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REVIEWS

Evaluation of potential of molecular and physical techniques in studying biodeterioration

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Abstract Recently, researcher's abilities to elucidate the biogeophysical and biogeochemical mechanisms of complex biodeterioration processes occurring at monumental sites has been greatly revolutionized by use of molecular, physical and highly sophisticated so called high throughput next generation sequencing techniques. Such achievements are obvious in several areas of biotechnology and environmental science including geomicrobiological studies related to biodeterioration and bioconservation of ancient historic architectural monuments and artworks. Application of these techniques in studying architectural monuments and artworks is not just limited to predict the prevalence microbial diversity and identifying the mechanism of biodeterioration caused by inhabiting microorganisms, but also to provide in-depth molecular, biogeophysical and biogeochemical basis of how microorganisms respond to different environmental conditions to accelerate the process of biodeterioration, which in turn will offer tremendous opportunities to environmental scientists and researchers to formulate or device preventive and remedial safeguard techniques to control undesirable growth and survival of microorganisms on monuments and work of arts. Further evaluation studies and investigations are currently in progress to upgrade these molecular and physical strategies and to develop reliable approaches to better explain the various processes of biodeterioration and related phenomenon. A comprehensive description of techniques being successfully incorporated and applied in this regard is described in this review. Taken together, it can be anticipated that these techniques possess an astounding potential to turn around research related to geomicrobiological studies related to biodeterioration and bioconservation of monuments.

Keywords Molecular techniques · Geomicrobiology · Geophysical changes · Geochemical changes · Biodeterioration · Bioremediation · Bioconservation · Community fingerprinting

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1 Introduction

Cultural heritage and architectural monuments are exposed to environmental pollutants (inorganic and organic), climatic factors (temperature, humidity, rain and other precipitation, sun exposure) and chemical treatments (biocides, surfactants and hydrophobic compounds) that facilitates the microbial growth

resulting in physical, chemical and aesthetical changes in them. These changes cause irreversible damage of value of an object of art and the phenomenon is known as Biodeterioration.

Biodeterioration of cultural heritage and architectural monuments is the principal field of interest for the researchers all over the world. Physical or mechanical alterations (Warscheid and Braams 2000; De los Ríos et al. 2004), chemical changes to mineral matrix (Ortega-Morales 2006; Grbić et al. 2009; Grbić and Vukojeviã 2009) and aesthetical loss (Brimblecombe and Grossi 2005; Toniolo et al. 2009) are the primary symptoms of the damage of cultural heritage and architectural monuments and caused by synergistic action of environmental factors (such as rain water, moisture, wind, sunlight and pollutants) and microbial activity on stone surface of monuments and civil structures (Videla et al. 2000; Kramar and Mirtič 2008; Scheerer et al. 2009). These alterations are sometimes irreparable or irremediable (Dakal and Cameotra 2012). Plentiful of investigation aiming at studying the phenomenon of biodeterioration and its causes were made (Kolo et al. 2007; Benzzi et al. 2008; De Felice et al. 2010). Owing to the complex nature of biochemical and biophysical changes causing biodeterioration of monuments and artworks, it has become very much difficult to understand the mechanism of deterioration and to develop appropriate remedial measures. Earlier researchers used culture-based methods for the detection of microbial growth and activity on monumental stones. The culture-based method was associated with many drawbacks which encouraged the researchers to use some sophisticated and specialized molecular techniques for geomicrobiological studies in biodeterioration (Dakal and Cameotra 2012). Recently, the use of molecular techniques for studying the mechanism of biodeterioration had gained considerable success. During this decade, researchers intensively practiced numerous molecular and physical techniques and routinely performed the analysis of proteins, biomarkers and nucleic acids for studying the process of biodeterioration (Welton et al. 2005; Berdoulay and Salvado 2009; Cuzman et al. 2010). The use of well documented molecular biological and physical sciences tools and several other techniques (such as next generation sequencing techniques (NGS) and bioinformatics tools) has greatly assisted the researchers in identifying the microbial diversity on monuments and studying the molecular mechanism of biodeterioration (Palla et al. 2003; Jroundi et al. 2010). Researchers also used the polyphasic approaches to increase their knowledge of microbial diversity involved in the process of biodeterioration (Gaylarde et al. 2004; Ortega-Morales 2006; Ramírez et al. 2010). A comprehensive evaluation of the potential of molecular techniques and the physical techniques which are successfully incorporated and applied in geomicrobiological studies related to biodeterioration of monuments are discussed in this review. Apart to this, a brief overview of culture-based techniques and the major drawbacks of the techniques have also been presented to allow readers to follow the hierarchy of techniques being used in geomicrobiological studies from beginning.

2 Culture-based approaches

Culture-based technique involves isolation of the microbes by enrichment the samples in liquid media. Microorganisms are sampled from the monumental sites using aseptic techniques (in accordance to cultural heritage ministry recommendation) and are immediately transferred to laboratories for microbiological analysis (Polo et al. 2010). If they are sampled from distant site, they are generally kept refrigerated or preserved during transportation (Zimmermann et al. 2005; Cuzman et al. 2010). The most common methods of sampling are scraping off sample using a sterile scalpel (Schabereiter-Gurtner et al. 2001a; Herrera et al. 2004), sterile glass–fibre brush (Gorbushina et al. 2002), velvet fabric (Kyi 2003), swabs (Kyi 2003; Grbić et al. 2009) and adhesive tape method (MAT) (Urzì and Albertano 2001; Grbić et al. 2009; Pangallo et al. 2009). A small amount of the sample is used for inoculation in media following homogenization and serial dilution in physiological saline solution (0.9% NaCl). Enumeration of cultivable microbial cells (colony forming units, CFU) is done using Helber counting chamber (Gómez-Alarcón et al. 1994; Welton et al. 2005), epifluorescence microscopy (Welton et al. 2005) or flow cytometry (Cappitelli et al. 2009). For isolating the pure culture and for testing the purity of the isolated microorganism streak test is generally performed (Saiz-Jimenez and Laiz 2000; Welton et al. 2005). A wide variety of culture media, both solid and liquid, have been used for this purpose based on type of microbes (Table 1).

Microbiological analysis is generally performed using certain staining procedures or using different microscopes (such as stereomicroscope, epifluorescence, light microscope and scanning electron microscope) (Cappitelli et al. 2007, 2009; Grbić et al. 2009, Wiktor et al. 2009; Cámara et al. 2011).

Some researchers applied Simpson Index to calculate the microbial diversity (different colony type) in environment samples (Kiel and Gaylarde 2007). Microbial diversity (D) in sample can be estimated using relation $D = 1 - \sum Pi^2$ where, Pi is the proportion of individuals of the same species in relation to the total number of individuals. A higher diversity is assumed if the Simpson Index is near to 1 (Kiel and Gaylarde 2007).

2.1 Limitations of culture-based methods

Culture-based methods, for long time have been used for identification of microorganisms thriving on monuments. Growing microorganisms, such as cyanobacteria, algae and fungi, in specific growth media

Table 1 Culture media for isolation and identification of microbes involved in biodeterioration of monuments

Microbes	Media	References
Green microalgae	Modified Bold's basal medium (MBBM) with 1.5% agar	Schumann et al. (2005)
Algae and cynobacteria	Chu and Detmer media with (for chlorophyta) and without (for cyanobacteria) glucose (10 g/L) and casein (5 g/L)	Cappitelli et al. (2009), Polo et al. (2010)
	Knöpp's media	Ortega-Morales et al. (2000), Crispim et al. (2003), Grbić et al. (2009)
	BG-11	Albertano and Urzì (1999), Cuzman et al. (2010)
	Bold basal medium	Barberousse et al. (2006)
	BRII, BBM and Zehnder media	Albertano and Urzì (1999), Gorbushina et al. (2002), Pangallo et al. (2009)
Bacteria	Nutrient agar (for total aerobic heterotrophic mesophilic bacteria); Postgate C medium (for sulfate reducing bacteria); differential reinforced clostridial broth (for sulphate-reducing bacteria); glucose broth (for acid-producing bacteria)	Herrera et al. (2004)
	R2A and skim milk	Pangallo et al. (2009)
	Skim milk agar	Gorbushina et al. (2002)
	EMB and cetrimide agar (Gram-negative bacteria), Mosel agar (spore forming bacteria) and Postgate B (anaerobic bacteria)	Videla et al. (2000)
Halotolerant and halobacteria	DSMZ media 372 and 1018	Laiz et al. (2009)
	Trypticase Soy-Agar (TSA) supplemented with NaCl and/or $MgSO_4 \cdot 7H_2O$	Saiz-Jimenez and Laiz (2000), Laiz et al. (2009), Pangallo et al. (2009)
	Thornton's media	Kiel and Gaylarde (2007)
Fungi	Malt agar	Gómez-Alarcón et al. (1994), Monte (2003), Cappitelli et al. (2007), Grbić et al. (2009), Wiktor et al. (2009)
	Malt extract broth	Wiktor et al. (2009), Cuzman et al. (2010), Polo et al. (2010)
	Potato dextrose agar	Wiktor et al. (2009)
	Yeast nitrogen broth	Wiktor et al. (2009)
	DBRC	Albertano and Urzì (1999), Gorbushina et al. (2002), DuPont et al. (2007)
	YGC (yeast glucose chloramphenicol)	Videla et al. (2000), Herrera et al. (2004)
	Dichloran Rose Bengal agar	Cappitelli et al. (2007), Sert et al. (2007)

remained a powerful tool for their isolation and identification. However, this method has some limitations, especially with the isolation and identification of cyanobacteria (Ortega-Morales et al. 2001; Crispim et al. 2003). This method generally yields artificially low numbers, because of the presence of inhibitory and predatory microorganisms such as fungi, protozoa and bacteria (Ward et al. 1990; Crispim and Gaylarde 2005). This situation can be apparently seen while culturing cyanobacteria which grow normally in dry environmental conditions but are lost in liquid culture media. The inhibition in their growth is mainly attributed to the presence of fungi in culture (Crispim and Gaylarde 2005). Additionally, cyanobacterial species often grow in association with other eubacteria, algae and fungi in a complex 3D biofilms which make their isolation and successive replication more tedious (Billi et al. 1998). The inability to isolate unicyanobacteiral cultures from their biofilms is an example of this situation (Ortega-Morales et al. 2001; Crispim et al. 2003). Sometimes culture-based techniques may overestimate a group of microbes with respect to other leading to disparity between the experimental results (Laiz et al. 2003). Unfortunately, inability to replicate the complex and dynamic culture conditions under which the microbes grow (as in biofilms) is another reason which made the culture-based method unreliable and unsuitable for biodeterioration studies (Kyi 2003; Herrera and Videla 2009). It is accepted that cultivation methods detect only 1% of the microbial diversity present in sample collected from monumental sites (Amaan et al. 1995; Ward et al. 1990; Gonzalez and Saiz-Jimenez 2004, 2005; Kiel and Gaylarde 2007). Use of selective media and culture conditions may lead to loss of diversity of strains detected in an environment sample (Crispim and Gaylarde 2005). Obligate-symbiotic and parasitic microorganisms flourishing under host and those which are never cultivated before or which had entered non-culturable state, could not be cultured using this approach. Thus, it becomes difficult to assess the contribution of culturable microbes and the in situ microbial diversity in the process of biodeterioration. Few researchers have proved that many microorganisms which remain undetected using culture-based techniques could be detected and identified using molecular biology based techniques (Rölleke et al. 1996, 1998, 1999, 2000). It also became apparent that for identification of a number of microorganisms in environmental sample, a common culture technique would not yield satisfactory and accurate results (Ferris et al. 1996). Though it is assumed that using extensive culturing few more microbes could be identified from environmental sample but it requires large amount of environmental sample even more than that could be collected from heritage sites. These limitations with culture-based technique presented a big challenge before researchers and consequently the use of molecular techniques appeared to be the viable solution (Ferris et al. 1996). Besides this, molecular techniques are the most promising mean by which microorganisms which cannot be cultured and identified using the culture-based methods can also be identified (Crispim and Gaylarde 2005). However, culture based techniques and development of new culture media is still encouraged because of advantages of having pure isolated to carry out physiological and metabolic studies.

