PATNA WOMEN'S COLLEGE 1940

IRIS
Journal for Young Scientists
ISSN 2278 – 618X **(Print)**
ISSN 2278 – 6384 **(Online)**

http://www.patnawomenscollege.in/journal

Isolation, biochemical characterization and antibiotic susceptibility pattern of bacterial isolates from heritage sites

• **Rakshita Govind** • **Swati Shubham**
• **Jaya Philip**

Received : November 2013
Accepted : March 2014
Corresponding Author : Jaya Philip

Abstract : *Ashoka Pillars, Patna, Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan sites of Patna are being rapidly deteriorated by various mechanical, chemical and biological deteriorating agents, which need special attention for protection. In the present study an attempt has been taken to isolate and identify some of the bacteria which probably can be involved in bio-deterioration. Further, the sensitivity of the bacterial isolates against different antibiotics has been tested. The obtained result could be helpful in application of antibiotics on monuments to check their growth and thus preventing the monuments from bio-deterioration to some extent.*

Key Words: *Ashoka Pillar, Tomb of Maner, Biodeterioration, Antibiotic susceptibiltity.*

Rakshita Govind
B.Sc. III year, Industrial Microbiology (Hons.),
Session : 2011-2014, Patna Women's College,
Patna University, Patna, Bihar, India

Swati Shubham
B.Sc. III year, Industrial Microbiology (Hons.),
Session : 2011-2014, Patna Women's College,
Patna University, Patna, Bihar, India

Jaya Philip
Assistant Professor, Deptt. of Industrial Microbiology,
Patna Women's College, Bailey Road,
Patna – 800 001, Bihar, India.
E-mail : jayaphilipmicrobio@gmail.com

Introduction:

Biodeterioration is the damage that is caused to any materialistic goods by any living organism right from microbes to mammals. This destruction causes economical loss, cultural as well as artistic losses of heritages, monuments, paintings etc. The world cultural heritages either in the form of mural paintings, stone artifacts or pre historic rock arts are usually under such environment which favours the growth of biodeterioration agents. Considering the microorganism, bacteria is supposed to be one of the major biodeteriorative agent but the exact mechanism of their biodeterioration is still under research.

Harmful microbial activities have generally been noticed on such rock based surfaces that are generally wet or exist in humid conditions. Their presence could be confirmed by the surface decolourization on any rocky substances, due to the alteration in the chemical composition that they bring on it. Bacteria usually damage stone by producing acids from their cell wall, which react with stone surfaces and form crusts, dissolve cations in the stone and stain the surface (Kumar and Kumar, 1999).

Many times biofilms get developed on the external walls of buildings depending upon the environmental conditions and physiochemical properties of the materials. Some investigations have begun to elucidate the essential role of biological agents that play in the deterioration of stones (Bock and Sand, 1993). It is also clear that many physical, chemical and biological factors act in both synergistic and antagonistic association to affect the durability of material (Koestler *et al.,* 1994).

The colourization of external surfaces of buildings by microorganisms causes the well known aesthetically unacceptable appearance of staining of the stone surfaces by biogenic pigments and the production of extra cellular polymeric substances that causes mechanical stresses to the mineral structure due to shrinking and swelling cycles of these colloidal biogenic slimes inside the pore system (Dornieden *et al.* 2000). This can lead to the alteration of pore size and distribution, together with changes in moisture circulation patterns and temperature response (Garty, 1991). Microorganisms may also alter the water permeability of the minerals by the deposition of surfactants (Gaylarde and Morton, 1999). It has also been shown that the early presence of biofilms on exposed stone surfaces accelerates the accumulation of atmospheric pollutants.

Thus the aim of the work was to assses the involvement of isolated bacteria in the biodeterioration of Ashokan Pillar and Tomb of Maner, India. Further, studying the biochemical and antibiotic susceptibility pattern for establishing the control measures.

Materials and Methods :

Study sites : The Ashoka pillar at Kumhrar, Patna and Tomb of Shah Makhdum Makhdum Daulat and Ibrahim Khan at Maner (Fig. 1) were the two sites having historical as well as archaeological importance located in Patna, the capital city of Bihar. The tomb and the pillar are the major tourists' spots of Patna. Various biological and physical deteriorative agents have already damaged the sites resulting the crust formation over the surfaces which fall abruptly and hence causing rapid deterioration.

