4. MATERIALS AND METHODS

The cultural diversity and historic past represented by monuments are under constant attack by different biological organisms. These include members of different groups of organisms like green algae, cyanobacteria, bryophytes and lichens which can act as a pioneer community (Crispim and Gaylarde, 2005). Members of cyanobacteria, bryophytes and lichens mostly constitute the biofouling organisms found on monuments. The present study focuses on these three groups of organisms and on their varied role in the deterioration process. The broadly outline plan followed in the current study is depicted in the chart below:

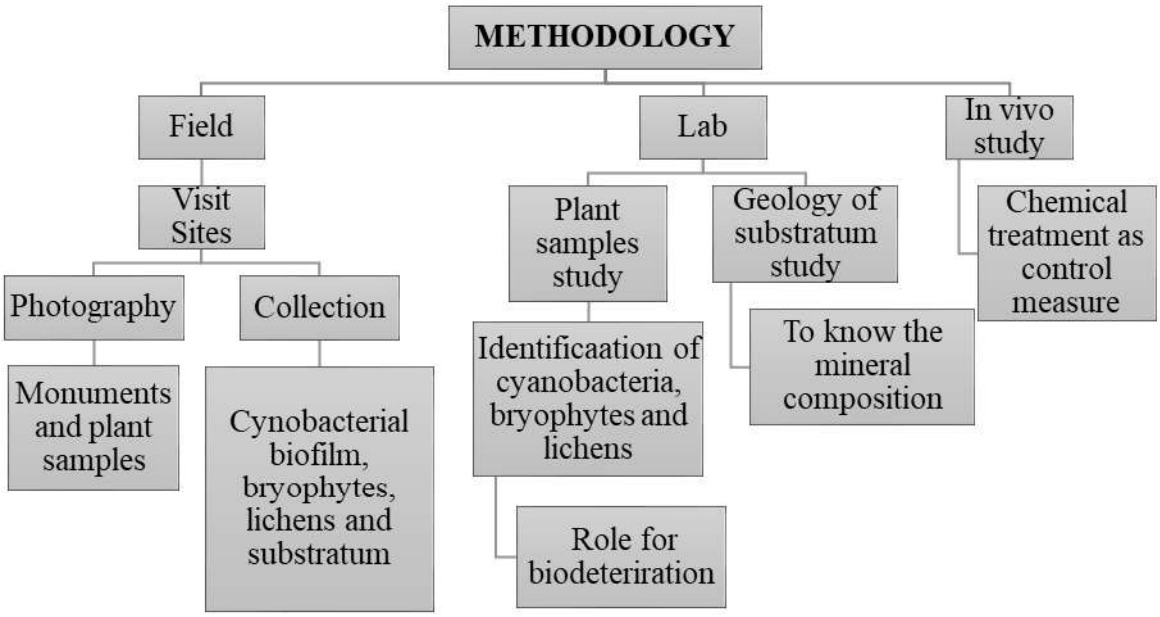


Fig. 4.1 – Brief outline plan followed during the current biodeterioration study

4.1 Site Selection

Central Gujarat has a large number of monuments which are in different stages of preservation (ASI, 2020). Several sites in Vadodara and Panchmahal districts were subjected to preliminary visits to check their suitability for the current study. They included the four gates of Dabhoi, Saptmukhi vav, Kayavarohan, Sevasi vav, Qutbuddin Hazira, Tamberkar Vada, several monuments of Champaner Pavagadh complex, old building of the M. S University etc. Majority of these sites were protected by ASI, which represented different substratum, different ages and different structural types. Among these, monuments of

Champaner Pavagadh complex have been designated as World Heritage Sites by UNESCO since 2004. The Baroda College was established in the year 1881 and in 1949 it was incorporated at The Maharaja Sayajirao University of Baroda. Several buildings of the university are more than 100 years old and are facing maintenance issues due to biodeterioration. Hence specific sites at the Champaner Pavagadh complex and two sites from the main campus of MSU were selected for the study. They have been enumerated in the table 4.1 below.

Table 4.1 – Selected study sites

Sr. No.	Sites Name	Landmark
1.	Makai Kothar	Pavagadh
2.	Navlakha Kothar	
3.	Jain Temple	
4.	Saher ki masjid	Champaner
5.	Mandavi	
6.	Amir Manzil	
7.	Dome of the Arts building	MSU campus
8.	D. N. Hall	

Permission to visit and work on the selected monuments of the Champaner Pavagadh Complex was sought from the Vadodara Circle office of the Archaeological Survey of India while for the buildings within the university campus, permission was sought from the respective Dean of the Faculty under which the buildings fell. These sites showed the presence of biofouing organisms. Figure 4.2 showing biofoulants on some selected study sites of Champaner Pavagadh.

Fig. 4.2 – Glimpses of Champaner Pavagadh monumets deteriorated by biological organisms

To achieve the objective mentioned in chapter 1 the current study was divided into different components. Firstly, the biological organisms damaging the different structure selected for the structure were observed, collected and identified. Later, specific roles in biodeterioration for the groups, cyanobacteria and bryophytes were studied as each group had different mechanisms. As restricted sampling of lichens was possible in the study area their role has been analysed differently. The methodology followed in all these have been sequentially described below.

4.2 Enumeration and Identification of Biofoulants on specific geological substrata

The identification of the diversity of biofoulants was the major task during the current study. It formed the crucial first step in the study of biodeterioration at the selected sites. The outline methodology followed to achieve this objective has been shown in figure 4.3 below.

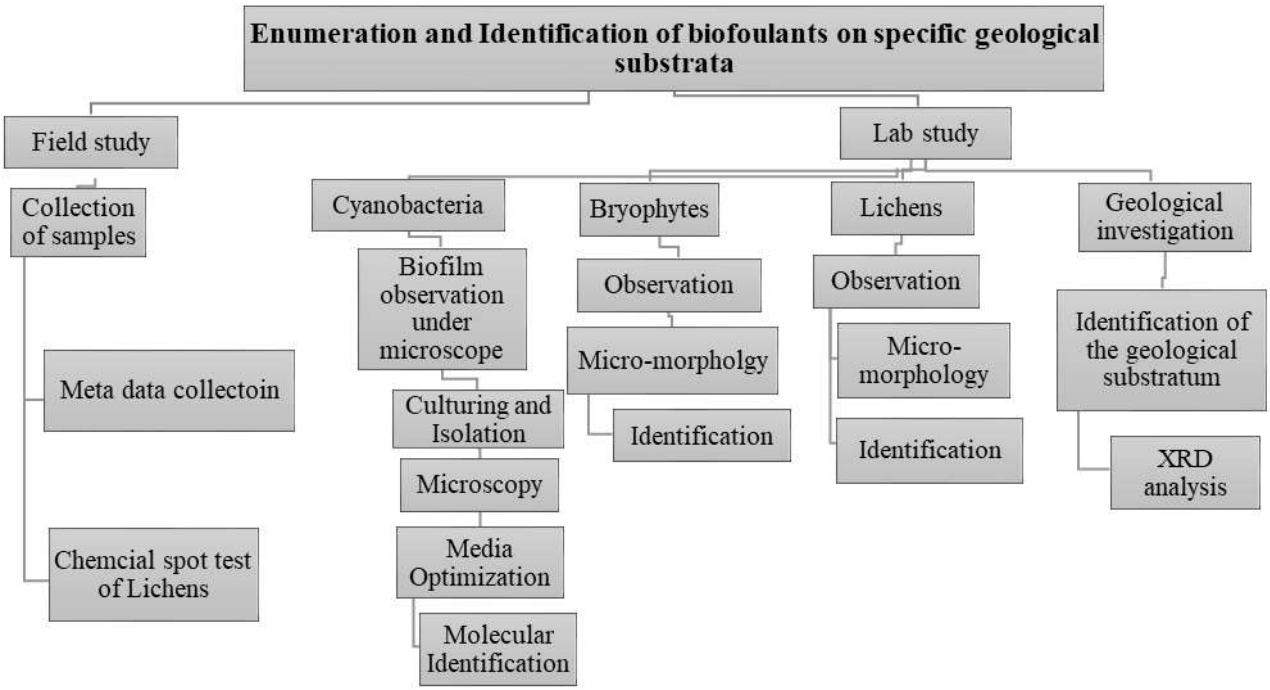


Fig. 4.3 – Chart showing detailed methodology for enumeration and identification of biological organisms and geological substrate analysis

4.2.1 Field study

Field work was carried several times between 2016 and 2019 to observe and understand biodeterioration process and collection of samples. Details regarding the site visits have been given in table 4.2.