3 Molecular techniques for microbial diversity survey

Recently, experimental approaches using molecular techniques have presented many findings demonstrating the cause and mechanism of biodeterioration of historic monuments. Use of molecular biology tools and techniques has opened new doors for the identification, characterization and description of microbial diversity thriving on monumental stones and artworks. Modern day biotechnological tools, techniques and instruments have unveiled the hidden microbial diversity in a wide variety of microbial consortium such as biofilms (Gurtner et al. 2000; Ortega-Morales et al. 2000; Schabereiter-Gurtner et al. 2001a; Gonzalez and Saiz-Jimenez 2004; Ortega-Morales et al. 2004). These techniques are relatively rapid and reliable as compared to culturebased methods. These tools and techniques are able to demonstrate the actual proportion of species present in an environment samples (Laiz et al. 2003). These specialized techniques presented many methods that have allowed microbes to be visualized and identified in situ or ex situ with high degree of specificity and sensitivity (De los Ríos et al. 2004; De los Ríos and Ascaso 2005; Welton et al. 2005; Cámara et al. 2011). The detection using these techniques is previously seemed to be hampered by the small amount of sample, which is often less than 1 mg (Gurtner et al. 2000;

Gonzalez and Saiz-Jimenez 2005). Nowadays, detection is rarely constrained by sample quantity due to adequate PCR techniques linked to confocal based verification or qPCR. Besides this, quantitative information yielded using some molecular techniques which rely on PCR are often affected by PCR biases (Anderson et al. 2003). To present a comprehensive overview of all molecular biology and related techniques, these techniques are grouped into five categories: (a) techniques used for visualization and identification of microbes and studying their activity using microscopes and fluorescent activity stains, (b) techniques rely on identification of certain biomolecules such as pigments, proteins, enzymes, lipids etc. obtained from microbes, (c) clone library construction based techniques which comprises of sequencing of small subunits (SSU) 16S, 18S ribosomal RNA, and ITS etc., (d) community fingerprinting or profiling techniques such as DGGE, TGGE, t-RFPL etc., and (e) emerging high throughput next-generation sequencing and bioinformatics techniques. These techniques are described in the following sections and in Tables 4 and 5.

3.1 Clone library construction-based techniques

3.1.1 16S and 18S ribosomal RNA gene sequencing

The SSU ribosomal RNA genes (16S in prokaryotes and 18S in eukaryotes) are universally present in all prokaryotes and eukaryotes and offer an efficient mean to identify microorganisms sampled from monumental sties (Gonzalez and Saiz-Jimenez 2005; Schabereiter-Gurtner et al. 2001a) and to reconstruct their phylogenies. These ribosomal sequences possess variable and highly conserved regions, which are used as phylogenetic markers (Woese 1987) to identify and distinguish between microorganisms on all phylogenetic levels. These ribosomal sequences are first transcribed to produce cDNA which is then cloned (Ward et al. 1990). Alternatively, the DNA extracted from environmental sample containing the ribosomal gene fragment is PCR amplified using appropriate primer set (Table 2) and the amplified fragments are then cloned. As a result of both, a clone library is formed which contain ribosomal gene as inserts. Thereafter, the cloned gene is sequenced and analyzed. The analyzed sequence is then compared with the complete DNA database of the rRNA gene (Maidak et al. 1999) which facilitates the identification of species involved in biodeterioration (Gonzalez and Saiz-Jimenez 2005). Analysis of the DNA sequence can be accomplished using BLAST server of NCBI (www.ncbi.nlm.nih.gov) using Blastn program which is specifically designed for comparing query nucleotide sequence with nucleotide sequences in database. Several heritage sites and artworks such as mural paintings (Saiz-Jimenez and Laiz 2000; Gorbushina et al. 2002; Saarela et al. 2004), degraded wall painings (Rölleke et al. 1996, 1998; Schabereiter-Gurtner et al. 2001c), frescoes (Cappitelli et al. 2009), historic stained glass (Carmona et al. 2006), fountains (Cuzman et al. 2010) and monuments (Laiz et al. 2009) were also analyzed for studying microbial diversity using this technique.

3.1.2 Internal transcribed spacer (ITS) region sequencing

Sequencing of the ITS region located between 18S and 5.3S rDNA has become a diagnostic tool for identifying fungal (Anderson et al. 2003; DuPont et al. 2007; Giacomucci et al. 2011, Cámara et al. 2011) and bacterial contamination (Pangallo et al. 2009) in environmental samples collected from heritage sites. The technique involves the extraction of DNA using standard protocols, for instance, CTAB method for total DNA extraction from fungi (DuPont et al. 2007; Lan et al. 2010; Cámara et al. 2011). The DNA coding for ITS region is PCR amplified using a combination of different primers such as NS1, NS4, ITS1 and ITS4 (White et al. 1990; Cámara et al. 2011) and PCR products were cloned followed by analysis of cloned sequence.

3.1.3 Automated ribosomal-RNA intergenic spacer analysis (ARISA)

Automated ribosomal-RNA intergenic spacer analysis is a fingerprint molecular technique for microbial community analysis in environmental samples including biofilms (Cuzman et al. 2010). During previous years, ARISA have been widely used for characterization of bacterial and fungal species in environmental samples. The technique is based on isolation of whole genome of the sample followed by extraction of 16S-rRNA genes from it. The 16S-rRNA gene is then subjected to PCR amplification using specific primers sets such as AGT CGT AAC AAG GTA GCC GTA CC and CTT CGC CTC TGT GTG CCT AAG T

Table 2 PCR primer sets used in studying microbial diversity on monuments

Microbes studied	Primer set	Primer sequence (5′–3′)	Technique applied	References
Archeae	109AF	AC(G/T)GCTCAGTAACACGT	ARDRA	Lan et al. (2010)
	912AR	CTCCCCCGCCAATTCCTTTA		
	344F-GC	ACGGGGCGCAGCAGGCGCGA	16S rRNA gene sequencing	Raskin et al. (1994), Gonzalez and Saiz-Jimenez (2004)
	518R	ATTACCGCGGCTGCTGG		
	ARC344	ACGGGGAGCAGCAGGCGCGA	16S rRNA gene sequencing	Piñar et al. (2001d)
	ARC915	GTGCTCCCCCGCCAATTCCT		
	ARC344	ACGGGGAGCAGCAGGCGCGA	DGGE	Piñar et al. (2001d)
	518-GC clamped	ATTACCGCGGCTGCTGG		
Cyanobacteria	Cya106F Cya781R	CGG ACG GGT GAG TAA CGC GTG A GGG GAA TYT TCC GCA ATG GG	16S rRNA gene sequencing	Sanchez-Moral et al. (2005), Miller et al. (2009)
	1492R	TACCTTGTTACGACTT	16S rRNA gene sequencing	Polo et al. (2010)
	27F	AGAGTTTGATCMTGGCTCAG		
	CYA359F	GGG GAA TYT TCC GCA ATG GG	16S rRNA gene sequencing	Cappitelli et al. (2009)
	CYA781R[a]	GAC TAC A(or T)GG GGT ATC TAA TCC CAT T		
Bacteria	27BF	AGAGTTTGATC(A/C)TGGCTCAG	ARDRA	Weisburg et al. (1991), Lan et al. (2010)
	1492BR	TACGG(C/T)TACCTTGTTACGACTT		
	341F-GC	CCTACGGGAGGCAGCAG	16S rRNA gene sequencing	Muyzer et al. (1993)
	518R	ATTACCGCGGCTGCTGG		
	341F	CCTACGGGAGGCAGCAG	16S rRNA gene sequencing	Gurtner et al. (2000), Schabereiter-Gurtner et al. (2001a), Piñar et al. (2001c)
	907R	CCCCGTCAATTCATTTGAGTTT		
	341F-GC	CCTACGGGAGGCAGCAG	DGGE	Gurtner et al. (2000), Schabereiter-Gurtner et al. (2001a), (2001c), Piñar et al. (2001c)
	518R	ATTACCGCGGCTGCTGG		
	GC-357 F	CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGG	16S rRNA gene sequencing	Polo et al. (2010)
	907 R	CCTACGGGAGGCAGCAG		
	27F	AGAGTTTGATYMTGGCTCAG	16S rRNA gene sequencing	Gonzalez and Saiz-Jimenez (2005), Echigo et al. (2005), Laiz et al. (2009)
	1510R	CCCCGTCAATTCATTTGAGTTT		
	27F	AGAGTTTGATYMTGGCTCAG	16S rRNA gene sequencing	Schabereiter-Gurtner et al. (2001a), Carmona et al. (2006)
	907R	GGCTACCTTGTTACGACTT		
	HOL189F	AGA GTT TGA TCC TGG CTC AG	16S and 23S rRNA gene sequencing	Zimmermann et al. (2005)
	1037R	CCC CGT CAA TTC ATT TGA GTT T		
	616F	GGA AGT GAA CCA TCT CAG	16S and 23S rRNA gene sequencing	Zimmermann et al. (2005)
	AciD571R	CGA CAA GGA ATT TCG CTA C		
	27F	AGA GTT TGA TYM TGG CTC AG	16S rRNA gene sequencing	Schabereiter-Gurtner et al. (2001c)
	1492R	AAC TAG CCR GCT CAT TAT		
	L1	AGA GTT TGA TCM TGG CTC AG	Fluorescent-ITS	Pangallo et al. (2009)
	G17-(VIC labeled)	TAC GGY TAC CTT GTT ACG ACT T		
	27F	CAA GGC ATC CAC CGT	16S rRNA gene sequencing	Sanchez-Moral et al. (2005), Cappitelli et al. (2009)
	1522R	GTG AAG TCG TAA CAA GG		
	616F	AGA GTT TGA TCM TGG CTC AG	16S rRNA gene sequencing	Miller et al. (2009)
	907R	AAG GAG GTG ATC CAG CCG CA		
	344F	CCT ACG GGA GGC AGC AG	16S rRNA gene sequencing	Gonzalez and Saiz-Jimenez (2004)
	518R	ATT ACC GCG GCT GCT GG		

Table 2 continued

Microbes studied	Primer set	Primer sequence (5′–3′)	Technique applied	References
Eukaryotes	82EF	GAA(G/A/T)CTG(C/T)GAA(C/T)GGCTC	ARDRA	Lan et al. (2010)
	1391ER	GGGCGGTGTGTACAA(A/G)G(A/G)G		
	378 EF	CGGAGA(A/G)GG(A/C)GC(A/C)TGAGA	ARDRA	Lan et al. (2010)
	1492ER	ACCTTGTTACG(A/G)CTT		
	Euk1209F-GC	GCAGGTCTGTGATGCCC	18S rRNA gene sequencing	Diez et al. (2001)
	Uni1392R	ACGGGCGGTGTGTRC		
	EukA	AACCTGGTTGATCCTGCCAGT	18S rRNA gene sequencing	Diez et al. (2001), Carmona et al. (2006)
	EukB	TGA TCC TTC TGC AGG TTC ACC TAC		
	Euk1209F	CAG GTC TGT GAT GCC C	18S rRNA gene sequencing	Gonzalez and Saiz-Jimenez (2004)
	1392R	ACG GGC GGT GTG TRC		
Fungi	NS4	CTTCCGTCAATTCCTTTAAG	DGGE	Cámara et al. (2011)
	ITS4	TCCTCCGCTTATTGATATGC		
	NSO	TAT CTG GTT GAT CCT GCC	18S rRNA gene sequencing	Sert et al. (2007)
	ITS4	TCC TCC GCT TAT TGA TAT GC		
	NS1-GC	GC Clamp-CCAGTAGTCATATGCTTGTC	18S rRNA gene sequencing and DGGE	Cappitelli et al. (2007), Polo et al. (2010)
	NS2	GAATTACCGCGGCTGCTGGC		
	EF-1H	ATGGGTAAGGAAGACAAGAC	PCR-RFLP of partial translation elongation factor 1α gene (EF-1α)	DuPont et al. (2007), Cappitelli et al. (2009)
	EF-2T	GGAAGTACCAGTGATCATGTT		
	NS1	GTAGTCATATGCTTGTCTC	18S rRNA gene sequencing	Polo et al. (2010)
	EF3	TCC TCT AAA TGA CCA AGT TTG		
	27F	AGA GTT TGA TYM TGG CTC AG	18S rRNA gene sequencing	Schabereiter-Gurtner et al. (2001a), Carmona et al. (2006)
	907R	CCC CGT CAA TTC ATT TGA GTTT		