(a) (b)

Fig. 1. Site of Sample collection (a) Ashoka Pillar, Kumhrar and (b)Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan at Maner, Patna

Isolation and Identification : With the help of sterile spatula the crusts of the pillar and the tomb were picked and kept in different sterile vials. Similarly the soil samples of nearby environment of the pillar and tomb were kept in different sterile vials using sterile spatula (permission was procured from Archaeological Survey of India (ASI), Patna Circle, Patna for sample collection). The samples were brought to the laboratory and processing was started within two to three hours of arrival.

The isolation of bacteria was carried out using spread plate technique for which serial dilution (Aneja, 2003) was performed by dissolving 100 mg of sample in 9.9 ml of normal saline from which 1 ml was transferred in 9 ml normal saline. The process was repeated upto 10^{-8} dilution. One ml of 10^{-8} dilution was taken and poured in pre-prepared nutrient agar plates (Peptone 5g; beef extract 3g; NaCl 5g; agar 20g; distilled water 100 ml; pH 7.2) and uniformly spread. The whole process was

carried out in sterile environment. The plates were incubated in incubator (Hicon) at 37°C for 24 hours. After incubation the selected colonies were streaked on nutrient agar slant under sterile condition and incubated at 37°C for 24 hours for getting the pure culture.

The obtained colonies were characterised for cultural characteristic (on the basis of colour, margin, texture, elevation and opacity of the colony), morphological characteristics (Gram's staining was done and slides were observed in light microscope, make Merck under 10X and 40X magnification so as to characterise the isolates on the basis of their Gram's reaction) and different biochemical tests were performed (catalase test, indol production test, methyl red, voges proskauer tests, citrate utilization test, hydrogen sulphide production test, urease test, nitrate production test, starch hydrolysis test, sugar fermentation test, gelatin liquefaction test).

Finally, bacteria were identified using the Advanced Bacteriological Identification Software (ABIS online).

Antibiotic susceptibility test : Antibiotic susceptibility test was performed to detect the susceptibility of isolates against the panel of antibiotics by disk diffusion method (Bauer *et al* 1996). Twelve antibiotic discs *i.e* Nalidixin acid (Na30), Nitrofurantoin (Nf300), Cephalothin (Ch30), Ampicillin (A25), Co-Trimoxazole (Co25), Norfloxacin (Nx10), Oxytetracyclin (O30), Cefuroxime (Cu30), Amoxyclav (Ac30), Gentamicin (G10), Tetracycline (T), Erythromycin (E) were tested against the isolates. Cotton Swab was prepared by taking the sticks and smoothened with the help of knife. Thereafter, cotton was wrapped on the stick and fixed properly. A sterile cotton swab was dipped into the nutrient broth and rotated firmly against the upper inside wall of the tube. The inoculum was swabbed uniformly on Mueller Hinton (Hi Media) agar plates under aseptic conditions. The inoculum was allowed to dry for 10 minutes with the lid in place. The antibiotic disc was impregnated on the surface of the plates using sterile forceps. The plates were then incubated at 37°C for 24 hours in incubator. The zones of inhibition were measured in millimeters.

Results and Discussion :

A total of 14 bacterial colonies were obtained on the Nutrient Agar plate from site Ashoka Pillar, Kumharar. Out of the 14 isolates 2 isolates were randomly selected for further characterization. The isolates were named as K I and K II. A total of 17 bacterial colonies was obtained on the Nutrient Agar plate from site Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan at Maner, Patna. Out of which 2 isolates were selected for further characterization and were named as M III and M IV.

Cultural Characteristics: The cultural characteristics of the isolates obtained on Nutrient Agar were studied. The cultures were classified on the basis of colour, shape, texture, margin, elevation and opacity as shown in Table 1.

Table 1. Cultural characteristics of bacterial isolates from sites Ashoka Pillar, Kumhrar and Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan at Maner, Patna.

S. No.	Characteristics	K-I	K – II	M–III	M–IV
1.	Colour	Greyish white	Greyish white	Gray yellow	Greenish
2.	Texture	Glossy	Glossy	Granular	Smooth
3.	Margin	Smooth	Undulate	Smooth	Smooth
4.	Shape	Small round	Round	Round	Spreaded
5.	Elevation	Flat	Convex	Flat	Convex
6.	Opacity	Opaque	Translucent	Opaque	Opaque

Gram Reaction and Morphological Characteristics: The Gram's Reaction and morphological characteristics of bacterial isolates were studied by preparing slide and observing them under 10X and 40X magnification of light microscope. Both Gram positive and negative rods and cocci were isolated as shown in Table 2.