Table: 4.2 - Details of the sites visit

Sr. No.	Study site	Month and year to visit the sites	Permission letter no.
1.	Monuments of Champaner Pavagadh	October, 2016	36/13/MIS/T&A-2015-16/1/22
		July, 2017	36/13/MIS/T&A-2015-16-4480
		August, 2017	36/13/MIS/T&A-2016-17-3771
		April, 2018	36/13/MIS/T&A-2018-19-2701
2.	D. N. Hall	December, 2016	Verbally communicated
		July, 2017	
3.	Dome of the Arts faculty building	October, 2019	
		September, 2020	

4.2.1.1 Field Photography

Photography in the field was accomplished with a Canon Powershot A480 camera having 13 MP resolution. Additional photographs were also taken using mobiles. Photographs of the monuments, parts of monuments which had presence of biofoulants and those that didn't have the biofoulants, close ups of the different biofoulants encountered and identified in the field were taken.

4.2.1.2 Sample collection

The sample collection strategy differed with different groups of organisms. This has been elaborated group wise below,

4.2.1.2.1 Cyanobacteria

Cyanobacterial biofilm was collected using a non-destructive sampling method (La Cono and Urzi, 2003). In this method, single-side transparent adhesive tape having thickness 40 µ was pasted on surface of the monument having biofilm and then gently removed as shown in figure 4.4 (A). Biofilm organisms adhered to the tape which were then stored in individual sterile plastic boxes and brought back to the laboratory on the same day.

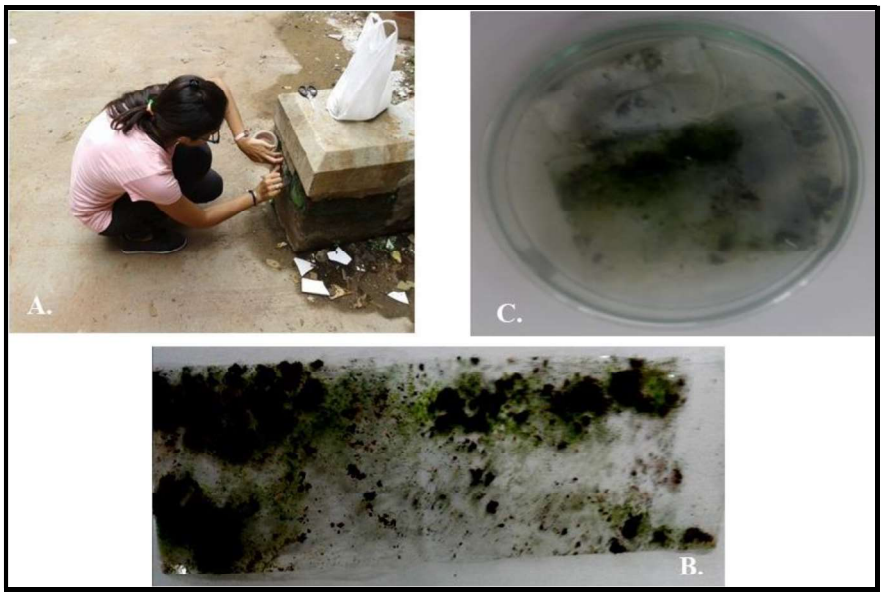


Fig. 4.4 – Cyanobacterial biofilm collection and process

4.2.1.2.2 Bryophytes

For bryophyte sample collection the methodology suggested by Glime (2013) with necessary modifications was followed. A knife or scalpel was used to remove the specimen from the substratum (Fig. 4.5). Samples were collected for lab study and herbarium preparation. The collected samples were kept in small plastic zipper bags or brown paper bags and necessary field information like date of the collection, location of the site, substratum, name of the samples, group (liverwort, hornwort and mosses) of the samples were recorded in a field diary. Wherever available, reproductive parts including the sporophyte were preferably collected.

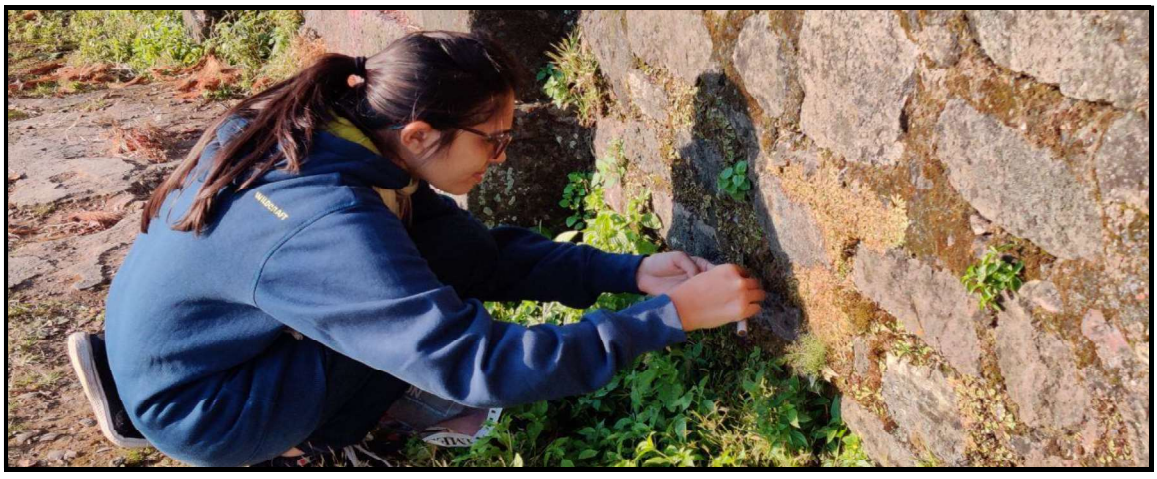


Fig. 4.5 – Collection of Bryophyte sample

4.2.1.2.3 Lichen

Lichen samples collection with substratum was difficult. The major morphological lichen type encountered in the study area was of crustose type, where it is specifically difficult to sample the lichen without the substrate. Since the sites were protected monuments, sampling would have damaged the monument surface. The same species were collected along with their substratum from the surrounding area of the sites. A sharp or pointed chisel and hammer were used to collect lichen specimens (Bajpai and Upreti, 2014). Due to restricted permission, some of the test like chemical spot tests like K test, KC test, C test, PD test required for lichens identification (Bajpai and Upreti, 2014) were conducted in the field directly as shown in figure 4.6. The lichens which were loosely attached to substratum were scraped out and collected Only a small amount of the sample needed for identification and herbarium preparation was collected. The collected samples were kept in sterile plastic boxes and then taken to the laboratory and then air dried under the fan.

Fig. 4.6 – Chemical spot test on the lichen thallus at the field location

4.2.1.3 Metadata collection

In addition to sample collection and field photography, additional information regarding the samples and its surroundings was noted in a field diary. The major metadata points collected have been enumerated in table 4.3 below.

Table 4.3 – Meta data collection

Sr. No.	Meta data
1.	The aspect (direction) of the samples
2.	Type of substratum
3.	Plane of the sample collected
4.	Location of the sample
5.	Associated species

4.2.2 Lab study

The collected samples which belonged to different groups of organisms were further analysed in the Laboratory. As depicted in figure 4.3 earlier they were processes differently for their specific objectives. The laboratory methodology followed for each group has been detailed below,

4.2.2.1 Cyanobacteria

The collected cyanobacterial biofilms on adhesive tapes were soaked in petriplates containing sterile distilled water for 72h (Keshari and Adhikary, 2013, 2014) as shown in figure 4.4 B & C. During this period the biofoulants got transferred to water. The tapes were the discarded and the biofilm was observed at different magnifications (100X, 200X and 400X) using the Carl Zeiss (Axio Lab.1 model) microscope. The cyanobacterial clumps had several species which were clustered together and hence they needed to be first isolated into their pure form before their identification could be carried out. This was achieved with in-vitro culturing and sub-culturing.

4.2.2.1.1 Cyanobacteria Culture

Biofilms clumps were grown in culture media to obtain pure culture and for further study. The culturing of cyanobacteria involved several requirements and steps that are described below.

4.2.2.1.1.1 Glassware and others

Borosil conical flask of 250 ml capacity were used as the culture container/vessel. They were plugged with non-absorbent cotton plugs. Borosil Petriplates (100 mm × 15 mm) and

bacterial loop were used during sub-culturing for isolation and purification of samples on agar media.

