Chapter Two

Experimental section
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Materials

2.1 Spores and sporopollenin

Spores of *Lycopodium clavatum* were purchased from Fluka Analytical, Switzerland and standard sporopollenin (isolated from *Lycopodium clavatum*) was purchased from Polysciences Inc. USA.

2.2 Chemicals and reagents

Chemicals of analytical grade, purchased from Qualigens Pvt. Ltd. India and SRL Pvt. Ltd., India, were used for isolation and purification of sporopollenin. Molecular biology grade chemicals were purchased from Sigma-Aldrich Inc., USA, Machery-Nagel, Germany and Roche Diagnostics, USA, for isolation, qualitative and quantitative analysis of DNA. Molecular weight markers, DNA ladders, reagents and buffers for restriction enzyme digestion and PCR were purchased from Bangalore Genie Pvt. Ltd. India.

Methods

2.3 Extraction of DNA

Plasmid DNA was extracted from *E. coli* DH5α using three methods, viz. alkaline extraction method (Engbrecht, et al., 1991, Heilig, et al., 1998), mini prep kit (Roche Diagnostics Inc., USA) and gigaprep kit (Machery-Nagel, Germany).

Genomic DNA was extracted from *E. coli*, *Saccharomyces cerevisiae*, *Triticum aestivum* and *Allium cepa* using standard method (Budelier, et al., 1998).
2.4 Isolation of sporopollenin microcapsules

Sporopollenin was isolated using the non-oxidative, extraction-hydrolysis method as originally described by Zetzsche (Zetzsche, et al., 1928, 1931a) and adopted by Guilford, et al., 1988. However, the protocol was partially modified to minimize the time required (Figure 2.1).

Spores of *Lycopodium clavatum* (100 g dry weight) were suspended in acetone (250 mL), stirred under reflux for 4 h and filtered through Whatman No.1 filter paper to get a solid residue. The residue was washed with fresh acetone (100 mL), resuspended in aqueous 6% (w/v) potassium hydroxide (KOH) solution (250 mL) and stirred under reflux (100°C) for 6 h. The residue was filtered, washed thoroughly with hot water (45-50°C) and again subjected to KOH treatment followed by washing with hot water (45-50°C), hot absolute ethanol (45°C) and cold water. The residue was stirred under reflux (50-70°C) in absolute ethanol for 2 h, filtered and washed sequentially with fresh absolute ethanol and dichloromethane. The resulting solid was re-suspended in fresh dichloromethane, stirred under reflux (50-55°C) for 2 h, filtered and dried in air at ambient temperature (25±2°C) to obtain sporopollenin powder.

**Figure 2.1:** Isolation and purification of sporopollenin. Illustration depicts different steps followed. (a) Protocol as per Zetzsche, et al., 1928, (b) Protocol followed in the present work.
Removal of cellulose and pectin from sporopollenin (after reaction with KOH) was carried out by two routes viz. (a) treatment with orthophosphoric acid as in Zetzsche's method (Figure 2.1, track 'a') and (b) enzymatic hydrolysis method as modified by the author (Figure 2.1, track 'b').

As per Zetzsche's method the filtered particles were suspended in 85% orthophosphoric acid, stirred under gentle reflux (25-30°C) for 5-10 days, neutralized using 1M NaOH, filtered and washed with copious amounts of hot water (45-50°C), ethanol (45-50°C) dichloromethane (45-50°C) and dried. The cycle of hot water-ethanol-dichloromethane treatment was repeated 2 - 3 times and finally, the powder was stirred under reflux in ethanol for 2 h, filtered, washed with dichloromethane and vacuum dried to get purified sporopollenin microcapsule preparation.

According to the modified protocol enzyme mixture of cellulase (1%) and pectinase (1%) prepared in (100 mL of 100 mM) citrate phosphate buffer (CP-buffer) at pH 4.8 was used. Sporopollenin (10 g) was reacted with the enzyme mixture at 45°C with stirring (100 rpm) for 24 h. The powder was filtered, washed with hot water, dichloromethane and vacuum dried to get sporopollenin microcapsule preparation.

2.5 Physical and chemical characterization of sporopollenin microcapsules

Sporopollenin microcapsules isolated by modified method are used for characterizing its properties. The sporopollenin isolated from Zetzsche's method was also characterized by FTIR and SEM for validation of the modified process.