Table 2. Gram reaction and morphological characteristics of bacterial isolates from sites Ashoka pillar, Kumhrar and Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan at Maner, Patna

S. No.	Gram's Reaction	Shape	Arrangement
K I	+ve	Cocci	Cluster
K II	-ve	Rods	Single
M III	+ve	Rods	Diplobacillus
M IV	-ve	Rods	Single

Biochemical characterisation: Based on the various biochemical test (Table 3), the isolates from Kumharar were identified as *Staphylococus aureus* (75 % as identified by ABIS online) and *Clostridium colicanis* (77% as identified by ABIS online).The isolates from Maner were identified as *Bacillus cereus* (77% as identified by ABIS online) and *Pseudomonas fluorescens* (77 % as identified by ABIS online).

The role of bacteria in the weathering of rock probably depends largely on the environmental conditions. While bacteria might evolve in humid environments and form biofilms within the porous space of building stone, in arid and semi arid environments their occurrence might be limited. Members of the *Actinobacteria* phylum inhabit stone more effectively than most of the single-celled bacteria. This fact can be attributed to their filamentous growth and also to their effective utilization of various nitrogen and carbon sources (Saarela *et al*. 2004). Heterotrophic bacteria include a variety of genera such as *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Flavobacterium*, *Pseudomonas*, *Micrococcus*, *Staphylococcus*, *Nocardia*, *Mycobacterium*, *Streptomyces* and *Sarcina*, which are the species most frequently isolated from wall paintings (Bassi *et al*. 1986; Ciferri 1999; Heyrman *et al*. 1999; Palla *et al*. 2002; Pangallo *et al.* 2012; Suihko *et al.* 2007)

Table 3. Biochemical characteristics of bacterial isolates from the sites Ashoka Pillar, Kumhrar and Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan at Maner, Patna

S.No.	Tests	K-I		K-II		M-III		M-IV	
1.	Catalase Test	-ve		-ve		-ve		-ve	
2.	Nitrate Production	+ve		+ve		+ve		+ve	
3.	H_2S Production	-ve		+ve		-ve		+ve	
4.	Indol Production	-ve		-ve		-ve		-ve	
5.	Methyl red	-ve		-ve		+ve		+ve	
6.	Voges-Proskaur	+ve		+ve		+ve		-ve	
7.	Citrate Utilization	+ve		+ve		+ve		+ve	
8.	Urease Test	-ve		+ve		+ve		-ve	
9.	Starch Hydrolysis	+ve		+ve		+ve		-ve	
10.	Gelatin Liquifaction	+ve		-ve		-ve		+ve	
11.	Fermentation Test	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
	a) Lactose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	b) Sucrose	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
	c) Dextrose	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve

Antibiotic Susceptibility Pattern: The antibiotics for which both the isolates from Kumrar, K-I and K-II were sensitive are Norfloxacin, Amoxyclav and Gentamicin. Although the isolate K-II was resistant to Nalidixic acid, K-I was highly sensitive.The antibiotics for which both the isolates from Maner, M-III and M-IV were sensitive were Norfloxacin, Tetracycline and Gentamicin. The antibiotic susceptibility pattern obtained for the selected cultures are tabulated below in Table 4 and 5.

Table 4. Antibiotic susceptibility profile of bacterial isolates from sites Ashoka Pillar, Kumhrar

S.No.	Antibiotics	K-1		K-2	
		Zone in mm	R/S/I	Zone in mm	R/S/I
1.	Nalidixin acid	20mm	S	12mm	R
2.	Nitrofurantoin	_	R	10mm	S
3.	Cephalothin	_	R	_	R
4.	Ampicillin	14mm	I	_	R
5.	Co-Trimoxazole	30mm	S	15mm	I
6.	Norfloxacin	25mm	S	36mm	S
7.	Oxytetracyclin	20mm	_	12mm	_
8.	Cefuroxime	16mm	R	13mm	R
9.	Amoxyclav	18mm	S	20mm	S
10.	Gentamicin	21mm	S	22mm	S
11.	Tetracycline	_	R	15mm	S
12.	Erythromycin	_	R	18mm	I

Table 5. Antibiotic susceptibility pattern of bacterial isolates from sites Tomb of Shah Makhdum Daulat Maneri and Ibrahim Khan at Maner, Patna.