4.2.2.1.1.2 Growth media

Different media like blue green algae agar (BG-11), bold basal (BBM), Z8 etc. have been used for the isolation and growth of biofilm microorganisms. Among these the BG-11 media has been preferred by several researchers (Rippka *et al*., 1979; Keshari & Adhikary, 2013, 2014; Rossi *et al*., 2012). Hence in the present study the BG-11 media was used. The components of the media (Rippka *et al*., 1979, Keshari and Adhikary, 2013, 2014 and Rossi *et al*., 2012) have been listed in table 4.4 below. All these chemicals used in media preparation were of analytical grade and belonged to either Merck, SRL or Loba. The weight/volume of each component were taken as per mentioned in the table 4.4 for one litre of the media. 1% agar-agar was added for solid media. Fresh media was prepared each time.

Table 4.4 – BG – 11 media composition

Media components	**Concentration (g/lit)**
$NaNO_3$	1.5
$K_2\ HPO_4$	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
$C_6H_8O_7$	0.006
$C_6H_8FeNO_7$	0.006
EDTA	0.001
Na_2CO_3	0.02
Trace metal mix	1 ml*

*Trace metal mix (See table 4.5 below for composition)

A separate stock solution for trace metals was prepared and its composition has been depicted in table 4.5 below. During final nutrient media preparation, the pH value was adjusted to 7.4 using 0.1N NaOH.

Table 4.5 – Trace metal mixture solution

Chemicals	Concentration (g/lit)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.006
$Co(NO_3)_2.6H_2O$	0.049

4.2.2.1.1.3 Sterilization

For sterilization, the culture media and culture containers were autoclaved in pressure cooker for 30 mins under 15 PSI (Swenson *et al*., 2018).

4.2.2.1.1.4 Culture conditions

All cultures were maintained between 27 – 30 ºC temperature in 2500 Lux white light for 16h and 8h in dark. In the light phase, the cultures were kept on a rotatory shaker at 100 RPM for 2h.

Isolation:

Isolation was done by streak plate method in laminar air flow. Small amount of sample was taken from cultured flask and streaked on agar plate by sterile bacterial loop. The petriplate was incubated in culture condition specified above.

Sub-culturing:

Sub culturing is the aseptic transfer of micro-organisms from a culture to fresh medium. Separated colonies were picked up with sterile loop and transferred to separate nutrient agar plates. Repeated isolation of culture broth on agar plates was carried out for obtaining pure cultures. The pure colony was grown in broth media and kept in culture condition. The cultures were shaken manually twice a day.

Morphological Identification:

The cultures were confirmed as pure by viewing the petriplates using the Leica SD6 Stereo-binocular microscope. After confirmation of pure species, the cells were transferred to a slide and observed under the compound microscope Leica DM 2000. The images were

captured at 450X magnification using the camera Leica DMC2900 (3.1 MP). Monoculture of organisms were identified by micromorphology by following standard monographs like Desikachary (1959), Komarek and Anagnostidis (1989) and (1999).

4.2.2.1.2. Optimization of media components

For obtaining higher biomass, the optimum nutrient media composition was optimized using the response surface method (RSM) model of the Design Expert 7.0.2 (state-ease, USA) (Wang *et al*., 2007; Mehta *et al*., 2020). RSM was developed by Box and Colleagues in late 50's (Gilmour, 2006; Bruns *et al.,* 2006). RSM has several statistical and designed mathematical model which uses best fit model obtained for the given experimental data and experimental design (Teofilo and Ferreira, 2006). RSM has different experimental designs like central composite design (CCD), the box behnken design, the one factor design, the miscellaneous design, the D optimal, the user defined design, the distance based design and the historical data design.

4.2.2.1.2.1. Experimental design for optimization

Central Composite Design (CCD) of RSM was selected to optimize the main two components of media. The increase the culture biomass of the species requires nitrogen and phosphate in optimum concentration because both of these components are the backbone of the growth factor (Taiz and Zeiger 2002). Considering this, work was carried out to optimize the salts components $NaNO_3$ and K_2HPO_4 of BG 11 media for increasing the biomass of *Chroococcidiopsis* sp. The experiment was designed by the Design Expert 7.0.2 (stat-ease, USA) (Mehta *et al*., 2020). The range of the variables of $NaNO_3$ and K_2HPO_4 were selected from preliminary study. The lowest and highest values of the variables have mentioned in table 4.6. Central composite design with 2^2 factorial gives 4 star points and axial points having 5 replicate at the centre as central point leading to 13 runs were engaged for the optimization of the concentration (Bradley, 2007).

For statistical calculation, the variables were coded by equation 4.1:

$$x_i = {}^{x_1 - x_0}/_{\Delta x} \quad (i = 1, 2, \ldots q) \qquad \ldots\ldots\ldots \text{Equation 4.1}$$

Where x_i is the dimensionless value of variable 1, x_1 is real value of variable 1, x_0 is centre point value of x_1 and Δx is step change.

CCD is a second order level design. In this design, the dependent variable gives information about interaction between factors in their relation to the dependent variable using 2nd order polynomial equation 4.2:

$$\hat{Y}_i = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i.j=1}^{2} \beta_{ij} x_i x_j$$

…….. Equation 4.2

Where $\hat{Y}_i$denoted predicted response, x_i and x_j input variables, β_0 intercept term, β_i linear effect, β_{ii} squared effect and β_{ij} interaction term. The Design expert software was run for solving the regression equation and analysis of the response surface contour plot (Zheng *et al*., 2008; Mehta *et al*., 2020).

Table 4.6 – Range and Levels of variables

Variables		Range and Levels				
		-2	-1	0	1	2
X_1	K_2HPO_4 (g/l)	5	7	9	11	13
X_2	$NaNO_3$ (g/l)	10	13	16	19	22

4.2.2.1.3. Molecular identification

Molecular identification is an extensively used method as it facilitates the confirmation of species based on specific markers. For biofilm organisms, the 16S rRNA gene sequence is commonly used (Nubel *et al*., 1997; Tillett and Neilan, 2000; Keshari and Adhikary, 2013 and 2014).

Genomic DNA extraction

DNA was extracted using the xanthogenate-SDS method (Tillett and Neilan, 2000) with minor modification. The protocol followed for extraction of DNA is given in figure 4.7 below:

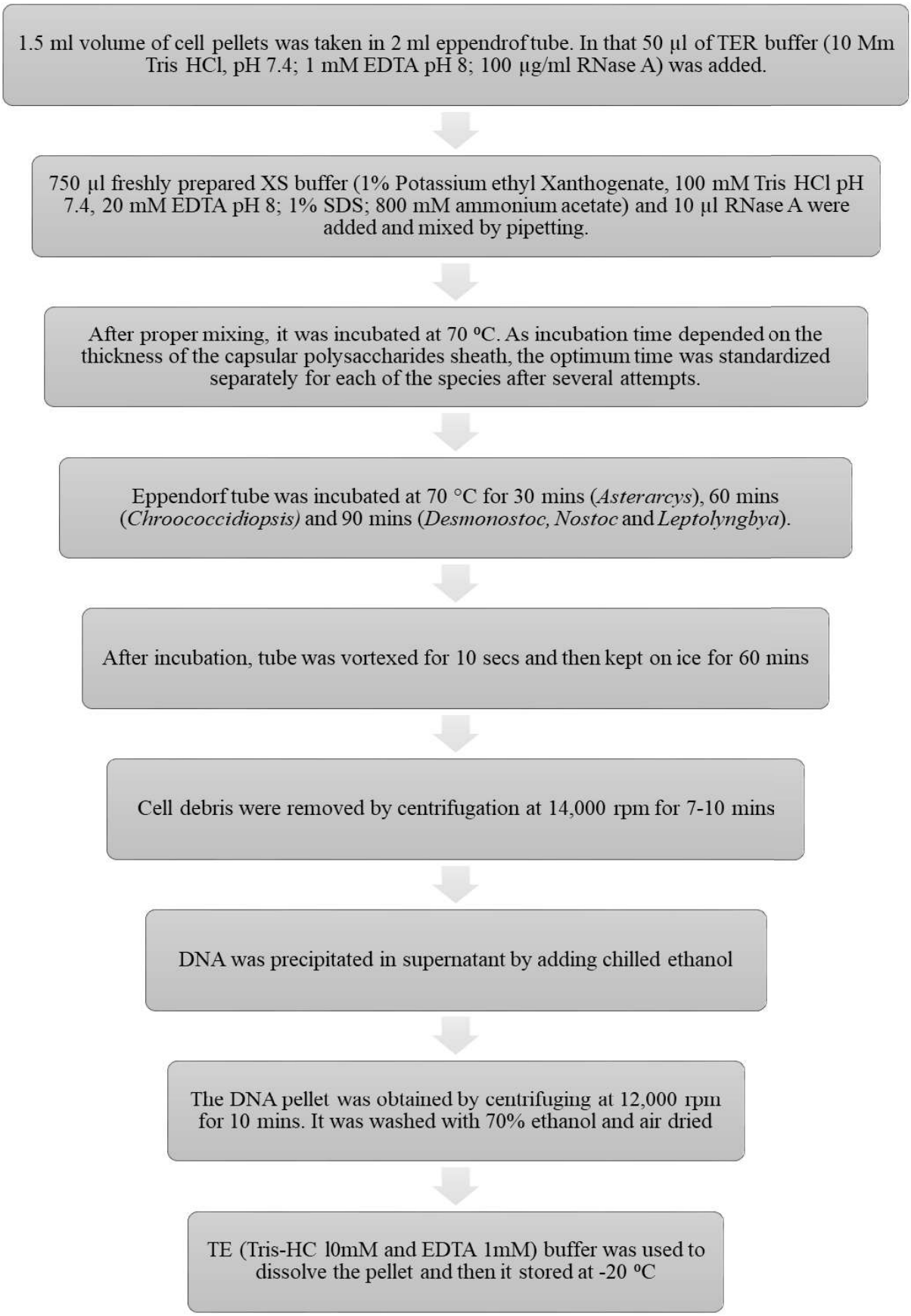


Fig. 4.7 – Protocol for DNA extraction

PCR amplification

The PCR amplification process was carried out by NCBI Database-based designed primers. Primer selection and design was based on GC content, Tm value and ideal number of nucleotides. In the current study, the primers used and their sequences are mentioned in table 4.7.

Table 4.7 – 16S rRNA primers used for amplification

Primers	Sequences
DNM1	F "GAAAGCCTGACGGAGCAATA" R "CGGGACTTAACCCAACATCT"
DNM2	F "GCGGTGAAATGCGTAGAGAT" R "GTAAGGGGCATGCTGACTTG"
DNM3	F "AGAGGATGAGCAGCCACACT" R "GGGATGTCAAGCCTTGGTAA"

20 µl PCR reaction mixture was prepared and used for amplification. The PCR reaction mixture used has been given in table 4.8 below.

Table 4.8 – Composition of PCR reaction mixture

Ingredients	Volume in µl
Dream Taq master mix	10
Forward primer	0.5
Reverse primer	0.5
Template DNA	1
MilliQ water	8

35 PCR cycles were performed, initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 secs, elongation at 55°C for 45 secs, extension at 72°C for 2 min and final extension 7min at 72°C. 2% Agarose gel was prepared for gel electrophoresis and the amplicon band with DNA ladder (Thermo scientific) on the loaded gel was observed. The

exact match DNA band was purified for sequencing. DNA purification was carried out by GeneJET Gel Extraction (Thermo scientific). The purified amplicon quality and quantity was checked using nano photometer (IMPLEN NP80). Nano photometer was used for testing the quality in the form of 260/280 ratio (acceptable ration was1.8 to 2) and concentration of DNA in ng. Based on that samples were further processed. The obtained amplicon was subjected to Sanger sequencing which was outsourced to Agrigenome pvt. Ltd. (Kochi, Kerala). The sequences were obtained in FASTA format. They were the subjected to BLAST analysis in NCBI Database for identification purpose.

4.2.2.2. Bryophytes

In laboratory, the collected bryophytes samples were used for identification, voucher preparation and calcium uptake analysis. The bryophytes study was done in several steps which are enumerated below,

4.2.2.2.1. Processing of collected samples

The samples were air dried under the fan for better preservation. For air drying of the samples, paper boat was prepared and the samples were kept overnight or for a few days openly in air as per the specimen. The sample needed for analysis was separated from the group and kept in water filled petriplate for cleaning. Dust and debris were removed with brush and this cleaned sample was used for observation. Some part of the sample was analysed fresh.

4.2.2.2.2. Isolation and photography

The fresh samples were analysed under the stereo binocular microscope and a few individuals were separated out on a slide or a watch glass. The isolated individuals were then subject to photography either directly or under the stereo binocular microscope.

4.2.2.2.3. Morphology and Micro-morphology analysis

Samples were observed under Leica SD6 Stereo-binocular microscope. During observation, morphology including plant or thallus size, branching, leaves morphology, their size, shape, arrangement, colour, costa, cells appearance was noted. For reproductive morphology the presence or absence of reproductive structure was first noted. The reproductive structures' information like size of the seta, shape and position of the capsule like erect, pendulous and semi pendulous, shape of calyptra was observed and recorded. After that micro-morphology like presence of appendages on thallus, mark on leaf cells, sheath of the leaf, cells of the costa, excurrent or percurrent, margin of the leaf etc. were recorded. If

the sporophyte was present, the size and colour of the spores, appearance of the peristome was observed and noted.

4.2.2.2.4. Photography of micro-morphological features

Photographs of above mentioned features of morphology and micromorphology were taken by Tucsen camera (3.2 MP) connected with Zeiss microscope. All these images were captured by IS Capture software. Sometimes images were clicked by digital microscope suite 2.0 software of the Celestron camera (1.2 MP) which was attached to one of the eye pieces of the SD6 Stereo-binocular microscope.

4.2.2.2.5. Identification of specimens and their confirmation

Based on above mentioned features, specimen was identified using dichotomous keys and characters from the flora or monographs like Chaudhary and Deora (1993) and Chaudhury *et al*. (2006, 2008), Gangulee (1969-72, 1974-78), Aziz and Vohra (2008), Bapna and Kachroo (2000). etc. Confirmation of some doubtful samples were done with the help of experts at the Bryology Laboratory of the CSIR -National Botanical Research Institute, Lucknow.

4.2.2.2.6. Preparation of Herbarium specimens

Herbarium vouchers were prepared from A4 size paper. The preparation of vouchers involves three fold on the paper. Directions given by Glime (2013) were followed in the preparation of the herbarium specimens. The herbarium specimens were stored in plastic boxes.

4.2.2.3. Lichen

The lichen specimens which could be collected from the nearby antiquities were brought back to the lab for analysis. For specimens which could not be collected from the monuments directly, were studied in field only. High resolution photographs were taken using the super macro mode of the Canon A480 powershot camera along with standard scales.

4.2.2.3.1. Morphology and micromorphology analysis

Samples were observed under Leica SD6 Stereo-binocular microscope. During observation, morphology including plant size, shape, lobes arrangement, texture of thallus, structure of thallus, shape margin, colour of thallus and margin, pattern of branching, presence of fruiting body was noted. For micro-morphology, features like appearance and

structure of the cortex, medulla and attachment of fruiting body, its colour, size, texture of the margin and disc were observed.

4.2.2.3.2. Photography of micro morphological features

Photographs of above mentioned features of morphology and micromorphology were taken by Tucsen (3.2 MP) and Celestron (1.2 MP) camera connected with Zesis and Leica SD6 Stereo-binocular microscope respectively. Observed images were captured by IS Capture software and Digital microscope suite 2.0.

4.2.2.3.3. Identification of specimens and their confirmation

Identification was done standard literature like Awasthi (1991), (2007) and Bajpai and Upreti (2014). Some of the lichen specimens were carried to a field workshop conducted by NBRI at Nainital where the identity of the species as well as its confirmation was done by lichenologist of CSIR- National Botanical Research Institute, Lucknow. The samples were deposited in the herbarium of NBRI, Lucknow and the accession numbers were obtained.

4.2.2.4. Geological investigation

During field study, sites were visited for purpose of observation and collection of the samples along with their substrate. Collection of geological substratum at every site was not possible because most of the monuments were protected structures. So wherever geological substrate samples were available for collection (either at the site or from the surrounding antiquities) they were collected. At places where it was not possible to collect the geological substrata information available in the literature survey was utilized. The mineral composition of the collected rock samples was analysed by microscopic analysis of the thin section of the substrate samples and powder XRD analysis.

4.2.2.4.1. Sample preparation for Microscopy

Thin section of the samples was prepared with the help of staff at Geology department and mounted on slides (Fig. 4.8). The slides were observed under Leica microscope in 10X magnification. During observation, phase contrast and polarized light microscope (Leica DM EP) were used for identification. The minerals present within the samples were identified by the experts available in Geology Department of the University. This information was recorded and their images were captured by Leica MC 120 HD camera (2.5 MP).