2.5.1 Particle size distribution

Sporopollenin microcapsules (5 mg) were suspended in deionized water (1 mL) and ultrasonicated (Ultrasonicator of 0.5-3W, Sakova Pvt. Ltd., India) for 1 min to uniformly disperse the particles. The mean particle size and particle
size distribution of the sporopollenin microcapsules was obtained using a laser particle analyzer (HELOS H1004, Sympatec GmbH, Germany).

2.5.2 Scanning Electron Microscopy

Samples (1 mg) were suspended in ethanol (500 µL), dip coated onto the aluminum stub and sputter coated (Sputter Coater JSM 820, JEOL Ltd., Japan) with gold and platinum for imaging by SEM (JSM 6360-A, JEOL Ltd., Japan) coupled with elemental analysis by EDXA (JED 2300LA, JEOL Ltd. Japan).

2.5.3 Transmission Electron Microscopy

Samples (1 mg) were fixed in 3% glutaraldehyde at 4°C for 1 h, washed with 0.1 M sodium cacodylate buffer (three washes of 10 min) and stained with 1% osmium tetroxide at 4°C for 1 h. The excess stain was washed with 0.1 M sodium cacodylate buffer followed by dehydration with ethanol gradient series (50 - 100%). The samples were resuspended in absolute ethanol-Araldite-A (1:1) mixture and incubated at 60°C for 1 h. They were then incubated in Araldite-A at 60°C overnight and mixed with Araldite-B (at 60°C for 48 h) to allow polymerization. Ultra thin sections (60-70 nm) of the polymer embedded samples (obtained using LKB 2088 Ultra microtome, Bromma, Sweden) were collected on carbon coated copper grids and imaged with a transmission electron microscope (JEM 109, JEOL Ltd., Japan).

2.5.3 Atomic Force Microscopy

Glass cover slips cleaned with Piranha solution were coated (Photo Resist spinner, Ducom Ltd., India) with 500 µL aliquots of DPX mixture (a commercial preparation containing distyrene, a plasticizer and xylene). This was followed by spin coating sporopollenin suspension (1 mg in 200 µL absolute ethanol). The sample was then scanned with an Atomic Force Microscope (MultiView 1000, Nanonics Imaging Ltd., Israel) in tapping mode (10 nm optical fiber probes, 3 N/m Force constant, 25 – 80 kHz resonant frequency, 256 x 256 pixels). Acquired images were processed using AFM-
SPM (Atomic Force Microscopy - Scanning Probe Microscopy) software (WSxM 4.0, Nanotec Electronica S. L., Spain).

2.5.5 Mercury Intrusion Porosimetry

Sporopollenin sample (5-10 mg) filled in a capillary was subjected to mercury Intrusion Porosimetry (Pascal 440, Thermoquest Italia S.p.A, Italy) in the range of 0.1 pa – 400 KPa pressure.

2.5.6 Elemental Analysis

The elemental contents viz. Carbon, Hydrogen, Nitrogen, Sulfur and Oxygen were determined by a CHNS analyzer (Vario ELIII, Varian Inc., USA). sporopollenin (2 mg) was used in this experiment.

2.5.7 Fourier Transform Infrared Spectroscopy

Sporopollenin microcapsules (20 mg), mixed with an equal amount of KBr were pelletized and used for the IR measurements. The FTIR spectra of Lycopodium clavatum sporopollenin samples (standard procured from Polysciences Inc., USA and isolated preparation obtained in the present study) were recorded on a IR spectrophotometer (Spectra One, Perkin Elmer Inc., USA). The spectra were collected in a range between 4500 and 500 cm\(^{-1}\) with a resolution of 1 cm\(^{-1}\) (100 scans per sample).

2.5.8 Solid-state \(^{13}\)C Nuclear Magnetic Resonance Spectroscopy

Purified sporopollenin sample (5 mg) was placed in 7 mm diameter spinning rotors and spun at high speeds (3-14 kHz) to acquire \(^{13}\)C NMR spectrum (500 MHz NMR spectrometer, Bruker Corporation., Germany) with sufficient resolution.

2.5.9 Diffuse Reflectance Spectroscopy

Absorbance and reflectance spectra (in the range of 200-1200 nm) of pelletized sporopollenin microcapsules (100 mg) were recorded using UV-Vis spectrophotometer (UV 3600, Shimadzu Corporation, USA) operated in diffuse reflectance mode (DRS).
2.5.10 Solid-state-spectrofluorimetry

A pellet of sporopollenin (10-20 mg) was used to measure its autofluorescence using a spectro-fluorimeter (FluoroLog® 3.11, Horiba-Jobin Yvon Inc., Germany). Sample was excited with 366 nm light to measure its emitted light.

