INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE

ISSN: 2067-533X Volume 6, Issue 2, April-June 2015: 137-144 www.ijcs.uaic.ro

ANALYSIS OF MICROFLORA PRESENT ON HISTORICAL TEXTILES WITH THE USE OF MOLECULAR TECHNIQUES

Tomasz LECH[*1], Aleksandra ZIEMBINSKA-BUCZYNSKA[2], Natalia KRUPA[3]

[1] Department of Microbiology, Faculty of Commodity Science, Cracow University of Economics, Rakowicka 27, 31-510 Krakow, Poland
[2] Department of Environmental Biotechnology, Faculty Of Power and Environmental Engineering, The Silesian University of Technology, Akademicka 2, 44-100 Gliwice, Poland
[3] Textile Conservation Laboratory, The Pontifical University of John Paul II in Krakow, Bernardyńska 3, 31-061 Krakow, Poland

Abstract

One of the most important point for the restoration and protection of cultural heritage objects is the early identification of material deterioration caused by microbial colonization. Biodegradation has to be understood as a result of the activity of an entire microbial community, rather than that of individual members of such community. Fungi are among the most degradative organisms inducing biodeterioration processes of ancient and modern materials. Electrophoresis in denaturing gradient gel (DGGE) is the technique most often used to study the structure of the microbial communities colonizing artworks like painted art objects, mural paintings, historical window glass, historical limestone buildings and the like. The present article describes the usage of this method to analyze the diversity of fungal taxons on the sixteenth century textile material. Additionally the procedure of main taxon identification by sequencing was described.

Keywords: *DNA extraction; DGGE; textile heritage objects; fungal communities; biodeterioration*

Introduction

Biodeterioration is a considerable problem in protecting and storing items of cultural heritage. Biodeterioration may be defined as the process in which unfavourable changes affect historical items and contemporary materials that are important for economic reasons, as a result of enzymatic activity of microorganisms [1]. Bacteria and fungi, which play the main role in the process of biodegradation, destroy items made of organic materials, such as paper, wood, leather, as well as cellulose, silk and woollen textiles etc., but they also pose a hazard to modern polymer materials [2-5]. The process of biodeterioration should be understood as a consequence of the activity of a group of microbes present in a given environment rather than as the activity of individual species. Hence, in order to eliminate microbial hazard, the examination and exploration of their diversity in a given environment or on a given item of cultural heritage has become significant [6]. The conventional culture-based methods used so far have become insufficient. It is estimated that less than 1% of bacteria in a given environment is recovered with culture-based methods. As for fungi, approximately 70% of environmental taxa can be determined [7]. Therefore, molecular methods, such as PCR (Polymerase Chain Reaction) or

* Corresponding author: lecht@uek.krakow.pl

PCR-DGGE (Polymerase Chain Reaction -Denaturing Gradient Gel Electrophoresis) are applied and considered sensitive and accurate alternatives to conventional techniques [8]. The DGGE technique allows the fingerprint (pattern of bands) to be created. It pictures the diversity of taxa in the investigated environment based on the analysis of sequences of proper fragments in the genome of microorganisms. The DGGE has become the most common method for analysing diversity of microorganisms that colonise works of art and historical items. An additional advantage of this method is the possibility of collecting samples in a non-invasive manner, which is of particular importance in items of cultural heritage [9-10]. The DGGE technique has been used with success in a diversity analysis of the microbial community of painted art objects, mural paintings, historical window glass, historical limestone buildings or historical paper and parchment documents [5-6, 9, 11-13]. Unfortunately, there are few reports describing the study microbial diversity on historical textiles. This paper proposes the application of the PCR-DGGE method for a diversity analysis of fungi on historical textiles. The object investigated in this study is one of the more valuable items stored in the All Saints parish in Krakow - a chasuble in red liturgical colour made of Italian silk that comes from the second half of the 16th century. The fabric was made in the technique of brocaded damask, gilded with ornamental thread. The analysis in terms of diversity of microbes present on the textile was performed prior to and following conservation in order to demonstrate potential changes in the microflora diversity. Additionally, an identification of the predominant taxon was performed using the sequencing technique.