[a] CYA781R contains an equimolar concentration of GAC TAC AGG GGT ATC TAA TCC CAT T and GAC TAC TGG GGT ATC TAA TCC CAT T

(respectively forward and reverse primer for eubaterial 16S rRNA gene amplication) and GTC GTA ACA AGG TAG CCG TA and GCC AAG GCA TCC ACC (respectively forward and reverse primer for cyanobacterial 16S rRNA gene amplification) (Lèpere et al. 2000; Cardinale et al. 2004; Cuzman et al. 2010). The PCR fragments are then separated using the capillary chromatography followed by fluorescent dye-labelled size standard addition and sequencing using automated capillary DNA sequencer (GeneScan 3100 ABI, Applied Biosystems). Using BioNumerics 2.5 software (Applied Maths, Belgium) an ARISA profile is generated. In order to evaluate the similarities in the microbial communities, generally PCA analysis of ARISA profile is performed using another software such as Unscrambler® Version 9.7 (Camo Software AS, Oslo, Norway) (Cuzman et al. 2010). ARISA electropherogram is been widely used in studying and identifying microbial diversity in environmental samples collected from artistic fountain (Cuzman et al. 2010). So far, the technique has been practiced for studying microbial diversity from different ecology such as mountains (Hutchens et al. 2010) and lakes (Jones et al. 2007) and has been less practiced for studying microbial population inhabiting monuments. However, few studies were performed using ARISA technique such as examination of eubaterial and cyanobacterial species isolated from monumental fountains of Italy and Spain (Cuzman et al. 2010).

3.1.4 Amplified rDNA restriction analysis (ARDRA)

Amplified rDNA restriction analysis is an extension of restriction fragment (RF) length polymorphism (RFLP). Tomaselli et al. (2000) used ARDRA for studying the biodeterioration of Italian monuments. Like ARISA, the technique also involves the extraction of genomic DNA and PCR amplification of rRNA

genes. Using this technique, the 16S (Crispim and Gaylarde 2005; Berdoulay and Salvado 2009; Lan et al. 2010) or 18S rDNA sequence (De Felice et al. 2010; Lan et al. 2010) is amplified by PCR using specific primers set. The amplified rDNA is subjected to restriction digestion using appropriate restriction enzymes followed by separation of fragments using DNA gel electrophoresis. Following this, RAPD analysis with the commercially available RAPD analysis kit is performed and ARDRA patterns are obtained. The pattern obtained is considered as the representative of the species analyzed and hence could be used for comparing different samples. According to ARDRA patterns, clones with identical restriction patterns were grouped into one operational taxonomic unit (OTU) (Lan et al. 2010). Clones representing each distinct ARDRA pattern are then selected for sequencing. Sequencing is necessary to establish phylogenetic identification of the isolated clones. Reconstruction of phylogenetic tee is done using software MEGA and PHYLP with the methods of maximum parsimony, neighbor joining and minimum evolution.

3.2 Advance molecular techniques for microbial community fingerprinting or profiling

The visualization and identification of microbial diversity in a community is called as community fingerprinting or profiling and is performed using following techniques. This technique emerged as an alternate technique to circumvent the limitations associated with clone library based approach. The technique is also useful for profiling of uncultivated or inactivated microorganisms (Gurtner et al. 2000; Gonzalez and Saiz-Jimenez 2005). The major disadvantage with the PCR-based techniques is biases associated with them (Anderson et al. 2003).

3.2.1 Denaturing DNA gel electrophoresis (DGGE)

Of the various molecular biology techniques currently used for genetic fingerprinting of the microorganisms associated with biodeterioration of monuments, the PCR-DGGE is most commonly used one. This technique also relies on the amplification of the ribosomal gene fragments as usually performed in case of 16S and 18S rRNA gene, but instead of performing sequencing, the PCR-DGGE is performed in a different way. The ribosomal gene fragments are amplified using a set of primers, such as 341F-GC (5′-tail-CC TACGGGAGGCAGCAG) and 518R (5′-ATTACC GCGGCTGCTGG) (Muyzer et al. 1993) for bacteria; 344F-GC (5′-tail-ACGGGGCGCAGCAGGCGCGA) and 518R (5′-ATTACCGCGGCTGCTGG) for archaea (Gonzalez and Saiz-Jimenez 2004); Euk1209F-GC (5-tail-GCAGGTCTGTGATGCCC) and Uni1392R (5′-ACGGGCGGTGTGTRC) for eukaryotes (Diez et al. 2001); and ITS4GC and ITS3 for fungi (Cámara et al. 2011). Primers 341F-GC, 344F-GC and Euk1209F-GC also possess a 40-bp long GC-rich tail (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGG), which facilitates stabilized migration of the DNA fragments during DGGE (Muyzer et al. 1993; Gonzalez and Saiz-Jimenez 2004; Schabereiter-Gurtner et al. 2001a). Using this technique PCR amplified products are subjected to DGGE which results in separation of partial ribosomal DNA amplified fragments. These fragments are equal in length but differ in sequence as they behave differently (due to different melting behavior) in gel containing a gradient of chemical denaturants (formed by urea and formaldehyde). GC pair offer more stability to sequence than AT. A difference in a single base pair results into significant difference in the melting behavior of the sequence in gel. DGGE profiling, so far, has been carried out for studying microbial diversity consisting of cyanobacteria (Cappitelli et al. 2009), bacteria (Rölleke et al. 1996, 2000; Gurtner et al. 2000; Piñar et al. 2001c; Schabereiter-Gurtner et al. 2001a, c; Gorbushina et al. 2002), archaea (Piñar et al. 2001a, b, d; Rölleke et al. 1998) and fungi (Schabereiter-Gurtner et al. 2001b; Giacomucci et al. 2011) inhabiting prehistoric cave paintings (Schabereiter-Gurtner et al. 2001c), mural paintings (Rölleke et al. 1998; Gorbushina et al. 2002), window glass (Carmona et al. 2006), stones (Cámara et al. 2011), building materials (Sanchez-Moral et al. 2005), and other architectural stoneworks and artworks (Gonzalez and Saiz-Jimenez 2004; Giacomucci et al. 2011). Some DGGE studies also pointed out that colonization starts at endolithic sites and epilithic sites get colonized by microorganisms later on (Cámara et al. 2011). For gaining better insights, some authors have coupled the PCR-DDGE with partial 16S and 18S ribosomal gene sequencing (Polo et al. 2010). However, as biases are associated with other PCR-based techniques (Suzuki and Giovannoni 1996; Anderson et al. 2003) and so with DGGE, these biases are also applicable (Schabereiter-Gurtner et al. 2001c).

3.2.2 *Temperature gradient gel electrophoresis (TGGE)*

Temperature gradient gel electrophoresis is a DNA barcoding technique commonly used in studying molecular ecology of microbes (Gonzalez and Saiz-Jimenez 2004). The technique relies on PCR amplification of gene pool of partial sequences of about 600 bp and subsequently segregating DNA fragments in a temperature gradient electrophoresis. At elevated temperature DNA fragments separates into two strands. The temperature at which melting or strand separation of DNA fragment occur depends up on the hydrogen bonding between nucleotides (two H-bond between A and T, three H-bonds between G and C) of the DNA (Gonzalez and Saiz-Jimenez 2004). GC pair is more stable to elevated temperature than AT because of presence of more H-bonds. Different sequences move differently under gradient electrophoretic conditions and their migration pattern in gel corresponds to their sequence composition (Gonzalez and Saiz-Jimenez 2004). Selected bands are then excised from the gel and sequenced to ascertain the diversity. However, the information obtained after TGGE analysis is less qualitative because of the small size (600 bp) of the DNA fragment used.

3.2.3 *Terminal restriction fragment length polymorphisms (t-RFLP)*

Besides, DDGE and TGGE, t-RFLP is also a techniques frequently used in geomicrobiological surveys for studying microbial community structure and dynamics (Osborn et al. 2000). In this technique the DNA extracts from whole microbial community is amplified using a primer pair (in which one primer is fluorescent). The resultant amplicons are subjected to digestion using restriction enzymes (commonly tetranucleotide recognizing) followed by size separation of RFs using electrophoresis on automated sequencers. The use of fluorescent primer makes the terminal ends of restriction fragments fluorescent (T-RFs) which aid in detection and analysis of fragments. This technique also offers an accurate and reproducible tool for microbial community fingerprinting and establishing their phylogenies (Osborn et al. 2000). However, as biases are associated with other PCR-based techniques (Suzuki and Giovannoni 1996; Anderson et al. 2003) and so with t-RFLP, these biases are also applicable (Von Wintzingerode et al. 1997).

3.2.4 *Single strand conformational polymorphism (SSCP)*

This technique is based on separation of single stranded nucleotides sequence of identical length but different conformations. Apart from sequencing, SSCP can also be used as a mean to identify new polymorphism in DNA. Using SSCP, the presence of actinobacteria strain, *Rubrobacter* was confirmed in the pink-stained area on the exposed walls of ancient Mayan sites of Uxmal (Ortega-Morales et al. 2004). Using the same approach together with phospholipids fatty acid (PLFA) analysis, the main cyanobacteria colonies in biofilms were also determined (Ortega-Morales et al. 2004; Ortega-Morales 2006).

3.3 Emerging techniques and bioinformatics

3.3.1 *High throughput next generation sequencing technology*

Usually generation and interpretation of biological sequence data of environmental samples collected from heritage sites is tedious, costly and time-consuming process. It involves a series of experiments to be performed in order to identify the cause and mechanism of biodeterioration. Nowadays, cutting edge technologies are enabling gene or DNA sequence information to become more valuable for identification of microbes thriving on the monuments. Here discussed is pyrosequencing technology, an automated and inexpensive sequence technology for rapid and high-throughput analysis of sequence data from environmental samples. The technology is first presented in 1998 enabling real-time monitoring of DNA synthesis using bioluminescence (Ronaghi et al. 1998). The technology is based on bioluminometric detection of inorganic pyrophosphate (PPi) released in a polymerization reaction catalyzed by four enzymes (Ronaghi 2001). The four enzymes used in this technology are the Klenow fragment of DNA Polymerase I, ATP sulphurylase, Luciferase and Apyrase (Fonseca et al. 2010). The enzyme substrate included in the reaction mixture is adenosine phosphosulfate (APS). Besides this, D-luciferin and the sequencing template with an

annealed primer to act as starting material for the DNA polymerase are also used. The sensitivity of this sequencing technology is mainly dependent on the concentration of luciferase which catalyzes photo emission reaction. The four nucleotides (dATP, dCTP, dGTP, and dTTP) are added one at a time, repeatedly, in a cyclic manner and the emission of light during reaction is detected by CCD camera (Ronaghi 2001). Like other sequencing techniques used in biodeterioration test, pyrosequencing also involves genomic DNA extraction from microbial samples followed by its PCR amplification and sequencing. Unlike sanger sequencing, pyrosequencing does not involves cloning steps thus eliminating the chance of cloning related artifacts and aberrant recombinants in surrogated host. Use of primer bar coding has allowed pyrosequencing to simultaneously analyze many samples at a time (Zhang et al. 2011). Although, pyrosequencing is a method of choice for exploring a majority of rare phylotypes in complex microbial community, but is not suitable for quantitative tracking of most critical phylotypes present within the community (Zhang et al. 2011). Another major disadvantage of using pyrosequencing technology are the short length of sequence fragments (average of 105 bp), and the need of whole genome amplification so as to generate sufficient amount of DNA for sequencing (Edwards et al. 2006). In the former case, it is difficult to correctly assemble genomes in the absence of a scaffold, while in the later case, it may bias these sequence analyses (Edwards et al. 2006). So far the technique has been successfully applied to metagenomic studies of deep mine microbial community (Edwards et al. 2006). The application of this technique has now extended to study geomicrobiology of biodeteriorated monuments.

