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Study of Microbial Diversity and their Role in the Deterioration of Tarkeshwar Temple, Jaipur, India

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ABSTRACT

Tarkeshwar Temple is an ancient temple of Lord Shiva in Jaipur, built-in 1784 A.D. Temple is mainly composed of limestone, sand powder and marble. In this article, we discussed microbial diversity and deterioration of the temple surface. In this study, a total of 135 bacterial and 125 fungal colonies were identified among them *Escherichia coli* and, *Aspergillus flavus* most abundant one. Importance value index (IVI) of bacterial species revealed that the *Escherichia coli* show maximum IVI value (88.77%) followed by *Pseudomonas aeruginosa* (77%), *Staphylococcus aureus* (75.55%) and *Staphylococcus haemolyticus* (58.64%) shows least IVI value; and for fungal species, Importance value index (IVI) disclose that *Aspergillus flavus* shows maximum IVI value (84.3%) followed by *Fusarium solani* (68.38%), *Aspergillus niger* (56.98%), *Aspergillus tubingensis* (46.27%) and *Aspergillus fumigation* (35.5%) shows least IVI value. The degenerative potential of isolated bacteria and fungi with the help of FE-SEM was also analysed. This study helps to find the culturable biodeteriogens mainly bacteria and fungi which excreted most of the enzymes, acids and pigments to deteriorate the site and appearance. This study also helps to plan a strategy to maintain this heritage site and for providing a healthy environment for people who come to visit for prayers and admire the beauty of the ancient temple.

Key words*: Biodeterioration, Bacteria, Fungi, FE-SEM Tarkeshwar temple.*

Introduction

Deterioration is an essential process in environmental cycling of matter but this may lead to loss of valuable cultural heritage. The deterioration classified as chemical, physical and biological but the biological deterioration plays a considerable role in cultural heritages. The deterioration caused by biological means is known as biodeterioration (Hueck, 1965). Biodeterioration studied for a long time to a better understanding of cultural heritage deteriorated conditions for their maintenance and longevity. An environmental factor affects the microbes associated with heritage site (Caneva and Salvadori, 1988). The material of heritage provides nutrient by degrading the fossil fuel derivatives, to the microbes for their survival (Gonzalesdel Valle *et al.*, 2003). The presence of microbes and complex biofilms on heritage sites accelerate decay (Uchida *et al.,* 2000). Microbes produced various organic, inorganic acids, chelating compounds, extracellular polymers and pigments due to metabolic activity create pressure to the surface results physical and chemical changes to cultural heritage. Fissures, cracking, exfoliation, desquamation and other alteration processes occur due to these various metabolic products produced by microbes (Sterflinger *et al.,* 1996; Gorbushina *et al.,* 1998). Endolithic microorganism colonizing to the interior part of porous stone (Walker and Pace, 2007) and utilize light, moisture, and shelter found

inside the stone (Caneva *et al*., 2008) and may also modify their surroundings of that shelter (McNamara *et al.* 2006). Bacteria often generate slime or extracellular polymeric substances (EPS) as part of a complex biofilm which causes change in pit morphology and dissolution rate of limestone (Perry *et al.* 2004). The filamentous structures of fungal hyphae favour their penetration into the monuments surface cause mechanical damage, with the expansion and contraction of the thallus. By taking advantage of penetration give a more favourable habitat for other organisms. Intact minerals can also perforate by fungi (Gadd, 2007). Jaipur is a world-famous heritage city of Rajasthan, India mostly known for its ancient forts and monuments. In Jaipur, there is an ancient temple namely Tarkeshwar Temple (fig.1) which is culturally very important and shows the tradition and legacy. Temple is mainly composed of lime stone, sand powder and marble. Tarkeshwar Temple is dedicated to Shankar Bhagwan "lord Shiva". Tarkeshwar Mandir was built in 1784 A.D. and black stone were used to make Shivling of Tarkeshwar Mahadev having a 9" diameter. The highlights of the temple is a Nandi which is made up of brass, Golden pictographs, marble floor and huge gongs and bells which were also made up of bronze. It's a situated in Chauda Rasta, Jaipur. By the side of the chowk is the Jagmohan (Assembly) hall. In this hall, four bronze gongs are hanging each weight of approximately 125 kilograms. The Ganesh Ji idols as shown here seated and has left turned trunk. In this article, identify the culturable biodeteriogens mainly bacteria and fungi which might be responsible for biodeterioration of Tarkeshwar temple of Jaipur which excreted most of the enzymes, acids and pigments to deteriorate the site and appearance. With the help of these identified bacteria and fungi, we can form a strategy to remove these microbes and helps to conserve and maintain the temple.