Materials and methods

The analysis of the state in which the textile was preserved demonstrated the presence of numerous off-white discolorations on the surface of the decorative silk fabric and darkening of the cellulose cotton fabric that lined the chasuble. These changes were the basis for microbial analyses (Fig. 1).

Fig. 1. Details of the textiles used in the experiment: a - front, b - back of chasuble from the collections of the Parish P.W. All Saints in Krakow, all before maintenance; c - fragment before wet cleaning; d - fragment after wet cleaning

The samples for analyses were mainly silk threads collected form the item. The first sample consisted of threads from the chasuble obtained prior to conservation. These were silk threads and a small amount of cotton threads dyed with an unstable synthetic dye with the total weight of 0.0270g. The second sample consisted of silk threads collected after conservation (wet cleaning) with the total weight of 0.0255g.

Biodiversity analysis with the PCR-DGGE technique

DNA extraction. The samples were placed in flasks with glass beads with 3-4mm in diameter and 3mL of water. Subsequently, they were shaken manually for 5 minutes. Following extraction, water from each sample was centrifuged in a sterile test tube of 1.5mL. The supernatant that was formed after 2-minute centrifugation in 13,000 rpm was removed and a new portion of water after extraction was added. In order to recover the greatest possible amount of material for analyses, the supernatant was centrifuged again. The final sample consisted of the pellet that formed during centrifugation and a slight amount of supernatant (approximately 50µL). 350µL of lysis buffer (100mM EDTA; 100mM Tris-HCl pH = 8; 0.5M NaCl; 3% SDS) and 400 U of lyticase were added to the samples. Subsequently, they were incubated for 16 h in 55^{o}C and shaken at 550 rpm. The DNA extraction was conducted with a standard phenol–chloroform method adjusted to fungal genetic material isolation [14].

PCR - Polymerase Chain Reaction. The genetic material obtained served as the matrix for a polymerase chain reaction (PCR). The nested PCR technique was applied for the purpose of the amplification of the fungal ITS1 region (Internal Transcribed Spacer). In the first PCR reaction, the ITS1F and ITS4 primers were used, as proposed in the studies of White et al. [15] and Grades and Bruns [16], thanks to which the multiplied ITS1-5.8S rDNA-ITS2 fragment was obtained (Table 1). The amplification was conducted in 30 µL of reactive mixture consisting of: water (for use in molecular biology, Sigma-Aldrich, St. Louis, MO, USA) – 21.9µL; 10X concentrated polymerase buffer (Sigma-Aldrich) – 3µL; $MgCl_2$ (50mM; Sigma-Aldrich) – 1.5µL; primers – forward and reverse 0.6µL each (with the concentration of 10µM/µL; Genomed, Warsaw, Poland); dNTP (mixture of four nucleotides with the concentration of 10mM/µL each; Sigma-Aldrich) – 0.9µL; Taq polymerase (5U/1µL; Sigma-Aldrich) – 0.25µL and genome DNA suspended in TE buffer – 1.5µL. The amplicons obtained were used as the matrix for the second PCR which was conducted with the use of primers for ITS1, ITS1F-GC and ITS2 regions. The reaction was also conducted in 30µL of mixture that consisted of: water (for use in molecular biology, Sigma-Aldrich) – 13.4µL; 10× concentrated polymerase buffer (Sigma-Aldrich) – 3µL; $MgCl_2$ (50mM; Sigma-Aldrich) – 1.5µL; primers – forward and reverse 0.6µL each (with the concentration of 10µM/µL; Genomed); dNTP (mixture of four nucleotides with the concentration of 10mM/µL each; Sigma-Aldrich) – 0.9µL; Taq polymerase (5U/µL; Sigma-Aldrich) – 0.25µL and the amplicons obtained with the volume of 10µL. Both the first and the second reactions were conducted in a T100 thermal cycler (BioRad, Hercules, CA, USA) with the use of the following programme: initial DNA denaturation at 94^{o}C for 5 minutes followed by 20 cycles consisting of 45-second DNA denaturation at 94^{o}C, annealing the primers to the matrix at the temperature of 68-58^{o}C for 180 seconds (lowering the temperature by 0.5^{o}C for each cycle), synthesis of a new complementary chain for 60 seconds at the temperature of 72^{o}C; and then 10 cycles consisting of DNA denaturation at 94^{o}C for 45 seconds, annealing the primers to the matrix at 58^{o}C for 180 seconds; synthesis of a new complementary strain for 60 seconds at the temperature of 72^{o}C and final elongation for 5 minutes at the temperature of 72^{o}C.