3.3.2 Bioinformatics tools

Many bioinformatics tools have got application in geomicrobiological research on deterioration of heritage monuments and artworks. Bioinformatics tools are mainly employed for phylogenetic analysis of 16S r-RNA (Piñar et al. 2001c) and 18S r-RNA genes, analysis of gene sequence having potential carbonatogenic activity using homology search through Blastn program of BLAST-version 2.0 (NCBI), establishing phylogenetic relationship using MEGA version 4.0 (Tamura et al. 2007) following multiple sequence alignment with CLUSTAL-W 1.8 (Thompson et al. 1994). Distance matrix and neighbor-joining (NJ) methods are mainly utilized for the purpose of tree construction (Schabereiter-Gurtner et al. 2001c; Piñar et al. 2001c; Zimmermann et al. 2005). Maximum likelihood (ML) and parsimony algorithms are also used by some researchers (Zimmermann et al. 2005; DuPont et al. 2007; Cuzman et al. 2010).

3.4 Detection of specific nucleic acids by fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization is a molecular diagnostic technique and has been the assay of choice for localization of specific nucleic acid sequence in natural context (Levsky and Singer 2003). Since its development, two decades ago, many modifications have been introduced to enable efficient detection of nucleic acids (Levsky and Singer 2003). It utilizes the potential of both the genetic probes and microscopy (Gaylarde et al. 2003). Both fluorescent dyes and enzymes can be used for specific detection of nucleic acid. For long time, though the underlying principle on which the technique works remained unaltered, many advancements such high sensitivity detection, simultaneous assay of multiple species (multi-target visualization), and automated data collection and analysis have significantly enhanced the efficiency of the technique (Levsky and Singer 2003) and made the technique accessible for a wide variety of applications including studies of microbial community composition at heritage sites. It has an obvious advantage over the use of radio-labeled probes based techniques for visualization of nucleic acids (Levsky and Singer 2003) in terms of speed, resolution and safety. When coupled with CLSM, FISH provides a mean to spatially localize the target microbial population in biofilms. So far the technique is used for studying microbial diversity of ancient caves (Baskar et al. 2006). Additionally, the potential of FISH is also evaluated for studying biodeterioration of building materials (May et al. 2008a).

4 Visualization of microbes and studying microbial activity

4.1 Microscopy

Microscopic visualization of environmental samples and activity studies has been used as a tool for studying microbial diversity since long time. Microscopy has

been traditionally used in culture dependent techniques and nowadays in some culture-independent microbial profiling techniques also. Not all microscopic techniques are considered as molecular techniques but microscopic examination of the biological sample is regarded as the preliminary step to be performed before going to more sophisticated one (Schumann et al. 2003). Microscopic examinations are generally done for morphological characterization of microbial community present in the collected sample (Cuzman et al. 2010) and for studying microbial mineral interaction (Ascaso and Wierzchos 1995). Numerous microscopic techniques like optical (Cuzman et al. 2010), light (Ascaso et al. 2002; Ramírez et al. 2010), phase contrast (Cappitelli et al. 2009), electron microscopy (Roldán et al. 2002; Cappitelli et al. 2009; Cámara et al. 2011) etc. are currently available to yield preliminary information regarding the microbial community inhabiting the historic monuments. Microscopy offers only visualization and to some extent identification, but for characterization of whole microbial consortium other molecular biology techniques, phylogenetic analysis and bioinformatics approaches need to be performed (Gaylarde et al. 2003; Ramírez et al. 2010).

4.2 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy is a non-invasive technique for in situ observation of surface and in depth structure of the sample, for obtaining high resolution optical images and spatial information regarding microbial colonization (Roldán et al. 2004; Hernández-Mariné et al. 2004). It also offers 3-D localization of fluorescent organisms or items labeled with fluorescent dye (Hernández-Mariné et al. 2004). The sample preparation and disturbance to structure is minimal in this technique (Roldán et al. 2004; Hernández-Mariné et al. 2004). Using this techniques the phototrophic bacteria can be visualized by their in vivo pigment autofluorescence while EPS and DNA by certain fluorophore and dyes (Concanavalin-A conjugated with the fluorophore Alexa Flu or 488 and Hoechst 33258 respectively) (Ramírez et al. 2010). A unique feature of the techniques (called optical sectioning) is that it is capable of using multiple excitation and detection wavelength for taking images from desired depth (depth sensitivity) which enable researchers to reconstruct the 3D topology of complex objects such as biofilms. Biofilms have a typical 3D structure which generally gets disturbed when biofilms are submerged in fixatives (Hernández-Mariné 2003). The use of this technique had an obvious advantage that, it neither requires fixing and slicing of biofilms, nor isolation and culturing of microbes. Besides this, biofilms can be observed by multi-channel detection (Ramírez et al. 2010). CLSM in combination with 166 appropriate stains such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC) can be utilized for identifying representative species of stone-inhabiting microorganisms like bacteria and fungi (Bartosch et al. 2003). Using digital image processing algorithm, the information regarding the presence of specific elements and molecules (DNA, phototrophic pigments), structures (matrix, sheath and filaments) and properties (monitoring microbial cell growth, cell division and senescence) can also be obtained. From this perspective, the technique is among one of the most important approaches for studying the spatial distribution of microorganisms in biofilm, 3D structure of biofilm, distribution of EPS, biofilm adaptation to seasonal changes and biofilm interaction with the mineral surface of the stone (Ramírez et al. 2010).

4.3 Studying microbial activity with use of fluorescent activity stains

Use of many fluorescent dyes has allowed the visualization of microbes under microscope on opaque background. Epi-illumination and fluorescent stains such as acridine orange and DAPI (4′,6-diamidino-2-phenylindole) can be used for this purpose (Welton et al. 2005). The use of fluorescent dyes has enabled us to count, visualize and distinguish the cells without separating them from their natural and original substrate (Welton et al. 2005). For measuring the total cell count of the fluorescent stained cells, a small aliquot is fixed with formaldehyde solution (concentration up to 2%) so as to kill cells. For estimation of viable cells in the sample, serially diluted aliquots are plated on the solid media and incubated at appropriate culture conditions. Acridine orange has been successfully used for the estimation of bacteria from a range of archaeological sites such as Portchester Castle and Tintern Abbey (Albertano and Urzì 1999; Tayler and May 2000). Use of DAPI as a counterstain for counting bacteria (in situ) may be unsuitable because of autoflourescence from stone particles which may makes the fluorescence from bacteria indistinguishable

(Tayler and May 2000). Some microorganisms being autofluorescent (exhibit fluorescence), the use of activity stains is not required for their detection. Other microorganisms are made autofluorescent by inserting fluorescent protein gene (such as GFP, green fluorescent protein) for their visualization and detection (Welton et al. 2005). Few fluorescent activity stains facilitate distinguish between viable, dead and active cells and thus enabling us to study their viability and activity following treatment with anti-microbial agents (Welton et al. 2005). For instance, these stains allowed the evaluation of the efficacy of the applied anti-microbial agent treatment on biofilms. The method was successfully applied for studying the microbial population inhabiting in biofilms isolated from biodeteriorated sites (Alakomi et al. 2006a). Evaluation of the efficacy using microscopic and physiological assessment of biofilms proved that the staining and fluorescent measurement is a promising method for studying how biofilms respond to in vitro anti-microbial treatments (Alakomi et al. 2006a). A detailed list of fluorescent dyes and stains used in biodeterioration studies of monuments is presented in Table 3.

5 Detection of biomolecules

Detection and analysis of specific biomolecules such as protein (Prieto et al. 2007), enzymes (Laiz et al. 2009), phospholipids (Ortega-Morales et al. 2004; Ortega-Morales 2006), photosynthetic pigments (Schumann et al. 2005; Cappitelli et al. 2009), and nucleic acids (Gonzalez and Saiz-Jimenez 2005) have also presented many direct and indirect ways for detection and quantification of microbes. Although, these techniques are not always destructive in nature, however in certain case where biomolecules (extractives) are present inside cells (such as chlorophyll and lipids), disruption of cells becomes compulsory. Assays of some enzymes in particular those of respiratory enzymes are much important as they give details about the metabolic state or activity of cells, rather than merely giving information about their capabilities to reproduce and grow to form more colonies on solid media.

5.1 Measurement of enzyme activity

A simpler way to assessing the microbial activity is to estimate the electron transport in cells via dehydrogenase enzyme (Gómez-Alarcón et al. 1994). The method facilitates the examination of spatial distribution of the microbial population over the rock surface. The reduction of compound such as TTC (2,3,5-triphenyltetrazolium chloride), a colourless soluble compound into an insoluble formazan derivative and INT [2-(4-iodophenyl)-3-(4nitrophenyl)-5-phenyl tetrazolium chloride], followed by reduction to INT-formazan by the active bacteria forms the basis for measuring dehydrogenase activity (Warscheid et al. 1990; Tayler and May 2000; Laiz et al. 2009). Following incubation, the probes are examined under a binocular microscope. Additionally, calorimetric assay of fluorescein diacetate (FDA) has also been used for examining activity of microbial population on stones (Tayler and May 1995; Kumar and Kumar 1999). Unlike INT which measures metabolic activity of microbes, FDA measures the enzyme activity (Kumar and Kumar 1999). However, FDA has greater sensitivity and rapid reaction rate with natural stones and requires less incubation time (Kumar and Kumar 1999). The amount of INT reduced and FDA cleaved for pure bacterial culture and mixed microbial population showed that FDA can also be used as an indirect method for assessing microbial activity on stones (Kumar and Kumar 1999). The major disadvantage of this method is that the enzyme released by the inactive cells and dead cells may lead to overestimation of the activity of the microbial population (Tayler and May 1995).