Fig. 1. Tarkeshwar temple of Jaipur.

Materials and Methods

Sampling

Sample collection was done from Tarkeshwar Temple under aseptic conditions (Fig.2), without harming the site in the month of November. The weather of Jaipur is semi-arid type. The average temperature of November month is maximum-29°C, and minimum-13 °C and the average annual rainfall is 601 mm (23.7 inch). From Tarkeshwar Temple ten samples (entrance, Ganesh Ji's idol, Bharonath Ji's idol, Shivling Gate, Shivling Surrounding, Shivling, Mata Mandir, 400yr Old Mata Murti, Mata Mandir Gate and shila) were collected from deteriorated sites and idols with the help of sterilized cotton swab (Gorbushina *et al.*, 2004) and scrapping off with a sterilized scalpel and collected in a sterile plastic airtight pouch for its microbial analysis. The adequate amount of sample was collected aseptically with sterilize tools.

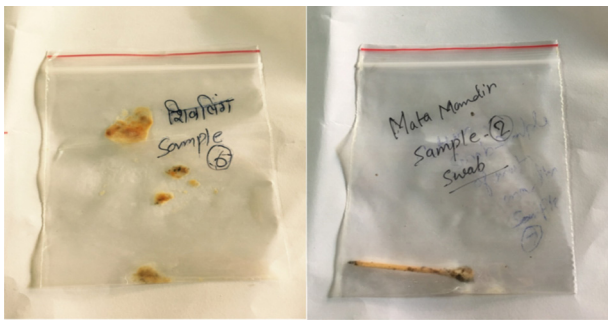

Fig. 2. samples were taken from different sites of Tarkeshwar Temple.

Cultivation of bacteria and fungi

The inoculated swab samples were suspended in labelled plain bottles containing 4 ml of peptone water (pH 7.0) to dislodge microbial cells from the swab sticks. Subsequently, an aliquot (0.1 ml) of the suspension was inoculated on respective media for bacteria and fungi (Obidi, and Okekunjo, 2017). The sterile spreader was used to spread the inoculum entirely on the plates. For fungi, samples were cultured on Potato Dextrose Agar (PDA) media (Potatoes, 200g, Dextrose 20g, Agar 15g, distilled water 1000 ml at pH (at 25 °C) 5.6±0.2) and Czapek–Dox agar plates (30g sucrose, 1g K2HPO4, 0.5g MgSO4.7H2O, 0.5g KC, 0.01g FeSO4, 15g agar, distilled water 1000

ml at pH 7.3) containing chloramphenicol (500 mg/l) to control the bacterial growth in the same and for the bacteria, nutrient agar plates (5g peptone, 3g beef extract, 5g NaCl, 20g agar in 1L distilled water, pH7–7.1) having Fluconazole (1 mg/ml) as an antifungal agent were used. The fungi and bacteria plates were incubated at 28 ± 1°C for 3-5days, 37 °C for 24 h respectively (Rojas *et al.* 2012). At the end of the incubation period, well defined, pigmented colonies that developed were counted, routinely sub-cultured on fresh plates to purify and identified. The cultures were maintained on fresh agar medium and stored at 4 °C for further use. All tests were carried out in duplicates.

Morphological and microscopic observation of bacteria and fungi

To determine colony size, shape, elevation and pigmentation morphological studies were carried out (Cheesbrough, 2008). Pigment production was established by observation of coloration on nutrient agar and potato dextrose agar after incubation (Rojas *et al.* 2012). Microscopic observations to determine microbial viability were made using an optical microscope (Fig. 3). The isolated bacterium was identiûed according to Bergey's manual of systematic bacteriology (Krieg and Holt, 1984). Fungal isolates were identiûed morphologically according to authentic literature reviews and mycological manuals (Raper and Fennell, 1965; Ellis and Ellis, 1997; Samson *et al.* 2004, 2010).