S.No.	Antibiotics	M-III		M-IV	
		Zone in mm	R/S/I	Zone in mm	R/S/I
1	Nalidixin acid	20mm	S	13mm	R
2	Nitrofurantoin	_	R	16mm	I
3	Cephalothin	_	R	_	R
4	Ampicillin	_	R	_	R
5	Co-Trimoxazole	_	R	16mm	S
6	Norfloxacin	32mm	S	26mm	S
7	Oxytetracyclin	13mm	_	12mm	_
8	Cefuroxime	_	_	13mm	R
9	Amoxyclav	_	R	_	R
10	Gentamicin	18mm	S	16mm	S
11	Tetracycline	15mm	S	13mm	I
12	Erythromycin	24mm	S	10mm	R

The two heritage sites under study are made of stone. The bacteria attack the stone under aerobic condition by producing acids. Thus, Norfloxacin, Tetracycline and Gentamicin may be used as antimicrobial agents for the disinfection of the stone to prevent deterioration.

However, besides the compatibility with the materials of the treated artefacts, the most challenging aspect of antibiotic treatments is the fact that, in many cases, monuments are infested by a mixed community of microorganisms with different levels of susceptibility towards the chemical compound applied. It might therefore exert a selective pressure on the microbial community and, in the worst case, the community may be turned into one that is less sensitive or even resistant to the antimicrobial, and might become even more harmful to the object.

Acknowledgements :

This work was conducted under the permission of Superintending Archaeologist, Mr M. S. Chauhan. Authors are thankful to Dr.Sister Doris D'Souza, Principal, Patna Women's College , Patna University for giving an opportunity to do this research work and for the facilities provided in carrying out this work.

References :

Aneja KR (2003). Experiments in Microbiology, Plant Pathology and Biotechnology. Fourth edition. New age International Limited Publishers, New Delhi, p.607.

Bassi M, Ferrari A, Realini M, Sorlini C. (1986) Red stains on the Certosa of Pavia a case of biodeterioration. *Int Biodeterior Biodegrad.* 22 :201–205.

Bauer, AW, Kirby WMN, Sherris JC and Turk M (1996). Antibiotic Susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45:493-496.

Bock, E and Sand, W (1993) The microbiology of masonry biodeterioration. *J Appl Bacteriol* 74: 503–514.

Ciferri O. (1999) Microbial degradation of painting. *Appl Environ Microbiol*.;65:879–885.

Dornieden, TH, Gorbushina, AA, Krumbein, WE (2000). Patina—physical and chemical interactions of sub-aerial biofilms with objects of art. In: Ciferri, O, Tiano, P, Mastromei, G (Eds.) of Microbes and Art: The Role of Microbial Communities in the Degradation and Protection of Cultural Heritage, Kluwer Academic, Dordrecht, pp 105–119.

Garty, J (1991) Influence of epilithic microorganism on the surface temperature of building walls. *Can J Bot* 68: 1349–1353.

Gaylarde, CC, Morton, LHG (1999) Deteriogenic biofilms on buildings and their control: a review. *Biofouling* 14: 59–74.

Heyrman J, Merga J, Deny R, Swings J. (1999) The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by microorganisms. *FEMS Microbiol Lett.* 181:55–62.

Koestler, RJ, Brimblecombe, P, Camuffo, D, Ginell, W, Graedel, T, Leavengood, P, Petushkova, J, Steiger, M, Urzi, C, Verges-Belmin, V, Warscheid, TH (1994) Group report: How do external environmental factors accelerate change? In: Krumbein, WK, Brimblecombe, P, Cosgrove, DE, Staniforth, S (Eds.) Durability and Change, Wiley, Chichester, UK, pp 149–163.

Kumar, R and Kumar AV (1999). Biodeterioration of Stone in tropical environments. USA : *J. Paul Getty Trust*, pp.1-2.

Palla F, Federico C, Russo R, Anello L (2002). Identification of *Nocardia restricta* in biodegraded sandstone monuments by PCR and nested-PCR DNA amplification. *FEMS Microbiol Ecol.* 39:85–89.

Pangallo D, Krakova L, Chovanova K, Simonovicova A, de Leo F, Urzi C. (2012) Analysis and comparison of the microflora isolated from fresco surface and from surrounding air environment through molecular and biodegradative assays. *World J Microbiol Biotechnol.* 28:2015–2027.

Saarela M, Alakomi HL, Suihko ML, Maunuksela L, Raaska L, Mattila-Sandholm T (2004). Heterotrophic microorganisms in air and biofilm samples from Roman catacombs, with special emphasis on actinobacteria and fungi. *Int Biodeterior Biodegrad*. 54:27–37.

Suihko ML, Alakomi HL, Gorbushina A, Fortune I, Marquardt J, Saarela M. (2007) Characterization of aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments. *Syst Appl Microbiol.* 30:494–508