2.5.11 Differential Scanning Calorimetry

Melting temperature (Tm) of sporopollenin microcapsules was measured using a Differential Scanning Calorimeter (Pyris 1, Perkin Elmer Inc., USA). Sample was heated from ambient temperature 25 ± 2°C to 500°C in air with 10°C min⁻¹ scan rate.

2.5.12 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis of oven dried sporopollenin sample was carried out (at 50 mL min⁻¹ airflow, temperature range: ambient to 900 °C, ramp rate 10°C min⁻¹) using a thermal analyzer (Star system 851, Mettler-Toldeo International Inc., Switzerland).

2.5.13 Differential Thermal Analysis

Thermogravimetric Analysis (TG) and Differential Thermal Analysis (DTA) of the samples were carried out using a thermal analyzer (Diamond TG/DTA, Perkin Elmer Inc., USA) under air from 45°C – 1200°C with 10°C min⁻¹ scan rate.

2.6 Qualitative and Quantitative Analysis of DNA

2.6.1 Spectrophotometric DNA estimation

Spectrophotometric estimation and quantification of DNA were carried out using a cuvette-less spectrophotometer (ND 1000, Nanodrop Inc., USA) with xenon lamp as a light source and CCD array detector. Absorbance of DNA in Tris-EDTA buffer (pH 8.0), considering the latter as a blank, were measured in
the range of 200–800 nm. Absorbance at 260 nm and ratio of absorbance at 260/280 were used to calculate the DNA concentration (ng/µL) with the inbuilt software (ND-1000 Ver 3.1.0, Nanodrop Inc., USA).

2.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA samples was performed using 0.75%, 1% and 1.5% agarose in 1 x TAE for genomic DNA, plasmid DNA and PCR products, respectively at 80V-100V for 60-90 min (Voytas, 2000) using a submarine electrophoresis unit and a power unit (Bio-rad Laboratories, USA). The gels were stained with 10 µg/mL EtBr and observed using a gel documentation system (Alpha Innotech. Corp., USA).

2.6.3 Restriction digestion pattern of plasmid DNA pUC18

pUC18 (100 ng) was mixed and incubated with the restriction enzyme Bgl I (Bangalore Genie, India) in 1x assay buffer, at 37°C for 2 h (Bloch, et al., 1995). It was then subjected to gel electrophoresis (see section 2.6.2).

2.6.4 Transformation efficiency

The functional efficiency of DNA was investigated by transformation. Transformation of 100 ng plasmid DNA (pUC18) was carried out using 50 µL competent cells of E. coli strain DH5α or JM109 by heat shock at 42°C for 2 min (Seidman, et al., 1997). The cell suspension (25, 50 & 100 µL) was spread on to the Luria Bertani agar plates supplemented with ampicillin (100 µg/mL). The plates were incubated at 37°C for 16–18 h. Efficiency of transformation was calculated by the formula: Transformation efficiency = Number of colonies x 1000 ÷ amount of DNA plated.

2.6.5 Real time Polymerase Chain Reaction

Real-time PCR experiments were performed using a Real Time PCR system (Light Cycler® 2.0, Roche Diagnostics, USA). The 10 µL PCR reaction mixtures contained 0.36 µM forward primer, 0.28 µM reverse primer, 2x SYBR Green I mixture (Light Cycler® Fast Start DNA Master Plus SYBR Green I) and template DNA.
Thermo cycling conditions were: an initial step at 95°C (15 min), 30-35 cycles of the following steps: 95°C (10 sec), 56°C (15 sec) with single fluorescence detection after 10 sec in each cycle, and 72°C (15 sec). Melt peaks were obtained by plotting the negative derivative of SYBR Green I fluorescence with respect to temperature (\( -dF/dT \)) against temperature. Standard stock DNA samples (25, 50, 75, 100, 125 and 150 ng/µL) were amplified to plot a standard curve and crossing point value (Cp) as well as copy number was calculated (See Annexure II).