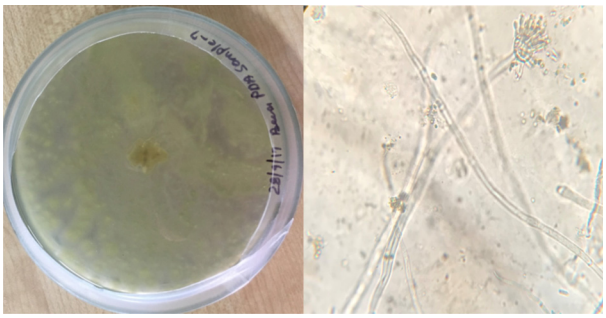

Fig. 3. Culuture plate and Microscopic view (400x) of *Aspergillus flavus.*

Biochemical identification tests

Pure, morphologically different bacterial colonies obtained from the incubated plates were initially evaluated by conventional Biochemical tests (table-1,2) such as Gram stain, catalase, oxidase, Coagulase, motility, indole production, MRVP and urease activity. Additional tests included nitrate reduction, citrate utilization, H2S production, Starch Hydrolysis (Cheesbrough, 2008). Purified fungal isolates were subsequently examined by a light microscope as described by Harrigan and Mc Cance (1976). Thus, all the isolates were identified to the genus level based on their morphology and sporing structures.1

Table 1. Result shows staining and motility test of isolated bacteria.

Isolates	Gram Reaction	Motility	Shape
TGM1	+ve	Motile	Rod
TBM2	-ve	Motile	Rod
TSG3	+ve	Non-Motile	Cocci
TSS4	-ve	Motile	Rod
TMS5	+ve	Non-Motile	Cocci
TMM6	-ve	Motile	Rod
TOM7	+ve	Non-Motile	Cocci
TMG8	+ve	Motile	Rod
TMS9	+ve	Non-Motile	Cocci
TME10	+ve	Non-Motile	Cocci

Table 2. Biochemical tests of isolated bacteria.

Isolates	Catalase	Oxidase	Coagulase	Citrate Utilization	Indole	Urease	MR (Methyl Red)	VP (Voges Proskauer)	H, S Production	Nitrate Reduction	Starch Hydrolysis
TGM1	+	+	-	+	-	-	-	+	-	+	+
TBM2	+	+	-	+	-	-	-	-	-	+	-
TSG3	+	-	+	+	-	+	+	+	-	+	-
TSS4	+	-	-	-	+	-	+	-	-	+	-
TMS5	-	-	-	-	-	-	+	-	-	-	+
TMM6	+	+	-	+	-	-	-	-	-	+	-
TOM7	+	+	-	+	-	+	+	-	-	+	-
TMG8	+	+	-	+	-	-	-	+	-	+	+
TMS9	-	-	-	-	-	-	+	-	-	-	+
TME10	+	-	-	+	-	+	+	+	-	+	-

(+) = presence; (-) = absence

Molecular Characterization of Bacteria and fungi

From bacterial and fungal samples, isolation of DNA was done by using the EXpure Microbial DNA isolation kit. For bacteria, amplification of 16S rRNA was done by using Forward Primer 27F (5' AGAGTTTGATCMTGGCTCAG 3') and Reverse Primer 1492R (5' TACGGYTACCTTGTTACGACTT 3') and for fungi, amplification of 18S rRNA was done by using Internal Transcribed Spacer (ITS) primers consisted of ITS-1 (52 -TCC GTA GGT GAA CCT GCG G-32) and ITS-4 (52 -TCC TCC GCT TAT TGA TAT GC-32). Amplified genomic DNA was purified, by using the Montage PCR Clean up kit (Millipore) and to get partial sequences 16S/18S rRNA gene the purified products were sequenced commercially (Yaaz Xenomics, Coimbatore, India). Then sequence was blast using NCBI (National Centre for Biotechnology Information; http://www.ncbi.nih.gov/) blast similarity search tool. Phylogenetic tree construction was performed (Fig. 4,5) using the program Tree Dyn 198.3 (Dereeper *et al*., 2008).