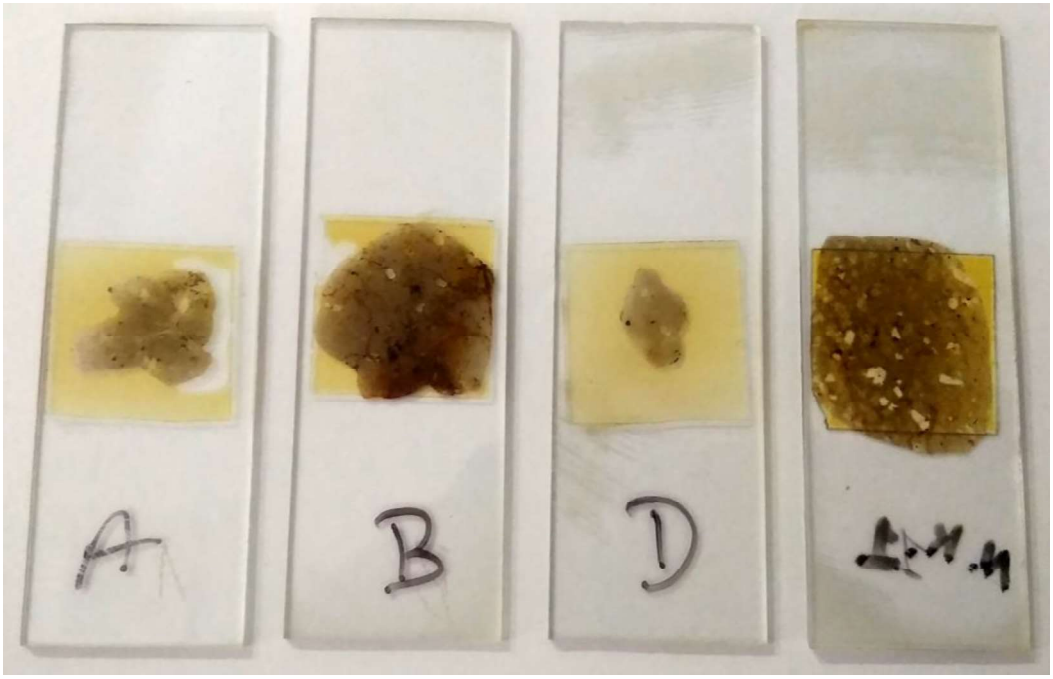


Fig. 4.8 – Prepared thin section slides of the substratum

4.2.2.4.2 XRD analysis of substrate sample

X-ray powder diffraction (XRD) analysis of four samples was carried out using the instrument facility of Faculty of Technology and Engineering (XRD model: X'pert Pro PANalytical). For XRD analysis, powder of each samples were prepared using steel mortal and pastel. The powder was sieved by mesh of size 200 ASTM (75 μ) size as very fine powder which is amorphous in nature was required for further analysis. These were analysed at 2 θ angle ranging from 0 to 80°. After completion of the run, various peaks representing the data were obtained in graph form (graphics format). Peaks were identified by JCPDS (Joint committee on Powder Diffraction Standards) software. This software contained a database of standard peaks which were used as reference for the purpose of identification in the XRD data obtained. After identification of all geological substrata, the biofoulants including biofilm organisms, bryophytes and lichens were enumerated with their substratum on different monument sites.

4.3 To study the specific role of cyanobacteria, lichen and bryophytes in biodeterioration.

An understanding of the mechanisms of biodeterioration by each group of organism, needs information on the specific role played by each group during the process. All groups of photosynthetic organisms have their own physiology and secrete various chemical that damage the substrate the monuments are made up of. Figure 4.9 below depicts the outline methodology followed in this component of the study.

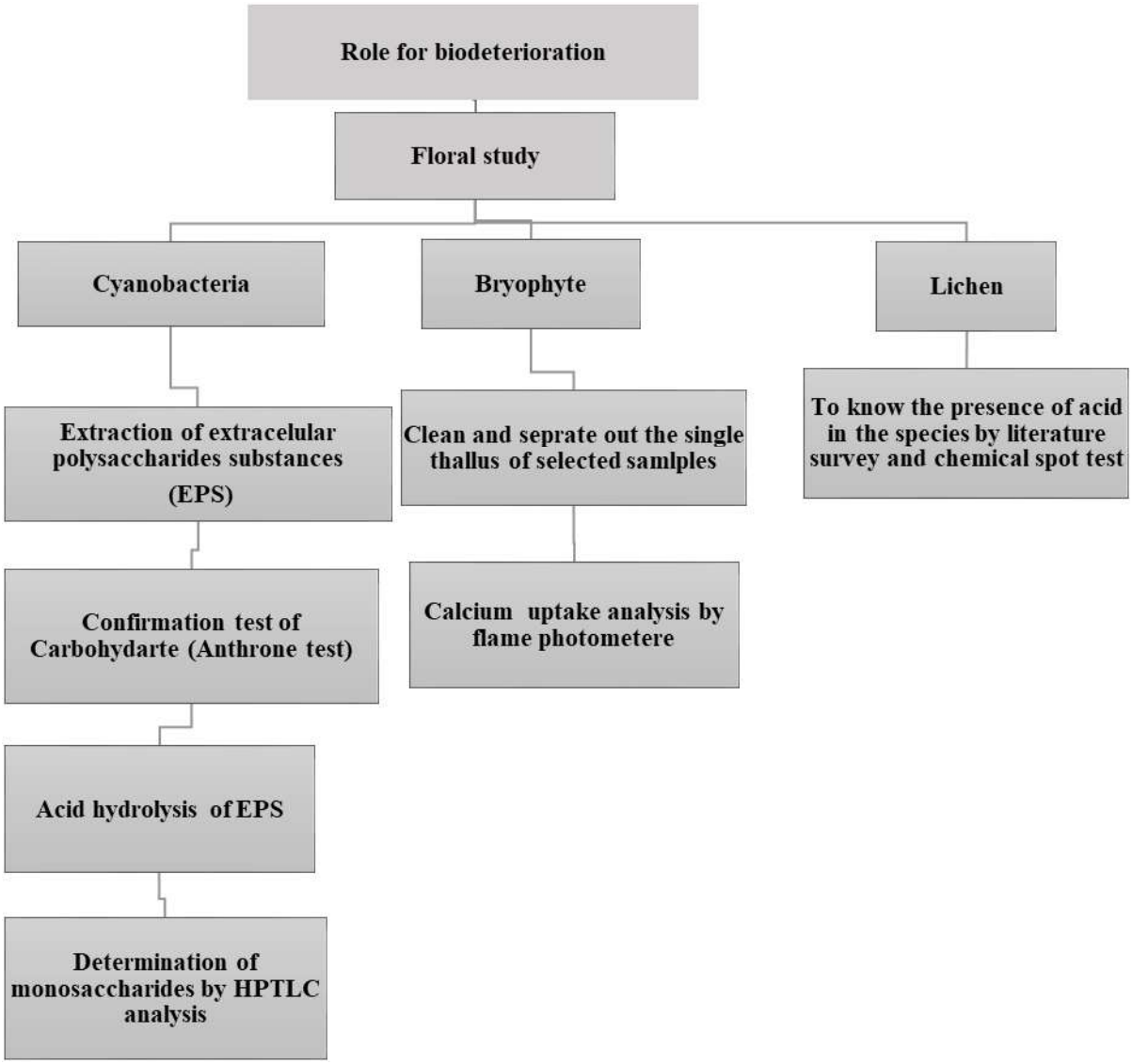


Fig. 4.9 – Outline methodology for role of biodeterioration of cyanobacteria, bryophytes and lichens

Cyanobacteria secrete EPS (extracellular polymeric substances), which is the key to the spread of phototrophic biofilms and also enhances the deterioration of stone (Crispim and Gaylarde, 2005). As for bryophytes, they possess a structure called rhizoids which physically

intrude the stone surface. However, studies on any mechanical damages caused by them is not yet reported from India and are also less documented in literature globally. The ability of mosses to uptake calcium point to their role in biodeterioration (Altieri and Ricci, 1997) but their specific role in biodeterioration of monuments and art works is still lacking. In case of lichens, the mixing of respiratory CO_2 with water in lichen tissues results in the formation of carbonic acid which enhance the solubility of rock surfaces by lowering the pH of the substratum (Seaward, 1988; Chen *et al*., 2000). Lichen acids have a relatively low solubility, but they are effective chelators, forming metal complexes with silicates, etc., derived from the substratum. Some of these characteristics of different groups have been investigated as shown below.