2.6.6 Apurinic / Apyrimidinic (AP) site quantification

AP site quantification was carried out using DNA damage quantification kit (Biovision Research Products, USA). The spectrophotometric avidin-biotin assay was carried out according to the instructions provided by the manufacturer as follows:

Sample DNA (5 µL) was mixed with 5 µL ARP solution in a 0.5 ml tube, and incubated at 37°C for 1 h to tag biotinylated ARP probe to the probable AP sites. Tris-EDTA buffer (88 µL) and glycogen (2 µL) was added to the reaction mixture and mixed well. Absolute ethanol (300 µL) was added and kept at -20°C for 10 min to precipitate the DNA. AP site-tagged DNA was pelleted down by centrifugation at high speed for 10 min. Pellet was washed with 500 µL 70% ethanol, and air dried. This biotin tagged (AP site) DNA was dissolved in DNA Tris-EDTA buffer to use for avidin-biotin assay.

AP site-tagged DNA samples and 0 to 40 ARP-DNA standards (0, 8, 16, 24, 32 and 40 ARP sites per 10^6 bp DNA), 250 µL each was mixed with 100 µL DNA binding solution and kept at ambient temperature (25 ± 2°C) overnight. DNA binding solution was discarded after 16 h and the wells were washed thrice with 250 µL 1x wash buffer provided with the kit. HRP-Streptavidin solution (100 µL) was added to each well and the plate was kept on a rocker for 1h at ambient temperature (25 ± 2°C). The solution was discarded and washed five times with 1x wash buffer. 100 µL of HRP developer (TMB-H₂O₂) solution was added in each well and the plate was incubated at 37°C for 1 h. Optical density was measured at 650 nm immediately after the reaction.
Calibration curve was plotted using the data obtained with standard ARP-DNA solutions. Absorbance values obtained from samples were applied to the standard curve to calculate the number of AP sites per 10^5bp in the DNA exposed to UV and high temperature.

2.7 DNA-sporopollenin interactions

2.7.1 Effect of sporopollenin on structural intactness of DNA

Effect of sporopollenin on structural intactness of DNA was investigated using Plasmid DNA (pUC18), Lambda DNA and Lambda DNA Eco RI/ Hind III double digest marker.

Experiment was performed in two sets, viz. Set-A: different concentrations of DNA (10, 25 and 50 ng/μL) incubated with a fixed quantity of sporopollenin (10 μg/μL) and Set-B: Fixed concentration of DNA (25 ng/μL) incubated with varying concentrations of sporopollenin (5, 10 and 50 μg/μL). The total reaction volume in both the experiments was 10 μL in T_{10}E_{1} buffer (pH 8.0) and incubations were carried out at 4°C for 24 h. Sporopollenin was separated from the mixtures by centrifugations and DNA samples in the supernatants were electrophoresed to check their structural intactness (See section 2.6.2).

2.7.2 Effect of sporopollenin on functional efficiency of plasmid DNA

The functional efficiency of DNA associated with sporopollenin was tested by transforming the plasmid DNA pUC18 (50 ng/μL) incubated with 10 μg/μL sporopollenin at 4°C for 24 h and the transformation efficiency was calculated as described in section no. 2.6.4.

2.7.3 Effect of sporopollenin on restriction digestion pattern

Restriction digestion of pUC18 (50 ng/μL) was carried out with Bgl I in the presence of sporopollenin (10 μg/μL) as described in section 2.6.3. The
samples were then subjected to gel electrophoresis as described in section 2.6.2.

2.7.4 Effect of sporopollenin on polymerase chain reaction

Three different concentrations of Sporopollenin 1, 5 & 10 µg/µL in deionized sterile water was added to the master mix of 800 bp template DNA (100 ng/µL), reverse primer, forward primer and Taq polymerase. Amplification by PCR was carried out for 30 cycles. After PCR, the mixture was electrophoresed as described in section 2.6.2.

2.7.5 Binding and release of DNA from sporopollenin microcapsules

The binding and release of DNA was studied based on DOE experiment based on 3 factors with 2 levels each viz. state of DNA-sporopollenin (dehydrated and in-solution), concentration of Lambda DNA Eco RI / Hind III double digest marker (50, 100 ng/µL), concentration of sporopollenin (5, 50 µg/µL), using L4 orthogonal array (Table 2.1 and 2.2). The DNA and sporopollenin were mixed and dried together. The Tris-EDTA buffer was added and the supernatant was electrophoresed to assess the DNA release.

