **1995**, *59*, 143.
[5] L. Laiz, G. Piñar, W. Lubitz, C. Saiz-Jimenez, in: "*Molecular Biology and Cultural Heritage, Proceedings of International Congress on Molecular Biology and Cultural Heritage*" Sevilla, C. Saiz-Jimenez, Eds., Balkema Publishers, Lisse, **2003**, 23.
[6] I.M. Head, J.R. Saunders, R.W. Pickup, *Microb. Ecol.* **1998**, *35*, 1.
[7] S. Rölleke, G. Muyzer, C. Wawer, G. Wanner, W. Lubitz, *Appl. Environ. Microbiol.* **1996**, *62*, 2059.
[8] S. Rölleke, A. Witte, G. Wanner, W. Lubitz, *Int. Biodeter. Biodegr.* **1998**, *41*, 85.
[9] S. Rölleke, C. Gurtner, U. Drewello, R. Drewello, W. Lubitz, R. Weissmann, *J. Microbiol. Meth.* **1999**, *36*, 107.
[10] C. Gurtner, J. Heyrman, G. Piñar, W. Lubitz, J. Swings, S. Rölleke, *Int. Biodeter. Biodegr.* **2000**, *46*, 229.
[11] C. Schabereiter-Gurtner, J. Heyrman, G. Piñar, W. Lubitz, J. Swings, S. Rölleke, *J. Microbiol. Meth.* **2001**, *36*, 65.
[12] I.G. Wilson, *Appl. Environ. Microbiol.* **1997**, *63*, 3741.
[13] D. Daffonchio, S. Borin, G. Frova, R. Gallo, E. Mori, R. Fani, C. Sorlini, *Appl. Environ. Microbiol.* **1999**, *65*, 1298.
[14] R.J. Steffan, J. Goksoyr, A.K. Bej, R.M. Atlas, *Appl. Environ. Microbiol* **1988**, *54*, 2908.
[15] D.W. Cullen, P.R. Hirsch, *Soil Biol. Biochem.* **1998**, *30*, 983.
[16] D.J. Lane, B. Pace, G.J. Olsen, D A. Stahl, M. L. Sogin, N. R. Pace, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 6955.
[17] G. Ranalli, G. Alfano, C. Belli, G. Lustrato, M.P. Colombini, I. Bonaduce, E. Zanardini, P. Abbruscato, F. Cappitelli, C. Sorlini, *J. Appl. Microbiol.* **2005**, *98*, 73.