Table 1. PCR primers used in this study

Primers	Sequence	References
ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Gardes and Bruns 1993
ITS4	5'- TCCTCCGCTTATTGATATGC-3'	White et al. 1990
ITS1F-GC	5'-CGCCCGCCGGCGGCGGCGGGCGGGGCGGGGGCA-CGGGGGGCTTGGTCATTTAGAGGAAGTAA-3'	Gardes and Bruns 1993
ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	White et al. 1990

DGGE analysis. The DGGE of nested PCR products obtained with the use of the ITS1F-GC and ITS2 primers was carried out in the Dcode Universal Mutation Detection System (BioRad). The amplicons of 300bp with GC clamp were separated in 8% polyacrylamide gel (acrylamide: bisacrylamide, 39.5:1) containing 30-60% of the denaturation factor (urea). The electrophoretic separation was conducted for 17 h at the voltage of 40V and in the temperature of 60°C in 1×TAE buffer (Tris, acetic acid, EDTA, pH = 8.0). Following the electrophoresis, the gel was dyed in SYBR Green (1:10,000, Invitrogen) for 15 minutes and rinsed in the MilliQ water for 30 minutes. The fingerprint obtained was visualised with the use of the UV light and photographed. The densitometric analysis was conducted using the Quantity One 1D software (BioRad) and the Shannon biodiversity index was calculated as described previously [17].

DNA band extraction and sequencing. In order to present the whole procedure, i.e. fungal diversity analysis, and subsequently, identification of individual taxa, the dominant taxon present on the item after conservation was assayed. The selected band from the DGGE fingerprint was excised with a sterile scalpel and suspended in water for use in molecular biology and vortexed gently. A repeated PCR reaction with ITS1F and ITS2 primers was conducted in the same conditions as before, and the matrix was the material isolated from the band excised from the gel. The entire volume of the PCR product was placed on the gel and the electrophoresis was conducted. The band obtained was again excised with a sterile scalpel and the genetic material was extracted and purified with the use of the commercial Gel Elute Kit (Sigma Aldrich). The obtained product underwent sequencing.

Results and Discussion

Out of numerous types of historical objects, textiles belong to the group of sensitive ones that react to changeable conditions of the microclimate. The chemical composition and structure of natural fibres is associated with poor resistance to external factors that shape the microclimate. Short-lasting air humidity and temperature changes, sun exposure as well as external and organic contamination resulting from using the item have an influence on the level and pace of fibre degradation [18-24]. Fibre degradation also depends on the material, and each chemical reaction contributes to weakening of natural fibres and affects structural resistance of textiles. The main factor that influences textile degradation is the level of relative humidity in relation to temperature of the surroundings. Abrupt and irregular changes of these factors cause physical changes in fibres and create conditions favourable for microbial development [18, 21, 23, 25, 26]. Among the liturgical Parament, churches also store historical textiles used as liturgical hangings. Decorative silk textiles ornamented with metal threads, from which liturgical vestments were sawn, were stiffened by fabrics with cellulose fibres and lined with various fabrics. This classifies such textiles to the group of complex items.

The chasuble examined in this research is an example of such an item. The accelerated process of natural biodeterioration observed in this study concerns mainly cellulose

components, such as the linen stiffener strengthened with natural glue and the cotton lining adhering to the ornamental part. The risk also concerns silk fibres of the decorative textile dyed with natural red dyes. Their physical condition was weakened, which was associated with long exposure to increased levels of relative humidity. Moreover, the parts of the vestment brocaded with metal ornamental threads showed traces of corrosion. In order to protect such complex items and select appropriate remedies, it seems important to examine microbial diversity and identify individual taxa. The aim of this paper was to propose methods for conducting such an analysis with the use of molecular techniques. In the first stage of the study, the DNA of microorganisms present in the samples collected before and after conservation was successfully extracted. The genetic material was used to make a fingerprint of the band pattern that showing the diversity of fungal taxa present on the investigated item. The differences in the nucleotide sequence in the conservative ITS1 fragment of the fungal genome were used. The band profiles obtained (Fig. 2a) underwent densitometric analysis and the Shannon biodiversity index was calculated (Fig. 2b). The analysis revealed differences in both the number and diversity of fungal genotypes found on the textile before and after the process of conservation. Taking the Shannon index into account, it was demonstrated that before conservation the level of fungal biodiversity on the investigated object was lower than after wet cleaning (1.23 vs. 1.47, Fig. 2b). Differences were also observed in the qualitative composition of fungal genotypes in the samples.