5.2 Photosynthetic pigments analysis

Besides using molecular techniques, there are few other methods performing which the presence of particular microbial population can be ascertained. For instance, pigment analysis (chlorophyll *a* to chlorophyll *b* ratio) of the colored powdered substance present over the monumental stones and artworks. It is a routine technique performed in order to identify the presence of photosynthetic bacteria such as cyanobacteria and green algae (Cappitelli et al. 2009; Schumann et al. 2005). The detailed method for extraction and analysis of photosynthetic pigments is described by some researchers (Schumann et al. 2005). Discrimination between cells with or without photosynthetic pigments is achieved by comparing the autofluorescence of individual cells by flow cytometric analysis. Cholorophyll *a* estimation has indicated the dominance of phototrophic epilithic microorganisms on the

Table 3 Stains/dyes/fluorochromes used for microbial analysis in biodeterioration studies

Stains	Microbes/biomolecules stained	Analysis	References
Acridine orange	Bacteria	UV/VIS spectrophotometer and light microscopy	Tayler and May (2000), Pangallo et al. (2009)
Amann's lactophenol	Bacteria	Light microscopy	Urzì and Albertano (2001), Pangallo et al. (2009)
Concanavalin-A conjugated with the fluorophore Alexa Fluor 488	Cyanobacteria and algae (EPS of biofilms)	CLSM analysis of chlorophyll and phycobilins	Ramírez et al. (2010)
Crystal violet	Bacteria	Spectrophotometer reading at A_{595}	Alakomi et al. (2006b)
5-Cyano-2,3-ditolyltetrazolium chloride (CTC)	Bacteria and fungi	Fluorescence microscopy	Bartosch et al. (2003)
4′,6-Diamidino-2-phenylindole (DAPI)	Bacteria (DNA)	Fluorescence microscopy	Bartosch et al. (2003), Baskar et al. (2006), Cuzman (2009), Giacomucci et al. (2011)
Fuorescein diacetate (FDA)	Bacteria, algae and fungi (DNA)	Viability measurement using fluorescence microscopy	Gorbushina et al. (2002)
Fluorescein isothiocyanate (FITC), CY3, and CY5	Fungi and eubacteria	FISH and immunostaining technique	Piñar et al. (2001c), Cappitelli et al. (2007)
Gram staining	Bacteria	Compound microscope	Videla et al. (2000), Pangallo et al. (2009)
Hoechst (3325)8	Cyanobacteria (DNA)	CLSM	Hernández-Mariné et al. (2004)
L-13152	Micro-organisms	Viability staining for distinguishing live and dead cells under fluorescence microscope	Welton et al. (2005)
Lactophenol cotton blue	Algae and fungi	Light microscope	Caneva et al. (2005), Grbić et al. (2009)
Methylene blue	Nucleic acids	Microscopy	Caneva et al. (2005)
Propidium iodide (PI)	Bacteria, algae and fungi (DNA)	Cell viability measurement using fluorescence microscopy	Gorbushina et al. (2002)
Periodic acid Schiff (PAS) stain	Lichens, free living fungi (EPS, glycogen, starch, cellulose, chitin, mucin, protein-carbohydrates complexes, and glycolipids)	Petrographic stained thin sections analysis	Urzì and Albertano (2001), Caneva et al. (2005), Mohammadi and Krumbein (2008), Wiktor et al. (2009)
SYBER-green	Cyanobacteria and algae (DNA)	FACS analysis for chlorophyll (660–700 nm) and phycoerythrin (560–590)	Cappitelli et al. (2009)
SYTO series	Cyanobacteria, algae and fungi (DNA)	Cell viability measurement using fluorescence microscopy	Cuzman (2009)
SYTOX green S-7020	Cyanobacteria (nucleic acids)	CLSM	Hernández-Mariné et al. (2004)
Tetramethyl rhodamine iso-thiocyanate (TRITC)	Biofilm	Epifluorescence microscopy	Cuzman (2009)

Table 3 continued

Stains	Microbes/biomolecules stained	Analysis	References
Wheat germ agglutinin (WGA from *Triticum vulgaris*) conjugated with the fluorophore Alexa Fluor 488	Cyanobacteria (EPS of biofilms)	CLSM	Hernández-Mariné et al. (2004)

exterior and interior walls of the archaeological sites of Uxmal and Kabah, Mexico (Ortega-Morales et al. 2000). The occurrence of two microalgae, *Stichococcus* sp. and *Chlorella* sp., has been observed on the man-made surfaces such as roof tiles, concrete and building facades (Schumann et al. 2005). Besides this the biological origin of dark patinas on granitic outcrops has been confirmed by the estimation of chlorophyll *a* in the samples (Prieto et al. 2007).In environmental samples, the chlorophyll *a* and *b* ratio was quantified using the below formula (Schumann et al. 2005):

$$\text{Chl}\,a = (16.5 \times A_{665}) - (8.3 \times A_{650}) \quad \text{and}$$

$$\text{Chl}\,b = (33.8 \times A_{650}) - (12.5 \times A_{665})$$

A fluorescence technique called chlorophyll *a* fluorescence is continuously gaining importance in studying biodeterioration of monumental stones caused by phototrophs and in evaluating the efficacy of biocides treatment against them (Tomaselli et al. 2002; Delgado Rodrigues et al. 2004; Häubner et al. 2006; Tretiach et al. 2008, 2010). The fluorescence measurement of the sample is carried out using pulse amplitude-modulated fluorimeter Mini-PAM (Walz, Effeltrich, Germany) and generated results are used to ascertain the microbial diversity.

5.3 Phospholipid fatty acids analysis

Phospholipid fatty acids (PLFA) are the major components of the cell membranes of all cells except those of the archaea, therefore profiling of PLFA allow the examination of most of the important members of many microbial communities (Heyrman et al. 1999; Gurtner et al. 2000). It has been reported that, when cells die, the membrane phospholipids breaks down, and hence PLFA analysis offers an enumeration of viable cells in the environmental samples (White et al. 1979, 1997). The analysis involves extraction of PLFA from environment samples and its subsequent methylation (derivatization to form fatty acid methyl esters, FAMEs) and quantification using analytical chromatographic methods such as GC and HPLC (Heyrman et al. 1999). To make the FAME analysis more reliable, the FAME profiles is compared with several reference profiles from the TSBA4.0 library using UPGMA of the Euclidean distance coefficients or Canberra metric coefficients (Heyrman et al. 1999; Gurtner et al. 2000; Gorbushina et al. 2002). This

technique has been employed for the determining the major colonizers of the biofilms, cyanobacteria and epilithic phototrophs on the interior and exterior (illuminated) surfaces of Mayan archaeological sites of Uxmal (Ortega-Morales et al. 2000, 2004; Ortega-Morales 2006). Using this techniques bacterial and fungal diversity and biodeterioration caused by them in mural paintings of St. Martins church was also determined (Gorbushina et al. 2002). The total amount of PLFA (in pmol) in environmental samples is directly proportional to the number of cells. Thus, the analysis of PLFA serves as an indirect method for enumeration of cells in the environmental samples (Kuhlman et al. 2006). For instance, quantification of ergosterol (membrane component of fungi) using HPLC has served as a indirect method for assessment of fungal growth on the painted walls and for testing the efficacy of the fungicides treatment applied to painted films (Pasanen et al. 1999; Saad et al. 2003; Welton et al. 2005). The technique is based upon the indirect enumeration of cells by quantification of PLFA and hence not provides the accurate number of cells (living or dead) in an environmental sample. Besides this, PLFA is a technique used to determine the community composition and biomass at community-level. However, for microbial community analysis, PCR-SSCP of the single subunit rRNA (SSU rRNA) gene is preferred over PLFA analysis because of its better resolution. Furthermore, the nucleic acid sequence analysis of SSU rRNA allows the comparison of the sequences with previously found sequences in databases to allow establishment of phylogenetic relationship. For this reason, the PLFA analysis sometimes is performed in conjunction with PCR-SSCP of the single subunit rRNA (SSU rRNA) gene (Ortega-Morales et al. 2004; Ortega-Morales 2006; May et al. 2008b).

5.4 Scanning electron microscopy (SEM)

Scanning electron microscopy images a sample by scanning it with a high energy electron beam in a rastar scan pattern enabling the examination of the surface topography and distribution of specimen at a high magnification (Hernández-Mariné et al. 2004). Typically, the sample preparation for SEM includes air drying and chemical fixation followed by critical point drying or freeze fixation (Hernández-Mariné et al. 2004). Chemical fixation is done with 2–4% glutaraldehyde (1,5-glutar-di-aldehyde) in a 0.1 M cacodylate buffer overnight (or 0.1 M phosphate buffer) followed by dehydration in ethanol (or acetone) and critical point dried (Albertano and Urzì 1999; Hernández-Mariné et al. 2004; Herrera et al. 2004; Mohammadi and Krumbein 2008). The conductivity of the non-conductive biological samples can be improved by the impregnation or coated with heavy metal salts such as with osmium using variants of the OTA staining method (O-osmium, T-thiocarbohydrazide, O-osmium) (Hernández-Mariné et al. 2004). Impregnation of samples before imaging is necessary to prevent rise of local electron concentration (called charging) which may affects the formation of clear images (Herrera and Videla 2009). Specimens are then placed on stubs having coating of carbon or gold sputtered (50 Å of gold cover) (Gorbushina et al. 2002; Sanchez-Moral et al. 2005; Wiktor et al. 2009). Samples which are prone to be collapsed when submerged in liquid medium are prefixed in acrolein vapour followed by addition of osmium tetroxide vapour so as to adequately preserve the 3D structures of samples. SEM is widely used in studying biodeterioration of monuments. Using SEM, the crust (Herrera et al. 2004) and powdery substances (Cappitelli et al. 2009) collected from the decayed surfaces of stones was analyzed for microorganism's estimation. SEM has also made possible to visualize the fungal and bacterial biofilm over crystalline surface of substratum (Mohammadi and Krumbein 2008). Using SEM in backscattered electron mode (SEM-BSE) the epilithic, endolithic and lithobiontic microbial colonization was observed on exposed fronts of dolostone quarry (Redueña, Madrid, Spain) and carbonate rock of the Jeronimos Monastery (Lisbon) (Cámara et al. 2011; Ascaso et al. 2002). Using combinations of microscopic and SEM techniques, many bioalteration processes such as micromorphological characterization of dark patina, biodissolution of calcium from lithic surface, anti-desiccant role of EPS, hyphal intrusion, pits and cavities formation by lichen and fungal thalus have been demonstrated (Carmona et al. 2006; Prieto et al. 2007). SEM-BSE together with DGGE is also performed to assess the efficacy of the biocides treatment to dolostone quarry (Cámara et al. 2011). SEM-BSE in conjunction with EDX has been used for studying biochemical activity of microorganisms (De los Ríos et al. 2002; De los Ríos and Ascaso 2005). SEM analysis of few biological samples such as

biofilms requires extensive manipulation which may be destructive. To prevent destruction of the cellular structure of the microorganisms, biofilms are analyzed using environment scanning electron microscopy (ESEM) which yields clear images of the biofilms and gives information regarding spatial relationship and surface chemistry without having extensive sample manipulation (Urzì and Albertano 2001; Herrera and Videla 2009). Owing to its advantages, ESEM has been extensively used in conjunction with EDX in studying biodeterioration of monuments (Videla et al. 2000; Ascaso et al. 2002; Herrera et al. 2003).

6 Techniques used for studying building materials

Molecular biology techniques are of less use in identification and studying the type and mechanism of deterioration caused to stones by microorganisms. For studying building materials and deteriorated works of art, modern day analytical methods are generally used especially those which are used in physical and geological sciences such as spectroscopic methods, petrography etc. (Tables 4, 5). These tools and techniques have helped researchers in studying the biogeophysical and biogeochemical changes brought about the action of microbes on monumental stones and artworks. Following sections describe about the different material characterization and surface analysis techniques and present different examples of the use of these techniques in studying biodeterioration of heritage monuments and artworks. In order to make this review more lucid for interested readers, the techniques detailed in this section has been focused to provide better insights with respect to their applicability in biodeterioration studies, i.e. identification of: (a) type, (b) mechanism and, (c) extend of biodeterioration caused by microorganisms.