![Table 2.1: Factors and levels](image)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. State of DNA-sporopollenin</td>
<td>Dehydrated, In-solution</td>
</tr>
<tr>
<td>B. DNA (ng/µL)</td>
<td>50, 100</td>
</tr>
<tr>
<td>C. Sporopollenin (µg/µL)</td>
<td>5, 50</td>
</tr>
</tbody>
</table>

![Table 2.2: L4 orthogonal array layout](image)

<table>
<thead>
<tr>
<th>Trials</th>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>State of DNA-sporopollenin</td>
</tr>
<tr>
<td>2</td>
<td>Dehydrated</td>
</tr>
<tr>
<td>3</td>
<td>In-solution</td>
</tr>
<tr>
<td>4</td>
<td>In-solution</td>
</tr>
</tbody>
</table>
2.8 Sporopollenin microcapsules for DNA encapsulation

DNA encapsulation in sporopollenin microcapsules was attempted with the help of two different methods.

2.8.1 Microinjecting DNA in sporopollenin microcapsules

A single sporopollenin microcapsule was held with the vacuum hold in the micromanipulation system. Plasmid DNA (20 µL) in Tris-EDTA buffer (pH 8.0) was injected into it using a micro injector (Leica DM IRB, Leica Microsystems Ltd., UK).

2.8.2 Vacuum enabled DNA encapsulation by filtration through layered sporopollenin microcapsules (FTLS) method

This novel patented technique of DNA encapsulation uses a device ‘ηCap’ fabricated from routinely available laboratory plasticware (Figure 2.2). The device consists of three parts (a) 0.5 mL filter tube fitted with silica disc, (b) 1.5 mL microcentrifuge tube with holes made on the sidewalls, and (c) 5 mL pipette tip. Part (a) fits into part (b) which then gets encased in part (c) as shown in Figure 2.2. The tip of part (c) is connected to a vacuum pump.

About 100-200 µL sporopollenin stock suspension (20 mg sporopollenin microcapsules in 1 mL absolute ethanol) was pipetted into the ‘ηCap’ device and vacuum applied so as to get a uniform compact layer of sporopollenin on the silica disc. In the next step ca. 100-200 µL DNA stock in Tris-EDTA buffer (100 ng/µL) was filtered through the sporopollenin bed while the vacuum was on. After about 15-30 s the vacuum was cut off to avoid possible escape of DNA from the sporopollenin bed. The DNA loaded sporopollenin microcapsules thus obtained were then allowed to dry in a laminar air flow hood for 5-6 h.

2.8.2.1 Confocal Laser Scanning Microscopy

The encapsulated DNA was mixed and kept for few minutes to stain with 2 µg/µL 4',6-diamidino-2-phenylindole (DAPI) and mounted using an anti-fade mounting medium (Chemicon International, USA). The samples were
observed at 400 x magnification under a multispectral CLSM (Zeiss LSM510 ver. 2.01, Carl Zeiss AG, Germany).

Figure 2.2: qCap. The device consists of three parts (a) 0.5 mL filter tube fitted with silica disc, (b) 1.5 mL microcentrifuge tube with holes made on the sidewalls, and (c) 5 mL pipette tip. Part (a) fits into part (b) which then gets encased in part (c). The tip of part (c) is connected to a vacuum pump as shown in (A) and (B) shows the photograph of the device.

2.9 Thermal stability of sporopollenin encapsulated plasmid DNA

To assess the thermal stability of sporopollenin encapsulated plasmid DNA, Taguchi DOE with four factors and three levels each viz. concentration of pUC18 (25, 50 and 100 ng/μL), concentration of sporopollenin (5, 10 and 50 μg/μL), exposure time (15, 30 and 60 min) and exposure temperature (80, 100 and 120°C).

The different factors and their levels considered are shown in Table 2.3, while Table 2.4 shows the array layout. In addition, nine un-encapsulated DNA controls were also used. All DNA samples were encapsulated as per FTLS method.
DNA was released from the experimental samples using Tris-EDTA\(^1\) \((T_{10}E_{10})\) buffer \((pH 8.0)\). Subsequent analysis of thermal stability comprised of agarose gel electrophoresis and restriction digestion pattern to determine structural intactness and sequence intactness, respectively. The quality and quantity of the released DNA was estimated in terms of its transformation efficiency and Real Time PCR analysis. Real-time PCR of samples from nine trials with respect to their controls was carried out using a forward primer 5’-TCCCGGAGACGGTCAC-3’ and a reverse primer 5’-

\(^1\) Volume of Tris-EDTA buffer taken to release the DNA from sporopollenin microcapsule was equal to the volume of DNA solution used for encapsulation as well as in un-encapsulated controls
CAGGAAACAGCTATGACC-3' to amplify the 700bp MCS region of pUC18 (2686bp) as described in section 2.6.5.