4.3.1 Cyanobacteria

The EPS composition of six of the isolated biofoulants and their role in biodeterioration by cyanobacteria was studied.

4.3.1.1 Confirmation of EPS production

The production of exopolysaccharides was confirmed by Alcian Blue staining method (Fig. 4.10) given by Tamaru *et al*., 2005.

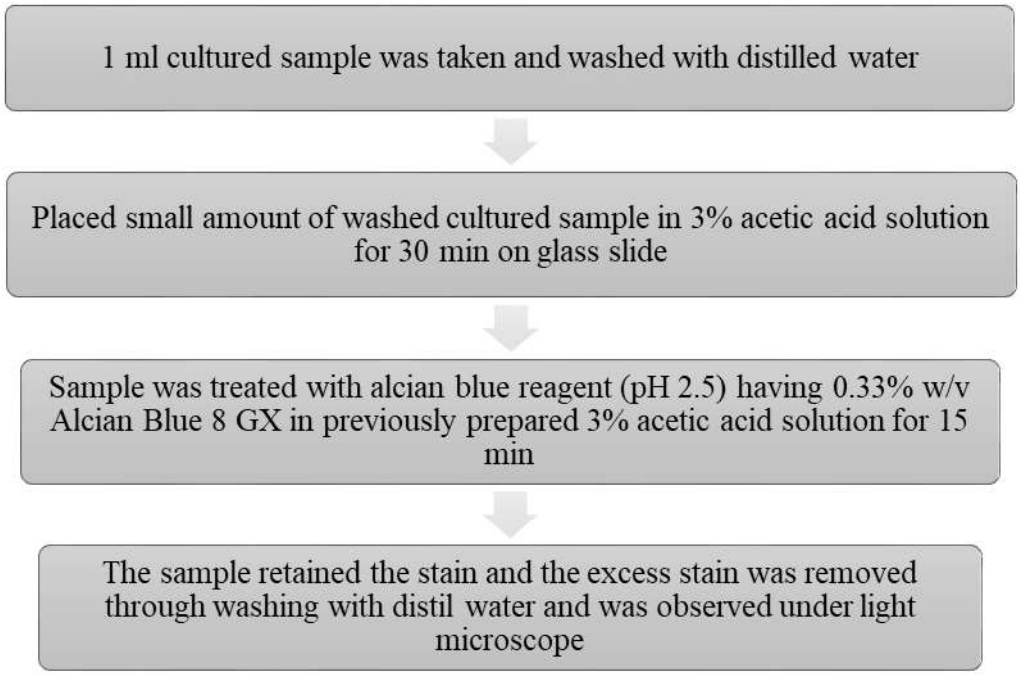


Fig 4.10 – Procedure followed for confirmation of EPS production

4.3.1.2 Extraction of EPS

Extraction of extracellular polysaccharides was done using the procedure specified by Sureshkumar *et al*., (2007) and Khattar *et al*., (2010) with minor modification. This has been outlined in figure 4.11 given below

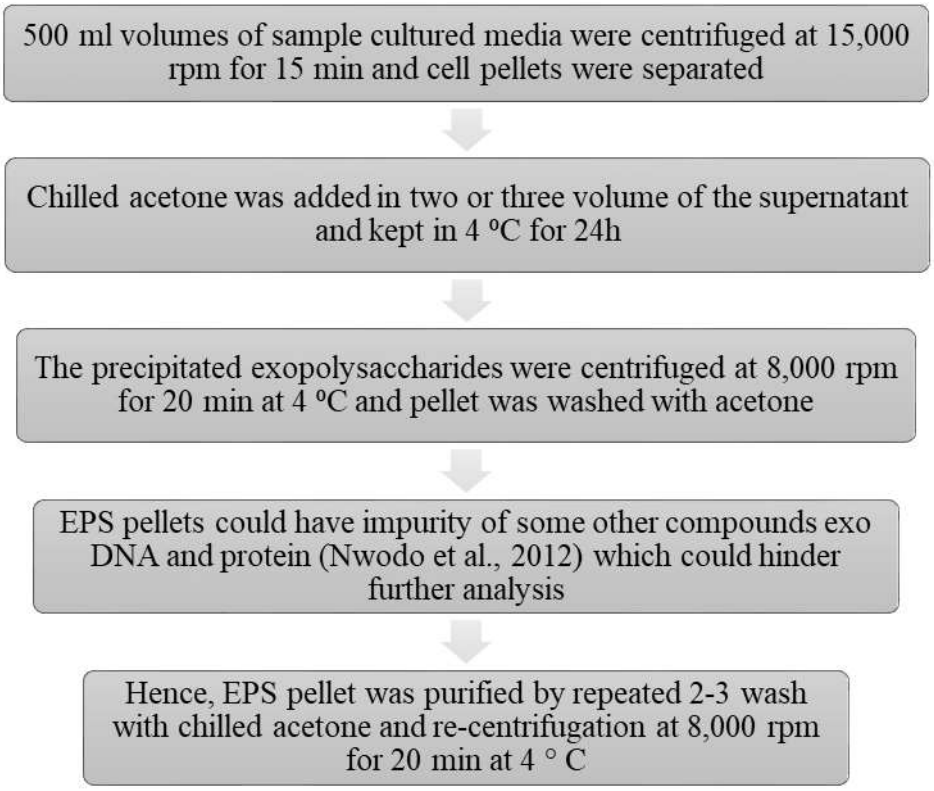


Figure 4.11 – EPS extraction procedure

4.3.1.3 Confirmation test of total carbohydrate

The purity of the pellet was checked by anthrone test (Delattre *et al*., 2016). The procedure has been outlined in figure 4.12 given below.