6.1 Petrographic studies, mineralogical calculations and X-ray diffraction

Surface techniques present evidences of alterations induced by microbial communities in the presence of surrounding environment. Using a combination of microbiological, surface analytical and mineral/chemical analysis techniques, an appropriate assessment of the biodeterioration process can be made (Herrera and Videla 2009; Cappitelli et al. 2009; Cámara et al. 2011). Thin section petrography studies involve the examination of the thin section of the constructional material under polarizing microscope (also called as petrographic microscope) (Cámara et al. 2011). The transformation of rock surface due to weathering process can also be studied by thin section petrography (Herrera et al. 2004). With thin section petrography studies it is also possible to assess the extent of microbial colonization of stones over different periods of time (Cámara et al. 2011). Using mineralogical calculations and thin-section petrography studies the chemical changes on the monumental stone surface and its possible provenance can be ascertained (Mohammadi and Krumbein 2008; Cámara et al. 2011). XRD usually adopt methods those are low invasive on the sample but crucial for morphological or surface analysis and for studying the changes caused by microorganisms and environmental factors (Herrera et al. 2004). For every crystalline structure analyzed, XRD generates a characteristic pattern. Like chemical analysis, mineralogical and petrography studies, XRD and other techniques yielded sufficient results related to the chemical and mineralogical composition of colored patina, encrusted sulfated crust, salt and deposited efflorescence that made it easier to ascertain and predict their possible provenance (Del Barrio et al. 2002; Sebastián et al. 2008; Toniolo et al. 2009). The technique is also used for analysis of stone samples scratched off from underneath the biofilms (Ramírez et al. 2010). X-ray diffraction analysis and thin section petrography analysis in conjunction with transmission polarized light microscopy (TEM) has been widely used for studying microstructural deformations caused by microorganisms (Del Barrio et al. 2002). Geologists and archaeologist generally use it for differentiation and classification of rocks, sands and soils and for investigation of its source and geological origin. The technique has been currently used in geomicrobiology to study mineral content and textural features of cultural materials made up of stones as found in sculptures and artworks. Using this technique the stone materials can be characterized and distinguished from other, type of deterioration can be interpreted and extend of deterioration caused to them can be monitored. Mineralogical calculation is much important in sense that it uses the data coming from thermogravimetry (TG) and chemical analysis (CA) and use it for explaining the mechanism of deterioration which in turn allow us to formulate the remedial methods to

Table 4 Molecular and physical techniques used for identifying microbes and studying biodeterioration of monuments

S. no.	Molecular and physical technique	Biodeteriorated monuments and artworks	Type of biodeterioration	Microbes detected	References
1.	SEM	Persepolis monuments (Iran)	Pits and etch marks	Fungi, bacteria and algae	Mohammadi and Krumbein (2008)
		Luca Signorelli frescoes in St. Brizio Chapel (Orvieto Cathedral, Italy)	Biofilm formation	Capsulated coccoid cells	Cappitelli et al. (2009)
		Mural paintings of St. Martin church in Greene-Kreiensen (Lower Saxony, Germany)	Brownish pigmentation, sporulation structures and hyphal growth on walls	Fungi and actinomycetes	Gorbushina et al. (2002)
		Monumental stones (Graphite crevices)	Biofilm	*Chroococcus* sp., *Synechococcus* sp., *Halospirulina* sp., *Leptolyngbya* sp., and *S. julianum*	Hernández-Mariné et al. (2004)
2.	LT-SEM	Jeronimos Monastery (Lisbon, Portugal)	Biochemical deterioration, pits, cavities and thallus intrusion	Lichens (*Thyrea*) and non-symbiotic cyanobacterial	Ascaso et al. (2002)
3.	ESEM	Roman Catacombs	Biofilm formation	Terrestrial epilithic cyanobacteria (*Eucapsis*, *Leptolyngbya*, *Scytonema*, and *Fischerella*), green algae and diatoms	Albertano and Urzì (1999)
		Historic stained glasses from the Cartuja de Miraflores (Spain)	Craters and pits (filled with whitish deposit) formed as a result of hyphae	*A. tamari* and other fungi and bacteria	Carmona et al. (2006)
		Saint Callixtus Catacombs and Domitilla Catacombs, Rome (Italy)	Green and white biofilm, chemical dissolution and pitting	Cyanobacteria and Actinobacteria	Sanchez-Moral et al. (2005)
		Uxmal and Tulum, Mayan archaeological sites (Mexico)	Microbial biofilms and crust formation	*Pseudomonas, Bacillus Cereus,* Hyphomycetes (*Monilia* sp.) and cyanobacteria (*Cyanocystis*) at Uxmal sites and *Dermocarpa, Myxosarcina, Gloeocapsa. Aspergillus ochraceus,* aerobic bacteria (*Pseudomonas* sp.) and anaerobic bacteria at Tulum sites	Videla et al. (2000)
4.	CLSM	Monumental Stones	Biofilm formation	*Cyanothece, Aphanothece* sp., *Gloecacapsopsis* sp., *Crinalium* sp., *Nostoc* sp., and *Scytonema julianum*	Hernández-Mariné et al. (2004)

Table 4 continued

S. no.	Molecular and physical technique	Biodeteriorated monuments and artworks	Type of biodeterioration	Microbes detected	References
5.	Fluorescent activity staining	Portchester Castle and Tintern Abbey	Decay of stones	Bacteria	Tayler and May (2000)
		Tacca's Fountain, Florence (Italy), Second Fountain from Villa la Pietra, Florence (Italy), Fountain from Patio de la Sultana, Generalife, Granada (Spain), Fountain from Patio de la Lindaraja, Alhambra, Granada (Spain)	Biofilm and patina formation	Autotrophic algae and cyanobacteria	Cuzman (2009)
6.	Respiratory enzyme measurement	Alcala de Henares (Madrid, Spain)	Biodeterioration of stones	Fungi (*Altematia, Penicillium, Phoma, Trichodenna, Mucor, Ulocladium, Dictyodesmium* and *Phialostele*) and Bacteria (*Bacillus, Micrococcus* and *Thiobacillus*)	Gómez-Alarcón et al. (1994)
		Portchester Castle and Tintern Abbey	Decay of stones	Bacteria	Tayler and May (2000)
7.	Photosynthetic pigment analysis	Luca Signorelli frescoes in St. Brizio Chapel (Orvieto Cathedral, Italy)	Rosy discoloration	Cyanobacteria	Cappitelli et al. (2009)
		Cathedral of Seville, Orologio Tower (Martano) and the Santa Clara-a-Velha Monastery (Coimbra)	Biofilm	*Chlorella* and *Gyrosigma*	Miller et al. (2009)
		Granite stones in historic cathedral	Discoloration	Green microalgae such as *Stichococcus* sp. and *Chlorella* sp.	Schumann et al. (2005)
8.	Phospholipid analysis	Mural paintings of St. Martin church in Greene–Kreiensen (Lower Saxony, Germany)	Discoloration	Heterotrophic bacteria	Gorbushina et al. (2002)
9.	16S/18S rRNA sequencing	Chapel of Castle Herberstein, Styria, Austria (Mural Paintings)	Efflorescences	*Bacillus* and *Halobacterium*	Saiz-Jimenez and Laiz (2000)
		Bayon Temple, Angkor Thom, Cambodia	Biofilm formation and color change of exterior stone surfaces	Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria; Alveolata, Fungi, Metazoa, Viridiplantae; Crenarchaeote, and Euyarchaeota	Lan et al. (2010)
		Demetra and Cronos sculptures of the Buonconsiglio Castle, Trento (Italy)	Crust and Green, black and green-black discoloration	Cyanobacteria, bacteria, fungi and eukaryotes	Polo et al. (2010)

Table 4 continued

S. no.	Molecular and physical technique	Biodeteriorated monuments and artworks	Type of biodeterioration	Microbes detected	References
10.	16S and 23S rRNA sequencing	Saint Callixtus Catacombs (Rome, Italy)	Biofilm formation	*Acidobacteria*	Zimmermann et al. (2005)
11.	ITS sequencing	Artistic Tiles from the Façade of the Grande Albergo Ausonia and Hungaria (Venice, Italy)	Colored alterations	Cryptoendolithic cyanobacteria	Giacomucci et al. (2011)
12.	ARISA	Tacca's Fountain, Florence (Italy), Second Fountain from Villa la Pietra, Florence (Italy), Fountain from Patio de la Sultana, Generalife, Granada (Spain), Fountain from Patio de la Lindaraja, Alhambra, Granada (Spain)	Formation of biofilm and patina of different colors and aesthetic loss	Algae, cyanobacteria and fungi	Cuzman et al. (2010)
13.	ARDRA	Historical tuff monument (De Francesco building) (Italy)	Discolouration and development of efflorescence and patina	Bacteria (*Salinibacter ruber*, *Acidovorax*, *Kocuria*, *Curtobacterium*, and *Actinobacterium*), and fungi (*Cladosporium, Aspergillus* and *Penicillium*)	De Felice et al. (2010)
		Bayon Temple Sandstone of Angkor Thom, Cambodia	Biofilm formation, chemical weathering and efflorescence	Bacteria, archae and eukaryotes	Lan et al. (2010)
14.	RAPD	Mural paintings of St. Martin church in Greene-Kreiensen (Lower Saxony,Germany)	Discoloration	Micromycetes and fungi	Gorbushina et al. (2002)
15.	PCR-RFLP of EF-1α	French Paleolithic painted cave of Lascaux	Alterations in the cave environment	*Fusarium solani*	Dupont et al.(2007)

Table 4 continued

S. no.	Molecular and physical technique	Biodeteriorated monuments and artworks	Type of biodeterioration	Microbes detected	References
16.	DGGE	Tomb of Postumio, Vilar de Frades Church	Efflorescences and cyanobacterial biofilms	*Rubrobacter*	Laiz et al. (2009)
		Chapel of Castle Herberstein, Styria, Austria (Mural Paintings)	Efflorescences	*Arthrobacter globiformis, Streptomyces griseus, Acinetobacter lwoffi* and members of the *Pseudonocardiaceae* and actinomycete lineage	Saiz-Jimenez and Laiz (2000)
		Cathedral in Milan	Blackening due to melanin pigment, intercrystal growth causing structural damage, biopitting etc	Dematiaceous meristematic fungi	Cappitelli et al (2007)
		Luca Signorelli frescoes in St. Brizio Chapel (Orvieto Cathedral, Italy)	Rosy discoloration	Cyanobacteria	Cappitelli et al (2009)
		Historic stained glasses from the Cartuja de Miraflores (Spain)	Staining of glass surface	Bacteria	Carmona et al. (2006)
		Orologio Tower in Martano (Italy), Santa Clara-a-Velha Monastery (Coimbra), Ajuda National Palace (Lisbon), and Cathedrals of Seville and Granada (Spain)	Biofilm	Cyanobacteria and Proteobacteria such as *Leptolyngbya, Cylindrospermopsis, Nostoc, Microcoleus* etc.	Miller et al. (2009)
		Saint Callixtus Catacombs and Domitilla Catacombs, Rome (Italy)	Green, white and brown biofilm formation	Cyanobacteria (*Cyanothece, Phormidium, Cyanobacterium, Aphanothece, Gloeothece*) and Bacteria (*Aquabacterium, Teichococcus, Devosia, Alcaligenes*)	Sanchez-Moral et al. (2005)
		Masonry and lime wall paintings from Herberstein, Austria Burggen, Germany	Red-pigmented biofilm	*Saccharopolyspora, Nocardioides, Pseudonocardia*, *Arthrobacter, Comamonas, Rubrobacter*, and *Kineococcus*-like bacterium	Schabereiter-Gurtner et al. (2001c)
17.	FISH	Demetra and Cronos sculptures of the Buonconsiglio Castle, Trento (Italy)	Crust and green, black and green-black discoloration	Cyanobacteria	Polo et al. (2010)