Analysis of stone sample deterioration by mixed culture isolates

An experimental set up was done to test the degenerative potential of dominant microbial isolates from Tarkeshwar Temple. A stone piece was collected from the same environment and wash and autoclave it for 121 °C (250 °F) at 100 kPa (15 psi) above atmospheric pressure for 15 minutes for sterilization (Wiktor *et al.*, 2009; Miller *et al.*, 2008) and for the record of the condition of the sample, FE-SEM images were taken. FE-SEM allows the surface characterization of materials and depth observation of field at a higher resolution with the help of conventional optical microscope. Prepared mixed inoculums by mixing bacterial and fungal pure colonies (mainly *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus haemolyticus*, *Staphylococcus aureus* and *Fusarium solani*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigation*, *Aspergillus tubingensis*) into 0.9% saline (0.5 McFarland). In a pre-sterilized container; the stone piece was placed and sprinkles the sterile water to maintain the moisture. And then the prepared mixed inoculums poured on the stone piece and closed the lid of that container (Miller *et al.*, 2008). This container was placed under natural environment. After every 24 hr sprinkle the sterile water to the stone piece aseptically. After every 6 months up to 2 years the degenerative potential of the sample was identified with the help of a Field emission scanning electron microscope (FE-SEM).

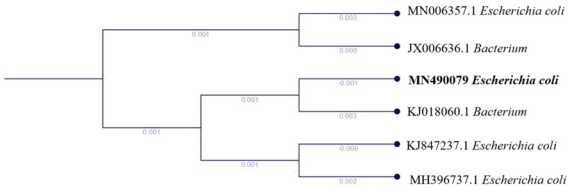

Fig. 4. Phylogenetic trees of TSS4

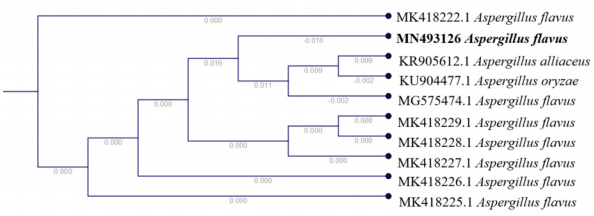

Fig. 5. Phylogenetic trees of TSG3

Results

Isolation of Bacterial and fungal Isolates

From 10 samples several bacterial and fungal colonies were recovered but the most frequent colonies which were present on all the samples were processed for further study. Total of 135 bacterial and 125 fungal colonies were identified from which dominated bacterial genera were *Escherichia*, *Pseudomonas*, *Bacillus*, *Acidobacter*, *Rhodobacter*, *Micrococcus*, *Staphylococcus and Streptococcus* and fungal genera *Penicillium*, *Aspergillus*, *Fusarium*, *Rhizopus*, *Trichoderma*, *Mucor*, and *Cladosporium*. The isolated bacterial species (Table 4) were *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus haemolyticus*, and *Staphylococcus aureus*, and isolated species of fungi (Table 5) were *Fusarium solani*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigation*, and *Aspergillus tubingensis* selected on the basis of dominance.

Molecular Characterization of the Isolates

The nucleotide sequences which were isolated from Tarkeshwar Temple deposited in the GenBank (NCBI database) and accession numbers regenerated (Table 3). These are:

Table 3. Accession number of samples sequence collected

Analysis of stone sample deterioration by FE-SEM

The degenerative potential of the sample was identified with the help of a Field emission scanning

Table 4. RF, RD, RA and IVI of different Bacterial species into Tarkeshwar Temple.