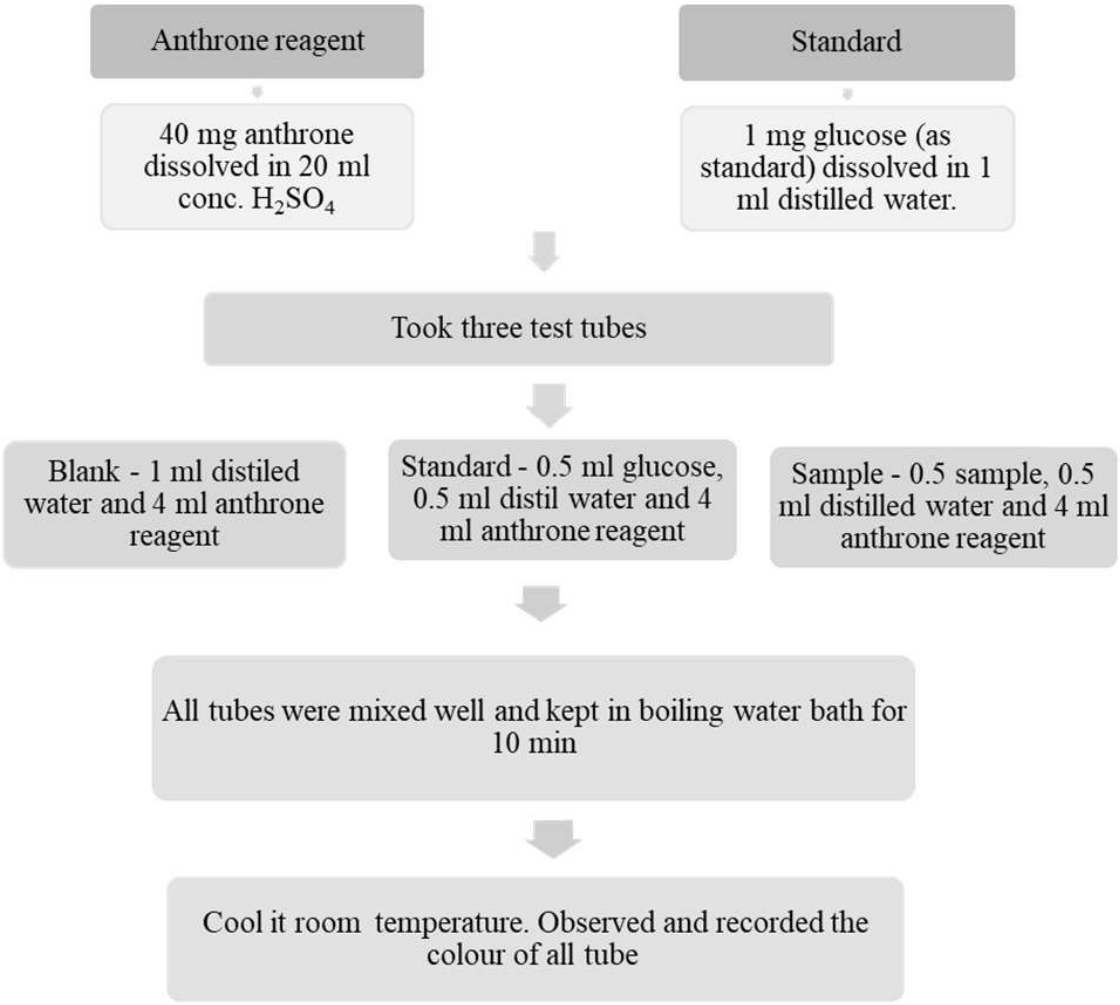


Fig 4.12 – Total carbohydrate confirmation test procedure

4.3.1.4 Acid hydrolysis

Acid hydrolysis of EPS was carried out by protocol described by Khattar *et al*., 2010 with some modifications. 1X mg exopolysaccharides pellet was hydrolysed by 3X ml 4 N TFA (Trifluoroacetic acid) at 100 ºC for 60 min. TFA was removed by evaporation along with drop by drop addition of equal volume of methanol. Fume hood was required for this process due formation of azeotrope along with methanol. This neutralized the sample but it could still have residues of TFA which would have hampered the separation study of the compounds. This was removed through rotatory evaporator (Heidolph Laborota 4000) and high vacuum pump.

4.3.1.5 Determination of monosaccharides by HPTLC analysis

The hydrolysed sample was used in HPTLC analysis for separation of monosaccharide present in exopolysaccharides. For HPTLC, CAMAG HPTLC system setup

was used with Linomat 3 as sample applicator, Scanner 3 for scanning the derivatized plate and winCATS planar chromatography manager software version 1.2.0. The standards and samples were applied using 100 µl CAMAG Linomat syringe. Separation as chromatogram were achieved on 20 X 10 cm TLC plates precoated with silica gel 60 F254 with support on aluminium sheet (Merck, India). The plate was saturated with 0.3 N NaH_2PO_4 solution and air dried. Saturated plate was laid out on X axis first position at 20 mm far from edge and Y axis position above 8 mm and 6 mm length of each band. Plate was developed using 4:5:1 ratio of n butanol: acetone: 0.3N NaH_2PO_4 as mobile phase. For development of TLC plate, CAMAG twin-trough glass chamber was used. The developed plate was derivatized by ADP (Aniline diphenylamine phosphoric acid). The derivatized plate was heated at 110 ºC for 10 - 15 min in oven. The plate was scanned by CAMAG TLC Scanner 3 at wavelength 366 nm. This analysis was carried out at the Department of Bioscience, Sardar Patel University, Vallabh Vidhyanagar.

4.3.2 Bryophytes

Role of bryophytes in biodeterioration was studied by calcium uptake analysis (Altieri and Ricci, 1997). In the present study, one liverwort species (*Astrella angusta*) and one moss species (*Hyophilla involuta*) that were commonly available and dominant on selected study sites were selected for calcium uptake analysis.

4.3.2.1 Analysis of Calcium from apoplast regions

40 mg (dry weight) 12-18 mm moss shoots (*Hyophilla involuta*) and 40 mg (dry weight) thallus of liverworts (*Astrella angusta*) were taken for Calcium studies. The samples were cleaned and washed in petri plate with distilled water. They were then taken in culture tubes and 20 ml deionized water was added to it. They were shaken for a few minutes and this wash was repeated for three times. 20 ml of 25 mmol $SrCl_2$ was added to the tubes having the samples (Altieri andRicci, 1997; Bates, 1992). These tubes were kept in two different temperatures at 20° C and at 37° C for 1 h. They were then centrifuged at 2000 rpm for 3 min. (Alteri and Ricci, 1997). The exchangeable ionic fraction (supernatant) was taken for estimating the calcium concentration using flame photometer (Systronics flame photometer 130).

Flame photometer measures the intensity of light produced (as a colour) when an element is exposed to the flame. Calibration was carried out using different 10 ppm - 150 ppm standard solutions that were prepared from a 1000 ppm stock solution. Both stock and standard

solutions were prepared in deionized water. Deionized water was used as a control which gave a flame of yellow colour. The filter was then adjusted for Calcium which gave an orange colour flame. The standard graph was prepared using the standard 10 – 150 ppm solutions. Three replicates each of *Hyophilla involuta* and *Astrella angusta* were then analysed and their responses noted. The final concentration of calcium was obtained using the standard graph.

4.3.3 Lichen

Lichens have the presence of secondary metabolites or acids and these compounds play a role in the deterioration of the substratum (Seaward, 1988; Chen *et al*., 2000). To know the presence of these acids, chemical spot tests and Thin Layer Chromatography (TLC) of samples in different solvents are generally performed. But in current study, samples material couldn't be collected in enough amount hence sample material was not sufficient to carry out TLC analysis. Thus, in current study, the chemical spot test and literature survey was used for knowing the presence of the secondary metabolites or acids. Chemical spot test was done on thallus. On thallus, surface pigments are not distributed evenly. Hence, sometimes the upper cortex was slightly scraped and then chemicals were applied for showing the red or orange anthraquinone derivatives. Some colourless substances depsides and depsidones were restricted to medullary rays. Chemical spot gave a clue for the localization of chemical substance on lichen thallus, by applying it on the thallus. In current study, there are four chemical spot test and literature survey were done to know the presence of acids.

4.3.3.1 Chemical colour spot test

- ✓ K test: 10-25 % aqueous solution of potassium hydroxide, when applied on thallus, the colour turned yellow then red for presence of O – hydroxyl aromatic aldehydes and the colour showed the bright red to purple for presence of anthraquinone pigments.
- ✓ C-test: A freshly prepared aqueous solution of calcium hypochlorite. It gives red colour for presence of m -dihydroxyl phenol and green colour shows for dihydroxy benzofurans.
- ✓ KC -test: At particular location on thallus, K reagent was applied then immediately C reagent applied. The different colour shows different substances like yellow for usnic acid, blue for dihydroxy dibenzofurans, red for depsides and depsidones.

- ✓ PD-test: p – phenylenediamine solution was prepared in ethanol in small quantity. This reagent was used in a day. The colour shows yellow, orange red for presence of aromatic aldehyde.

4.3.3.2 Literature survey for knowing the presence of acids

Standard references such as Bajpai and Upreti, 2014 and Shukla *et al*., 2014 were referred for knowing the secondary metabolites or acids in the species of lichens recorded in the current study.

4.4 To devise measures for the control of biodeteriogens

The in-vivo experiment on the control of biofoulants was carried out at the Arts Faculty Dome, one of the study sites in the area were permissions could be obtained. The D N Hall building was subjected to a major renovation during the study area and hence could not be taken up for the study. Fig 4.13 below gives an outline of the methodology employed for this objective.

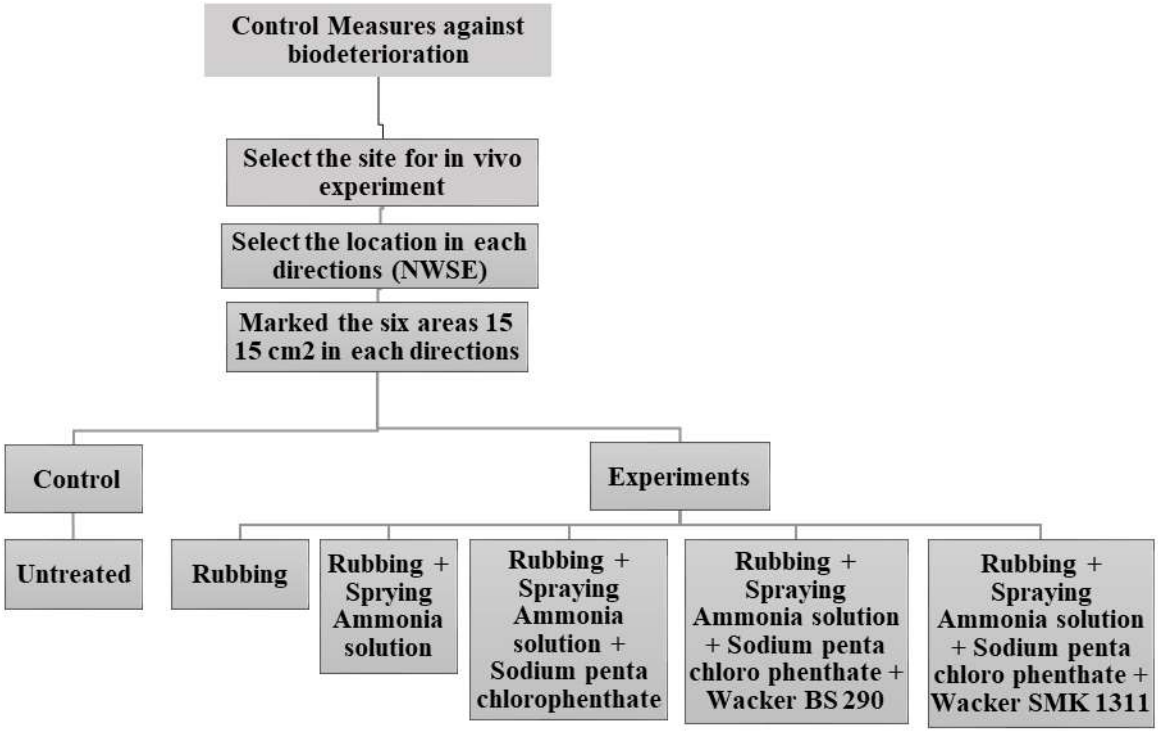


Fig. 4.13 – Outline chart showing the control measure for controlling the biofoulants growth