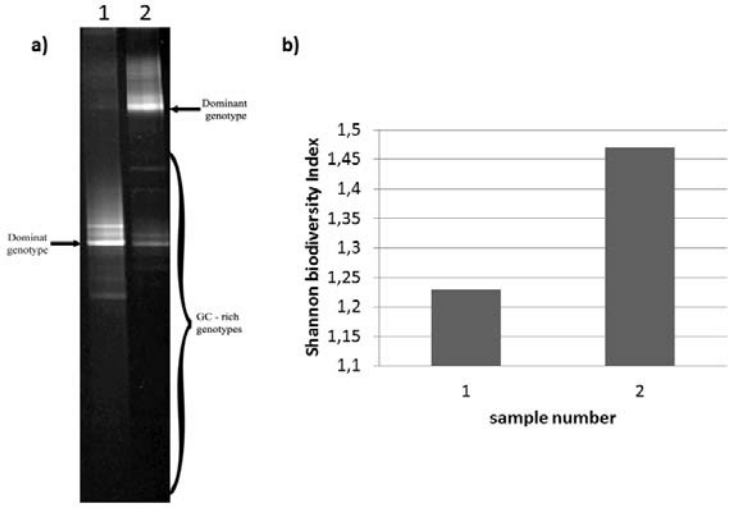

Fig. 2. Biodiversity analysis performed with PCR-DGGE; a) DGGE separation under UV light; b) Shannon Biodiversity index calculated on the basis of densitometric analysis form DGGE fingerprints;sample 1- before conservation process, 2- after conservation process

Samples 1 and 2 differed in terms of the dominant genotype and sample 1exhibited dominant genotypes in GC-rich region, while sample 2 in GC-poor part of the gel. Both qualitative and quantitative differences in genotypes found on the investigated object before and after conservation may result from its introduction into water environment during the process of conservation, which favours development of microbes that might have been present earlier but

in undetectable numbers. It is also possible that fungal spores that had been inactive prior to conservation germinated during the conservation process. On the other hand, following the removal of contamination in the form of organic and inorganic particles, numerous micro-niches, easily accessible to environmental microbes, appeared on the item. Therefore, it is important that after a conservation process objects should be stored under appropriate conditions which preventing them the secondary contamination. Furthermore, the study also included the identification of the dominant genotype in sample 2 after conservation (Fig. 2a). Sequencing revealed that the dominant species was *Aspergillus penicillioides*. It is a typical xerophytic species that may be found in places with low humidity where the development of other fungal species is inhibited. It has been isolated from various environments, including those with low water activity of $a_w = 0.68$. Among others, various isolates were obtained from the Dead Sea, food (grains, dried fruit, baked products), paper, glass lenses and human skin [27-28].

Conclusion

Historical textiles represent a large and important group of cultural heritage objects. It's significant part are ecclesiastical collections, especially chasubles and other liturgical textiles. They belong to complex objects made mainly of silk, as well as cotton, linen and wool. The variety of used material has a significant impact on their colonization by microorganisms and thus susceptibility to biodeterioration. In order to protect historic textile collections it is crucial to ensure appropriate climate conditions, i.e. temperature and humidity in which they are stored. It is also important to monitor the microbiological status of these objects. Therefore, it is important to explore and implement new methods to rapid microbiological analysis of the historical textiles. Methods must not be invasive and destructive for the textile, which are very delicate due to its age and state of preservation. The complexity of the microflora present on the cultural heritage objects can affect their destructive potential. The investigations reveal that the proposed methods may be used for determining the diversity of microbes presented in historical textiles and for identification of individual genotypes. Obtaining such information will facilitate implementation of appropriate actions aiming at protecting textile objects of cultural heritage.

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Received: October, 06, 2014
Accepted: March, 24, 2015