Table 4 continued

S. no.	Molecular and physical technique	Biodeteriorated monuments and artworks	Type of biodeterioration	Microbes detected	References
18.	SEM-EDX	Peridtlite, Cambodian cultural heritage	Release of metabolic acids and leaching of rock material, production of carbonic acid, oxalic acid and chelating agents and aesthetic loss	Cyanobacteria, algae and lichens	Herrera et al. (2004)
		Dolomudstones of the Terwagne Formation (Viséan, Bocahut quarry, in northern France) and limestones of the "Morrone di Pacentro" Formation (Lower Cretaceous, Italy	Diagenesis	Fungi	Kolo et al. (2007)
19.	Thin section petrography	Persepolis monuments (Iran)	Hyphal intrusion and biopitting	Lichens and fungi	Mohammadi and Krumbein (2008)
20.	XRD	Cathedral of Lleida	Bioactivity resulting in the formation of colored red-orange patina	Microorganisms	Del Barrio et al. (2002)
		Dolomudstones of the Terwagne Formation (Viséan, Bocahut quarry, in northern France) and limestones of the ''Morrone di Pacentro'' Formation (Lower Cretaceous, Italy	Diagenetic process	Fungi	Kolo et al. (2007)
		Robbás fountain statues, Ljubljana (Slovenia)	Crust formation, biofilm formation, mineral dissolution and decohesion between calcite grains	Cyanobacteria, green algae and lichens	Kramar and Mirtič (2008)
21.	MIP	Dolostones from Redueña, Madrid, Spain	Alterations in chemical composition and porosity of lithic substrate, intercrystalline penetration of fungi	Epilithic (lichens), Endolithic (fungi) and lithobiontic microorganisms	Cámara et al. (2011)
22.	FT-IR	Peridotite, Colombian cultural heritage	Biosolubilization of stone surface by metabolic acids	Bacteria and fungi	Herrera et al. (2004)
		Vallicelliana Library, Rome (Italy)	Chromatic alterations	Fungi	Monte (2003)

Table 4 continued

S. no.	Molecular and physical technique	Biodeteriorated monuments and artworks	Type of biodeterioration	Microbes detected	References
23.	FT-RS	Ecclesiastical buildings in the vicinity of Burgos, Castille y Leon, Spain	Calcium oxalate formation	Lichens belonged to genera *Caloplaca*, *Candelariella*, *Aspicilia* and *Xanthoria*	Villar et al. (2004)
		Dolomitic Stones	Metal oxalate formation	*Lecanora sulfurea, Aspicilia calcarea*, *Dirina massiliensis* f. *sorediata*, *D. massiliensis* f. *massiliensis* and *Tephromela atra*	Edwards et al. (2003)
24.	MS	Cathedral of Lleida	Bioactivity resulting in the formation of colored red-orange patina	Microorganisms	Del Barrio et al. (2002)
25.	Thermal analysis	Cathedral of Lleida	Bioactivity resulting in the formation of colored red-orange patina	Microorganisms	Del Barrio et al. (2002)
26.	RS	Dolomudstones of the Terwagne Formation (Viséan, Bocahut quarry, in northern France) and limestones of the ''Morrone di Pacentro'' Formation (Lower Cretaceous, Italy	Diagenetic process	Fungi	Kolo et al. (2007)

Table 5 Molecular and analytical techniques and strategies employed for studying microbial community composition and deteriorated artworks

Techniques	Monumental surface/ stone (Ca, Si etc.)	Painting and frescoes	Patina and crust	Biofilms	Polymers/ metals (plastics/ paints)	Wooden artworks	Composite materials cement/ mortar	References
Microscopy								
Light				√				Albertano and Urzì (1999)
Optical	√		√			√	√	Del Barrio et al. (2002), Popescu et al. (2005), Sarkar et al. (1994), Polo et al. (2010)
Electron			√	√		√		Popescu et al. (2005), Roldán et al. (2002), Cappitelli et al. (2006)
CLSM				√				Roldán et al. (2004), Hernández-Mariné et al. (2004), Ramírez et al. (2010)
Fluorescent activity staining								
Fluorescent dye	√			√				Alakomi et al. (2006a), (2006b), Welton et al. (2005)
Ribosomal RNA sequencing								
16S rRNA sequencing	√		√					Berdoulay and Salvado (2009), Polo et al. (2010), De Felice et al. (2010), Fonseca et al. (2010)
18S rRNA sequencing	√		√					Berdoulay and Salvado (2009), Polo et al. (2010)
ITS sequencing	√		√					Berdoulay and Salvado (2009), Anderson et al. (2003)
ARISA				√				Cuzman et al. (2010)
ARADA			√					Berdoulay and Salvado (2009)
PCR-based techniques								
DDGE	√	√						Rölleke et al. (1998), Polo et al. (2010), Gurtner et al. (2000), Piñar et al. (2001c), Fonseca et al. (2010)
TGGE	√							Gonzalez and Saiz-Jimenez (2004)
t-RFPL	√							Osborn et al. (2000)
Genetic probe-based								
FISH	√	√						Polo et al. (2010), Piñar et al. (2001c)
Biomolecules quantification								
Chlorophyll estimation	√							Cappitelli et al. (2009), Schumann et al. (2005), Fonseca et al. (2010)
Phospholipids analysis	√							Ortega-Morales et al. (2000), Ortega-Morales et al. (2004), Ortega-Morales (2006), White et al. (1997)
Respiratory enzyme analysis	√							Warscheid et al. (1990), Tayler and May (2000), Welton et al. (2005)

Table 5 continued

Techniques	Monumental surface/ stone (Ca, Si etc.)	Painting and frescoes	Patina and crust	Biofilms	Polymers/ metals (plastics/ paints)	Wooden artworks	Composite materials cement/ mortar	References
Studying building materials								
Chemical and mineralogical analysis	√		√				√	Sarkar et al. (1994), Del Barrio et al. (2002), Campos-Suñol et al. (2008), Polo et al. (2010)
Petrography			√				√	Sarkar et al. (1994), Campos-Suñol et al. (2008)
Water uptake and mercury porosimetry	√						√	Sarkar et al. (1994), Welton et al. (2005), Ruiz-Agudo et al. (2007), Cámara et al. (2011)
SEM		√	√		√	√	√	Del Barrio et al. (2002), Herrera et al. (2003), Herrera et al. (2004), Popescu et al. (2005), Sarkar et al. (1994), Chiavari et al. (2007), Robbiola et al. (2008), Campos-Suñol et al. (2008), Welton et al. (2005), Wiktor et al. (2009)
XRD	√		√			√	√	Del Barrio et al. (2002), Herrera et al. (2003), Herrera et al. (2004), Popescu et al. (2005), Sarkar et al. (1994), Perez-Rodriguez et al. (2011), Benzzi et al. (2008), Welton et al. (2005), Ruiz-Agudo et al. (2007)
FT-IR	√		√			√		Herrera et al. (2004), Popescu et al. (2005), Campos-Suñol et al. (2008), Polo et al. (2010), Monte (2003)
Mössbauer spectrometry			√					Del Barrio et al. (2002)
Raman spectrometry	√		√		√			Chiavari et al. (2007), Robbiola et al. (2008), Campos-Suñol et al. (2008), Prieto et al. (2007)
TG and DTA	√		√			√	√	Severiano et al. (2010), Sarkar et al. (1994), Perez-Rodriguez et al. (2011), Barcina et al. (1997), Welton et al. (2005), Monte (2003)
ICP-MS	√		√				√	Del Barrio et al. (2002), Welton et al. (2003), Adorni and Venturelli (2010)
Grazing incident diffraction			√					Benzzi et al. (2008)
LIF/LIDAR/other laser spectroscopy		√			√			Lazic et al. (2003), Ciupiński et al. (2010), Colao et al. (2005), Hällström et al. (2009)

stop or avoid further deterioration and degradation (Welton et al. 2005). In this technique, the sample is ground to a standard thickness (30 μm) (Prieto et al. 2007). Standard thickness gives known colors between crossed polarizers which distinguish different samples and identify them by using reference table. Mineralogical and chemical analysis of the patina confirmed that the composition of patina is similar to that of dolostone composition, and the formation of patina on stone suggests that they have some sort of protective role (Del Barrio et al. 2002). Among different microscopic techniques used in thin-section petrography analysis, fluorescence microscopy has been suggested better over other microscopic techniques. The sample to be visualized using fluorescent microscopy is first impregnated with epoxy resin containing a fluorescent dye which offers visualization (Welton et al. 2005). Welton et al. (2005) demonstrated that pores and cracks on stones surface (degraded by lactic acid excreted by bacteria) can be best visualized using fluorescence rather than in ordinary or polarized light microscopy.

6.2 Mercury intrusion porosimetry (MIP)

This method is used for determination of connected porosity (Pconc), pore size distribution, median pore size (rm) and bulk density (qbulk) of the biodeteriorated stones (Sanchez-Moral et al. 2005; Cámara et al. 2011). In Mercury porosimetry method, mercury is allowed to intrude inside the pores of the material under high pressure. The intruded volume of mercury is dependent upon the pressure applied and it gives structural information about the material. Before analysis the specimen is made granular and all liquids present in it are removed (dried overnight in oven at 1,100°C) (Ruiz-Agudo et al. 2007). As the technique needs specimen analysis under high pressure conditions it become crucial for us to know whether to what extend the specimen under investigation will be affected by high pressure conditions. Under these conditions, some specimen such as in case of plastic, permanent deformation may occur. For that reason the method is considered suitable for analysis of coal, sandstones and plastics. It has been demonstrated that materials with narrow pores which end up in a large cavity are less resistant and more prone to deformation and salt weathering (Welton et al. 2005; Benavente et al. 2004). As the technique is performed at high pressure, therefore it becomes necessarily important to know the behavior of the material undergoing test at high pressure conditions (Welton et al. 2005). Another concern is related to MIP size distribution measurement using Washburn model, which does not provide the actual pore size distribution (Welton et al. 2005). The experimentally obtained pore sizes are often smaller than their actual sizes. Some researchers have preferred considering the threshold diameter and the total intruded volume of mercury as only parameters for comparison with pore structure (Diamond 2000). The method so far has been used successfully for the analysis of composite materials such as cement, pastes, mortars, concrete, swelling damage in sandstone (Sebastián et al. 2008), building materials of historic monuments (Sanchez-Moral et al. 2005), weathered rocks and stones (Cámara et al. 2011), ceramics, corroded concretes etc. (Sarkar et al. 1994).

6.3 Scanning electron microscopy-EDX

Scanning electron microscopy is a microscopic technique which uses a high energy electron beam to scan the surface of a specimen and to collect the necessary images. This technique in conjunction with other microprobes like EDX (Energy Dispersive Detector) etc. has gained great importance in geomicrobiological studies related to biodeterioration (Ascaso et al. 2002; Del Barrio et al. 2002; Carmona et al. 2006). Scanning electron microscopy-EDX is a program for automated morpho-chemical characterization and analysis of elemental particles present on the exposed surface of the stones. It allows us to determine the chemical composition of the sample. Prior to EDX analysis, the sample is ground for XRD characterization and accomplished using conventional Bragg–Brentano method with parafocusing geometry (θ–2θ coupled) which requires very less amount of sample. Comparatively high detection threshold and subsequently the overlapping of the energies of different elements make the detection of all elements impossible. Generally light elements in low concentration are difficult to detect using EDX. For this reason, EDX is considered unsuitable for the detection of trace elements in the pigments (Goldstein et al. 2003). However, EDX analysis confirms the presence of elements but do not provide information regarding their phases. Till date, SEM-EDX has been successfully employed for visualizing crystals of gypsum and ettringite (Welton et al. 2005), effects of secreted acids

etc. (Welton et al. 2005), patina on rock surface (Del Barrio et al. 2002), corrosion product over glass and metal artifacts (Carmona et al. 2006) and metal monuments surface (Chiavari et al. 2007; Robbiola et al. 2008). Besides this, the microbial induced calcium carbonate layer formation (biomineralization) can also be studied using SEM-EDX (Welton et al. 2005; Dick et al. 2006). Although using this technique only small area of the sample can be studied, but a quantitiative information regarding the presence of microorganisms and the microstructural information related to biodeterioration caused by them can be yielded (Ascaso et al. 2002; Welton et al. 2005).