Transformation efficiency was the criterion used to calculate signal to noise (S/N) ratios often expressed using the logarithmic decibel (dB) scale on the basis of bigger-the-better condition and the influence of individual factors. These data were used to interpret the effect of sporopollenin on thermal stability of encapsulated plasmid DNA (See annexure III for a detailed introduction to S/N ratio and estimation of factor effect contributions).

2.10 Evaluation of heat and UV induced oxidative damage to sporopollenin encapsulated plasmid DNA

This experiment was performed using a representative DNA sample, viz. pUC18 (50 ng/µL, sporopollenin concentration: 10 µg/µL) encapsulated by FTLS method. This sample along with un-encapsulated control DNA samples were heated at 100°C for 30 & 60 min and exposed to UV radiation (254 nm) for 1, 6, 12 and 24 h. DNA released from the samples was used for AP site quantification as described in section 2.6.6.

2.11 Thermal stability of sporopollenin encapsulated genomic DNA

These studies were carried out on two different genomic DNA samples encapsulated in sporopollenin microcapsules by FTLS method and their thermal stabilities were assessed using two distinct sets of criteria:

• *S. cerevisiae* DNA (50 ng/µL in 10 µg/µL sporopollenin microcapsules) was exposed to 50, 60, 70, 80°C for 10, 30, 60 & 120 min. Controls (un-encapsulated DNA samples of same concentration and volume) were run simultaneously. DNA released from the samples was electrophoresed in an agarose gel to get qualitative assessment of thermal stability.
Allium cepa DNA (50 ng/µL in 10 µg/µL sporopollenin microcapsules) was heated from 45°C to 1200°C (ramp rate 10°C/min) in a TGDTA analyzer (Diamond TG/DTA, Perkin Elmer Inc., USA). Controls, viz. sodium salt of lyophilized Allium cepa DNA (5.7 mg) and sporopollenin microcapsules (3.031 mg) were also treated in a similar fashion. Thermal stability was assessed in terms of DNA degradation as revealed by Differential thermogravimetry (DTG), Differential thermal analysis (DTA) and Thermogravimetric analysis (TGA).

2.12 Shelf-life of the sporopollenin encapsulated DNA

2.12.1 Accelerated aging experiment to predict plasmid DNA quality after one year storage

In this experiment, DNA quality after one year of storage in sporopollenin microcapsules was assessed on the basis of accelerated aging test using Arrhenius equation (see complete description in Annexure III). According to accelerated tests, the samples stored at elevated temperature can be used to extrapolate the storage time at ambient temperature e.g. the shelf life of DNA samples stored at 60°C for one month can be extrapolated to shelf life of one year at ambient temperature (25 ± 2°C) based on the equations;

\[ r = \frac{\Delta T}{\Delta t} = \frac{Q_{10}}{T_{e} - T_{a}}^{10} \]

(Where \( r \) = accelerated aging rate, \( Q_{10} = 2 \), \( T_{e} = \) elevated temperature, \( T_{a} = \) ambient temperature)

\[ \text{Accelerated Aging Time} = \frac{\text{Desired Real Time}}{\text{Accelerated Aging Rate}} \]

Accelerated aging time or AAT (extrapolation) can be calculated by dividing desired real time by accelerated aging rate.

Example (i): When \( T_{e} = 60°C \), \( T_{a} = 25°C \) and \( Q_{10} = 2 \) (Industrial standard for accelerated aging studies of medical grade polymers)

Therefore, \( r = 11.31 \)

Hence, AAT = 365 days \( \div \) 11.31 = 32.27
Therefore, if a sample stored at 60°C for 32.27 days remains stable, it can be extrapolated to its stability at 25°C for 365 days (See section 4.5.1).