Isolated Bacteria	Number of Bacteria colonies										RF %	RD %	RA %	IVI
	TGM1	TBM2	TSG3	TSS4	TMS5	TMM6	TOM7	TMG8	TMS9	TME10				
Pseudomonas aeruginosa	-	1	6	2	4	5	3	-	5	9	25	25.92	26.08	**77**
Escherichia coli	4	3	2	5	3	6	5	4	3	8	31.25	31.85	25.67	**88.77**
Staphylococcus haemolyticus	5	6	2	-	1	5	-	-	4	-	18.75	17.03	22.86	**58.64**
Staphylococcus aureus	4	6	-	3	5	8	2	1	-	5	25	25.18	25.37	**75.55**

Table 5. RF, RD, RA and IVI of different fungal species into Tarkeshwar Temple

Isolated Fungi	Number of Fungi colonies										RF %	RD %	RA %	IVI
	TGM1	TBM2	TSG3	TSS4	TMS5	TMM6	TOM7	TMG8	TMS9	TME10				
Fusarium solani	-	5	6	2	-	7	5	-	-	4	16.21	23.2	28.97	**68.38**
Aspergillus flavus	4	6	3	8	5	6	2	9	3	1	27.02	37.6	28.19	**92.81**
Aspergillus niger	3	2	-	-	4	-	6	2	1	5	18.91	18.4	19.67	**56.98**
Aspergillus fumigation	-	2	1	3	-	1	-	-	-	4	13.51	8.8	13.19	**35.5**
Aspergillus tubingensis	2	1	3	1	1	-	1	2	1	3	24.32	12	9.95	**46.27**

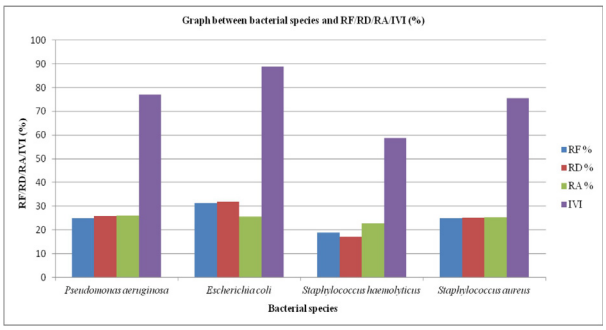

Fig. 6. Graphical representation of bacterial species and RF/RD/RA/IVI (%).

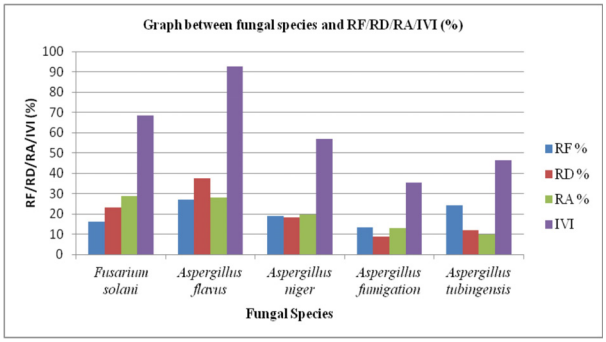

Fig. 7. Graphical representation of fungal species and RF/RD/RA/IVI (%).

electron microscope (FE-SEM). The difference between the before and after images of stone piece which were tested for the degenerative potential of isolated bacterial and fungal species, with the assistance of FE-SEM images (Fig. 8). The before FE-SEM image shows there was no visible deterioration as such but in the after FE-SEM image demonstrate the microbial growth on that stone piece reasons for damage. Damage can be aesthetical, mechanical and physical. The result described that bacteria and fungi are capable of degrading the material of heritage sites. Bacterial growth in the images (Fig. 8b-d) shows, biofilm formation because of *Escherichia coli* and *Pseudomonas aeruginosa* are having the potential to form a biofilm (Laverty *et al.*, 2014). Further, they provide the nutrient or perfect growth environment for fungi which can cause further deterioration. After FE-SEM image (Fig. 8) reveal the potential mechanical impact of biofilms on stone piece. FE-SEM images (Fig.8 b-d) showed the pit formation (with pointed arrow), hyphae penetration (with arrow), some rod and cocci colonies of bacteria and all over the growth of different microbes. The biofilms on stone piece has two actions physical and chemical. Due to microbial adhesion and penetration of the substrate, erosion and the breaking of surface layers