6.4 Fourier transform infrared spectroscopy (FT-IR spectroscopy)

Fourier transform infrared spectroscopy is an analytical technique that gives essential information regarding the structure and chemical bonding of both organic and inorganic materials. For obtaining good visualization and reflection, the cross-section of the surface must be planar (Boon and Asahina 2006). FT-IR spectroscopic analysis can be done using Nicolet Nexus spectrophotometer (Washington, DC, USA) which is coupled with a Nicolet Continuum Fourier transform infrared spectroscopy microscope and equipped with an HgCdTe detector cooled with liquid N2. Spectra were recorded by a Graseby-Specac diamond cell accessory in transmission mode between 4,000 and 700 cm^{-1}. The FT-IR analyses were performed on samples collected both before and after the treatments. The major drawback of the technique is associated with the detection of earth samples having mixed proportion of organic and inorganic matters, where the detection of organic compound is often hampered by the presence of inorganic compounds. As the reflectance and relative concentration of organic matter is low, the peak corresponding to inorganic matters predominates and that of organic are often not sufficiently visualized and signals reduced into heterogenous layers (Herrera et al. 2008). So far, the technique has been used for studying the chromatic alterations caused by fungi (Monte 2003), biogenesis of calcite, vaterite and aragonite crystal by bacteria (Saiz-Jimenez and Laiz 2000), and biosolubilization of stone surface by metabolic acids of bacterial and fungal origin (Herrera et al. 2004). The technique has also been used for studying the fungal biogenesis of oxalate patinas on marble stones (Monte 2003), black crusts collected from the Milan cathedral façade (Candoglia marble) (Toniolo et al. 2009).

6.5 Mössbauer spectrometry

Mössbauer spectrometry is a spectroscopic technique based on recoil-free, resonant absorption and emission of gamma rays in solid. This technique is highly sensitive and can sense subtle changes in the chemical environment of nucleus including changes in the oxidation state. This technique is usually performed following examination of specimen for the presence of oxidation products formed after XRD and FT-IR analysis (Videla and Herrera 2003; Herrera et al. 2003; Welton et al. 2005). It is also used for studying the weathering and biodeterioration effects on monumental stones and artworks (Del Barrio et al. 2002; Herrera and Videla 2009). Del Barrio et al. (2002) used this spectrometric technique in transmission geometry with conventional waveform spectrometer for the analysis of powdered sample (patina) at room temperature. Generally, a 57A Co/Rh source operating at an initial activity of 25mCi is used. The spectra analysis is usually done using a program called MOSF which is based on a non-linear least squares fitting procedure (Welton et al. 2005). The hyperfine parameters determined the nuclear interactions: isomer or chemical shift (IS) and quadrupolar splitting (QS) and using the least square fitting method of NORMOS program of R.A. Brand (Laboratorium fuer Angewandte Physik, Universitaet Duisburg) measured full width at half maximum (G) in mm/s (Del Barrio et al. 2002). Using this technique they demonstrated the explanation of the colored patina and proposed that the color formation is due to incorporation and deposition of dust particles (hydroxides or oxides of metals such as iron) from atmosphere and due to biogenic activity (Del Barrio et al. 2002). The technique has also been used for the studying the weathered peridotite stone (an igneous rock containing >90% Fe and Mg minerals such as olivine and pyroxene) of church of Veracruz in the urban city area of Medellin, Colombia (Welton et al. 2005).

Mössbauer spectrometry offers a complete, non-destructive, in situ and 3-D identification of corrosion product and measure accurately the fraction of each oxides present in corrosion product (Welton et al. 2005). There are two major drawbacks of the technique

are the limited number of gamma ray sources and the requirement that samples to be analyzed be in solid form so as to eliminate the recoil of the nucleus.

6.6 Induction coupled plasma-mass spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive technique based on principles of two highly specialized techniques ICP and MS. When coupled together inductively coupled plasma act as source for producing ion (by ionization) while mass spectrometer separates and detects individual ions. Using this technique a range of elements, both metals and non-metals, can be detected at concentrations below one part in 1,012 (part per trillion). With respect to geomicrobiological studies of deteriorated monuments and artworks, this technique provides a mean for chemical analysis of the sample or specimen (Adorni and Venturelli 2010) such as mortar and stones (Adorni and Venturelli 2010), stone masonry (Welton et al. 2003) and patina (Del Barrio et al. 2002).

6.7 Thermal analysis

Thermal analysis can be performed in two ways: thermogravimetry analysis (TGA) and differential thermal analysis (DTA). DTA may be used for studying mineral phases and organic matters. Analysis of powdered rock and patina is done at heating rate of 20°C/min from room temperature to 10,000°C (Del Barrio et al. 2002; Anastasiou et al. 2006). While techniques such as SEM and XRD are included at initial phase (first phase) of research, mineralogical calculation and thermal analysis are mainly performed later on (second phase of research). Thermal analysis in conjunction with mass spectrometry has been widely used for studying patina and has yielded much information which revealed that upon dehydration of weddellite/whewellite to form calcium oxalate, calcium oxalate transformed into calcium carbonate which in turn form calcium oxide via decomposition of the calcite (Perez-Rodriguez et al. 2011). TGA together with FT-IR was used for chemical composition characterization of plaster from wall paintings (Anastasiou et al. 2006) and for studying chromatic alteration caused by the formation of oxalate patina on carrara marble samples (Monte 2003). DTA/TG and mass spectrometry analyses presented the mechanistic overview of this deterioration mechanism (Perez-Rodriguez et al. 2011). Using DTA/TG with DSC (differential scanning colorimetry) qualitative and quantitative information regarding the biodeterioration caused to monumental stones such as limestone and dolomite can be yielded (Barcina et al. 1997). Additionally, they also detected a higher proportion of organic compounds which they predicted to be formed possibly through the degradation of the preservative products applied to the building materials and surface and claimed that degradation resulted due to microbial action or attack (Perez-Rodriguez et al. 2011). Mass spectrometric studies revealed that carbon monoxide and carbon dioxide gas evolve during the transformation of CaC_2O_4 to $CaCO_3$ (Perez-Rodriguez et al. 2011). TG/DTA studies together accompanied with other have been also applied for physical and chemical characterization of crust and salt efflorescence (Grondona et al. 1997).

6.8 Laser induced fluorescence (LIF) and fluorescence LIDAR

Laser induced fluorescence is a non-invasive, non-destructive, remote sensing technique used applied to studies of ancient frescoes, pottery and monumental stone surface characterized by the presence of atmospheric pollutants and degraded materials (Lazic et al. 2003; Comelli et al. 2004). It has many advantages in sense that it allows visual characterization of the degraded surface without moving the specimen from its natural location. The applications of LIF can be extended by integrating it with a fluorescence lidar system equipped with a scanning device which facilitates collection of larger images (Weibring et al. 2001; Lognoli et al. 2003). Fluorescence lidar is a non-invasive, remote sensing technique (Cecchi et al. 2000) that makes it possible to extend the application of the laser-induced fluorescence technique to the outdoor environment (Weibring et al. 2001; Lognoli et al. 2003). Although initially developed for the investigation of marine environment and vegetation, in the past decade this technique has been successfully included into the field-research related to geomicrobiology of the cultural heritage buildings (Hällström et al. 2009).

6.9 Raman spectroscopy

Use of physical instruments has become a routine procedure in geomicrobiological research in biodeterioration. Raman spectroscopy is one of them. It is

used for studying the rotational, vibrational and other modes of frequencies. The spectra can be obtained from samples in solid, liquid and gaseous form. It offers a quantitative analysis of the sample and gives information about the concentration of different compounds in the sample. The instrument has a microscope (such as Olympus BH2 microscope), gas laser (such as Coherent Innova 70C Argon/Krypton mixed gas laser), liquid nitrogen cooled CCD detector (1,024 × 256 CCD-3000 detector) and a TV camera for image viewing. The integration time may range from few seconds to 1 min for each spectral domain. For enhancing the readability, baseline subtraction and smoothing can also be done on the spectra. Use of Raman spectra for gaining knowledge of the presence of secondary minerals and encrusted salts on stone structure of monuments and artwork is one of the major tools in conservation and management. Raman spectra and other related techniques have been successfully applied for the identification of new mineral neoformation such as sulfates, carbonates, halides, and phosphates on stone surface (Ostrooumov et al. 2008; Edwards et al. 2003). Raman spectra analyses showed the presence of calcium oxalate monohydrate (by *Lecanora sulfurea* and *Aspicilia calcarea*) and dehydrate (*Dirina massiliensis f. sorediata*, *D. massiliensis f. massiliensis* and *Tephromela atra*) in the biomineral product of lichen bioweathering (Edwards et al. 2003). FT-Raman spectroscopy has been used for the characterization of secondary minerals formed by the fungal and lichen species on the stone surfaces (Prieto et al. 1999; Villar et al. 2004). Chiavari et al. (2007) using SEM–EDS and Raman spectroscopy studied the composition of pale green patina over the bronze monuments and confirmed the presence of a complex mixture of crystalline copper hydroxisulphate and of amorphous/nanocrystal Sn-containing compounds. Similar investigation was carried out by Robbiola et al. (2008) on pale green color patina over the surface of outdoor bronze metal monuments, which further confirmed the composition of patina.

7 Conclusion

The geoactive role of microorganism is of great impact in relation to deterioration of ancient monuments and cultural assets. The microbial colonization and certain environmental factors synergistically contribute towards deterioration of these architectural beauties of ancient times. Currently, the conservation of these cultural heritages is one of the many concerns before world. The major step towards conservation and preservation is to characterize the microbial population on the monumental stones before applying any remedial treatment. Besides this, studies related to biogeophysical and biogeochemical alterations brought about by microbial interaction with stone surface are also worth to study so as to devise appropriate conservation methodologies. While the tradition culture based methods provided useful but limited information regarding microbial diversity inhabiting stones, hence molecular biology techniques and other strategies were practiced and in fact their use in geomicrobiological studies unraveled the microbial diversity involved in biodeterioration and helped researchers in understanding the different processes of deterioration such as biogeochemical and biophysical alterations. Besides this, integration of data generated by various techniques may be utilized for designing appropriate conservation regime. For instance, data generated from TG and CA together with MC can be used for identifying the mechanism of deterioration caused to monumental stones and artworks. Similarly data obtained from SEM-EDX, FT-IR, MS, RS and various in situ microscopic techniques may also be used for developing appropriate conservation protocol. Nowadays, a multi-disciplinary approach and the use of two or more techniques in combination are generally practiced to identify the type and cause of biodeterioration. Studies aiming at finding cause-effect correlation between the microbial colonization on stones and resulting deterioration of the surface are in at the forefront of research which presented many models to explain the process of biodeterioration. The correlation between data collected from studies carried out on microbial community structure and on deteriorated building materials and artifacts was used for evaluating the authenticity of the proposed models, and then their use in formulating effective diagnostic and conservative strategy. Besides this, other new emerging techniques such as photogrammetry which prepares 3-dimensional sketches for the restoration projects of architectural heritages are concurrently gaining interest these days. The non-topographic application of photogrammetry in conjunction with new technologies

for data acquisition (CCD cameras, Photo-CD, photoscanners), data processing (computer vision), structuring and representation (CAD, simulation, animation, visualization) and archiving, retrieval and analysis (spatial information systems) has lead to novel systems, processing methods and results that enabled surveying and monitoring of heritage monuments, objects and sites in new fashion (Avşar et al. 2008). We can anticipate that the future research in this direction will generate better predictions regarding the mechanistic overview of the biodeterioration to better safeguard historic architectural and cultural heritages.

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