4.4.1 Selection of chemicals and site for *in vivo* study

Chemicals that are used as a control agent against biofoulants growth on any monument needs to take some characteristics. For example, the applied chemical should be transparent and non-hazardous. The ASI guidelines also mandate that the chemicals should work without damage to the structure of the monument. Considering all these points, based on literature survey and interaction with ASI conservationists (Dr. Deepak, ASI Aurangabad Circle, Personal Communication), two chemicals based on Silane siloxane compound were shortlisted for the *in vivo* study. They were having trade name Wacker BS 290 (Gupta and Sharma, 2011; Sadat-Shojai and Ershad-Langroudii, 2009; Tsakalof *et al*., 2007; Tewari, 2016) and Wacker SMK 1311 (Tewari, 2016; Soulios *et al*., 2019).

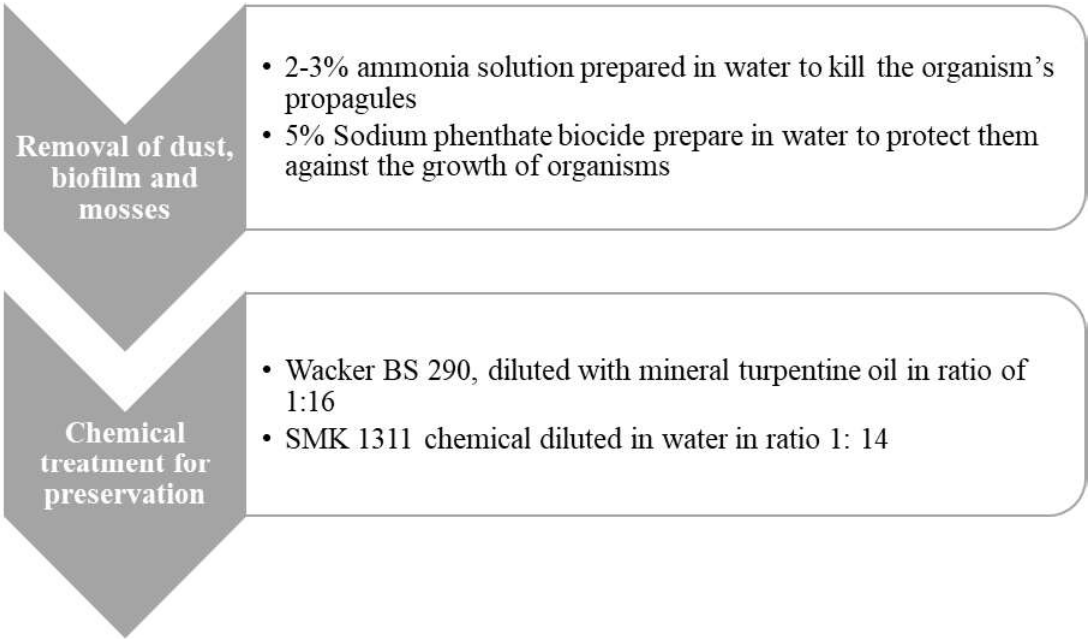
4.4.2 Selection of specific locations on the selected site

On the dome, specific areas were selected in the four cardinal directions and marked with pencil. On each area, 6 blocks of 15×15 cm^2 area each were marked. From that one block was used as control (where no treatment was done), The treatment protocol followed has been given in table 4.9 below.

Table 4.9 - The chemical treatment protocol for *in vivo* study

Block No.	Treatment
1	No treatment, only outline marked
2	Scrubbing with wire mesh
3	Scrubbing with wire mesh + Ammonia solution wash
4	Scrubbing with wire mesh + Ammonia Solution wash + coating with sodium penta chlorophenthate
5	Scrubbing with wire mesh + Ammonia Solution wash + coating with sodium penta chlorophenthate + coating with Wacker BS 290
6	Scrubbing with wire mesh + Ammonia Solution wash + coating with sodium penta chlorophenthate + coating with Wacker SMK 1311

4.4.3 Chemical preparation for experiment



4.4.4 Work plan for chemical conservation on site

Permission for visiting the dome of the arts building could not be obtained in the monsoon due to risk of accident as it becomes slippery in monsoon. Moreover, the chemicals required for the treatment could not be obtained due to COVID 19 pandemic. Hence, experiment was started in the month of October and continued till month of May (further study could not have carried out because restoration work already started on the same building of the arts faculty). Steps of the *in vivo* experiment are given below fig. 4.14 and some important steps of it showing in fig. 4.15.

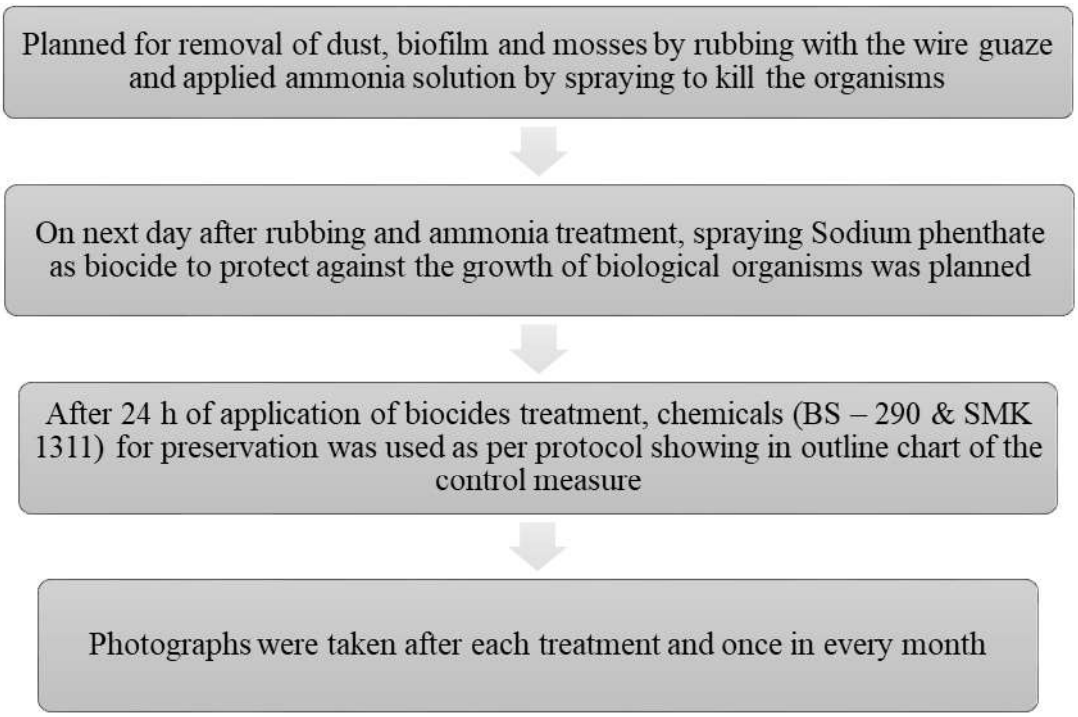


Fig. 4.14 – Steps of *in vivo* experiments on the dome of Arts faculty building

Fig. 4.15 – Experiment work on the dome of the arts building of the MSU campus

A. Marking the areas for the treatments, B. Spraying chemicals on the rubbed surface and C. Noting the monthly observation and capturing the images