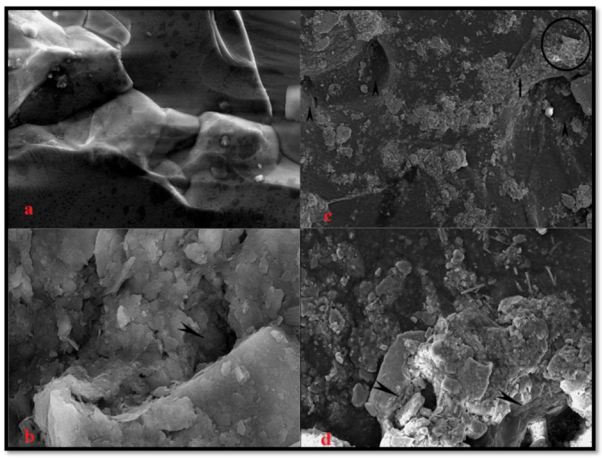

Fig. 8. FE-SEM image (a-d) of biodeterioration of stone piece by mixed inoculums (bacteria and fungi isolates) before (a) and after (b-d).

happen as physical actions and due to metabolic products and other substances produced by microorganisms dissolution and chelating processes occurs as chemical actions (Koestler, 2000; Hirsch, *et al.*, 1995). The chemical and physical changes take place simultaneously (Ascaso *et al*., 2002).

Discussion

During the screening of Tarkeshwar Temple, a total of 135 bacterial and 125 fungal colonies from which four bacterial species and five fungal dominated species were isolated. The composite results indicate that in all the ten (10-10) samples of bacteria and fungi each was mainly dominated by *Escherichia coli* and *Aspergillus flavus* respectively due to their high percentage relative values. The frequency and relative frequency of microorganism are directly or indirectly correlated with meteorological data and climatic conditions (Chandel, 1990). Study of importance value index of a species in the community provides idea of the relative importance. Importance value index (IVI) of bacterial species (Table 4) revealed that the *Escherichia coli* show maximum IVI value (88.77%) followed by *Pseudomonas aeruginosa* (77%), *Staphylococcus aureus* (75.55%) and *Staphylococcus haemolyticus* (58.64%) shows least IVI value; and for fungal species, Importance value index (IVI) disclose (Table 5) that *Aspergillus flavus* shows maximum IVI value (84.3%) followed by Bacteria can also cause severe problems like the excreted metabolic products such as organic and inorganic acids and exoenzyme such as coagulase, amylase, cellulases, etc. are responsible for the hydrolysis of material (Fig. 8) (Rölleke, 1996; Schabereitner-Gurtner, 2000). Due to deterioration of cultural heritage leading effects are stone dissolution, pigmentation or colour alteration, surface alterations, biocorrosion and transformations into smaller sized crystals, etc. (Chand and Cameotra, 2011). Bacteria also used the material components as a substrate for their metabolism. *Aspergillus* was able to solubilize powdered stone and chelate various minerals in a rich glucose medium because they produce organic acids such as gluconic, citric, and oxalic acids (Lapidi and Schipa, 1973). Fungal hyphae penetrate deeply into the material of heritage site (Fig. 8c arrow)and release extracellular enzymes, resulting in aesthetic deterioration and mechanical disintegration due to material loss, pigmentation, contamination, acid corrosion, and enzymatic degradation (Ettenauer *et al*. 2010; Sterflinger, 2010). Isolated bacteria and fungi both were responsible for health risk also some of them are pathogenic in nature such as *E.coli*, *Pseudomonas aeruginosa*, *Aspergillus flavus*. These cultural heritages attract a bunch of tourists; hence it is an important aspect of health and maintenance of cultural heritage. Due to health and socio-economic regions, this is an area of interest for restoring and maintenance (Górny and Dutkiewicz, 2002).

Conclusion

In this work, we have reported the various microbial strains that contribute markedly to the discoloration and biodeterioration of Tarkeshwar Temple, Jaipur. The role of the isolated strains in the observed discoloration which was clearly visible to the naked eye has been facilitated by virtue of their ability to produce pigments and deterioration which can be aesthetic or physical done due to excretion of enzymes, penetration of hyphae, biofilm formation. This study identified the microbial communities present on discoloured painted buildings and provides data for a more detailed study of the ecology and physiology of these groups of organism on the cultural heritage of Jaipur.

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