Example (ii): When Ta= 30°C considering all other parameters are as in example (i)

\[ r = 8 \]

Hence, \( AAT = 365 \text{ days } \pm 8 = 45.62 \)

Therefore, if a sample stored at 60°C for 51 days remains stable, it can be extrapolated to its stability at 30°C for 410 days (See section 4.5.2).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
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<tbody>
<tr>
<td>A. Conc. of DNA (ng/µL)</td>
<td>25 50 100</td>
</tr>
<tr>
<td>B. Conc. of sporopollenin (µg/µL)</td>
<td>5 10 50</td>
</tr>
<tr>
<td>C. Time (days)</td>
<td>1 7 32</td>
</tr>
<tr>
<td>D. Temperature (°C)</td>
<td>4 25 60</td>
</tr>
</tbody>
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Table 2.5: Factors and levels

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Control factors assigned to columns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>1</td>
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<td>8</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
</tr>
</tbody>
</table>

[Note: Nine trials as per L9 OA along with their controls were carried out in triplicates]

Table 2.6: L9 orthogonal array layout

45
Accordingly, DNA (pUC18) samples were encapsulated by FTLS method and stored at 4, 25 and 60°C for 1, 7 and 32 days. To get statistically valid and reliable data L9 orthogonal array was used with four factors and three levels as detailed in Table 2.5 and Table 2.6. In addition, nine un-encapsulated DNA controls were also used.

After the requisite period of storage as per the DOE, DNA was released from the samples using Tris-EDTA buffer. Subsequent analysis to assess the extent of preservation comprised of tests such as agarose gel electrophoresis, restriction digestion pattern and transformation efficiency to determine structural intactness, sequence intactness and functional efficiency, respectively.

Transformation efficiency was the criterion used to calculate signal to noise (S/N) ratios on the basis of bigger-the-better conditions and the influence of individual factors. These data were used to interpret stability of encapsulated plasmid DNA.

2.12.2 Predicting shelf-life of plasmid and genomic DNAs preserved in sporopollenin microcapsules - use of Artificial Neural Networks (ANN) based mathematical simulation

In this experiment quality of sporopollenin encapsulated plasmid and genomic DNA samples was assessed on the basis of accelerated aging test (Arrhenius model corresponding to 410 days storage). These data were then used to predict DNA quality with the help of Artificial Neural Networks (ANN) based mathematical simulation (see complete description of ANN in Annexure III).

The study comprised of L4 orthogonal array experiments carried out for 51 days as detailed in Table 2.7 and 2.8.

All the samples were encapsulated by FTLS method. In addition, four un-encapsulated DNA controls were also used. On every third day, DNA was released and tested for structural intactness using agarose gel electrophoresis and spectrophotometric estimation. The absorbances at 260
nm values were used for Taguchi S/N ratio analysis (nominal-the-best) to interpret DNA stability.

The 75, 15 and 10 % values (absorbance at 260 nm) were used for training, testing and validating the Artificial Neural Network, respectively. The experiment was based on L4 \( (2^3) \) orthogonal array as described above and involved a single input in ANN generated from multiple independent variables (two levels each of three factors). The single output absorbance at 260nm for every third day till 51 days was used in this study.

### Table 2.7: Factors and levels

<table>
<thead>
<tr>
<th>Control factors</th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Types of DNA</td>
<td>Calf thymus DNA</td>
<td>pUC18</td>
</tr>
<tr>
<td>B. Conc. of DNA (ng/µL)</td>
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</tr>
<tr>
<td>C. Temperature (°C)</td>
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<td>60</td>
</tr>
</tbody>
</table>

### Table 2.8: L9 orthogonal array layout

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Types of DNA</td>
<td>DNA (ng/µL)</td>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>1</td>
<td>Calf thymus DNA</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Calf thymus DNA</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>pUC18</td>
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<td>60</td>
</tr>
<tr>
<td>4</td>
<td>pUC18</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>

[Note: Four trials as per L4 OA along with their controls were carried out in triplicates]

In this modeling problem we have considered a multiple-input-multiple-output (MIMO) three-layer feed forward neural network, and the number of hidden nodes is chosen by increasing it from a small number until the learning performance in terms of fitness value is good enough.

This modeling problem was, a hybrid Taguchi-genetic algorithm (HTGA) is applied to solve the problem of tuning both network structure and parameters.
of a feed forward neural network using MATLAB. The HTGA approach is a method of combining the traditional genetic algorithm (TGA), which has a powerful global exploration capability, with the Taguchi method, which can exploit the optimum offspring. The Taguchi method is inserted between crossover and mutation operations of a TGA. Then, the systematic reasoning ability of the Taguchi method is incorporated in the crossover operations to select the better genes to achieve crossover, and consequently enhance the genetic algorithms. Therefore, the HTGA approach can be more robust, statistically sound, and quickly convergent.