CHAPTER III

MATERIALS AND METHODS

This chapter deals with the methods, procedure and standards required to develop a finish focused on anti-microbial and insect repellent finish to preserve the textiles. An extensive review and a survey were conducted at several textile museums and with individuals who are textile enthusiastic with their personal textile collection to understand the preservative practices used for the textiles. Later, an experimental approach was taken to understand the microflora environment of a preserved cellulosic and proteinic natured textile, isolation and identification of the microbes on a preserved cotton, wool, and silk fabric was performed. Finally, a finish was developed using different types of a two-step process, consisting of the microemulsion and ionic gelation method and emulsion and ionic gelation method to develop nano particles which were coated on two types of fabric with the help of a binder. The characterization and surface analysis of the nanoparticles and the coated fabric was conducted to test their structure, properties and its application for preserving textiles against microbes and insects. The study also compares the effectiveness of single and combined oil nanoparticles against microbes, and the efficacy of the finish on two types of fabrics used: cotton and polyester.

The chapter deals with the materials and methods followed for fulfilling the objectives of the study.

3.1 Studying the preservative practices followed at the museums and by the individuals at home

3.2 Studying the micro flora of the preserved cellulosic and protein textiles

3.2.1 Selection of the degraded textile

3.2.2 Isolating and identifying the microorganisms present on the preserved cellulosic and protein fabrics undergone deterioration.

3.2.3 Characterization and identification of bacterial isolates:

3.2.3.1 Morphological characterization

3.2.3.2 Biochemical characterization

3.2.3.3 Identification of fungal isolates

3.3 Preparation of nanoparticles (NPs)

3.3.1 Selection of core and wall material

3.3.2 Pilot work

3.3.2.1 Preparation of essential oil chitosan nanoparticles (CNPs)

- Oil in water (O/W) emulsion + Ionic gelation process of NPs
- Nano emulsion + Ionic gelation process of the NPs

3.3.2.1.1 Screening the parameters for developing neem essential oil nanoparticles.

3.3.2.1.2 Screening the parameters for developing cinnamon essential oil nanoparticles

3.3.2.1.3 Screening the parameters for developing clove essential oil nanoparticles.

3.3.2.1.4 Screening the parameters for developing carom essential oil nanoparticles.

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3.4.1 Effect of Chitosan concentration on the entrapment efficiency, loading capacity, and nanoparticle size

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3.4.4 Impact of % TPP on the entrapment efficiency, loading capacity, and the size of the nanoparticles

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3.5.2 Determination of encapsulation efficiency (EE%) and loading capacity (LC%)

3.5.3 Determining the particle size and the polydispersity index (PDI) of the prepared NPs

3.5.4 Determining the minimum inhibitory concentrations of the NPs (MIC)

3.5.5 Surface analysis of the prepared NPs using Scanning electron microscope (SEM)

3.6 Application of the optimized essential nanoparticles on the substrate

3.6.1 Selection of the substrate

3.6.2 Application of the single NPs and in combination of NPs

3.7 Assessment of the treated fabrics

3.7.1 Assessment of Antibacterial activity of the treated cotton and polyester fabric using standard AATCC 147

3.7.2 Determination of Antifungal test using standard AATCC 30: Antifungal Assessment and Mildew Resistance Test

3.7.3 Surface analysis of the uncoated and coated fabric using Energy dispersive X-ray spectroscopy (EDX)

3.7.4 Determining the physicochemical stability of the nanoparticles over time and storage conditions

3.7.5 Determination of resistance to insects using standard ISO 3998:1977

Research design:

The study is divided into three phases as shown below:

Phase I

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Studying the preservation practices followed at Museums and by individuals at home

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Content study of herbs and spices employed for textile preservation

↓

Identifying the microorganisms present on the deteriorated cellulosic and protein textiles

Phase II

Cotton fabric Polyester fabric

Preliminary Data

1. Fabric Count
2. Fabric GSM
3. Fabric Thickness
4. Fabric weave

Preparation of essential oil nanoparticles

Wall material:

Chitosan

Core material:

1. Neem oil
2. Cinnamon oil
3. Clove oil
4. Carom oil

Parameters:

- Chitosan %
- Polymer: Oil
- Surfactant %
- Crosslinking agent%
- Stirring rate

Analysing properties:

1. Determining the calibration curve of the essential oil.
2. Assessing the Size and Polydispersity index (PDI) of the nanoparticles
3. Determining the Encapsulation efficiency
4. Determining the Loading capacity
5. Determining the Minimum Inhibitory Concentration (MIC) of the nanoparticles against the selected microbial strains

Application of Nanoparticles on the substrate

- Spraying + Padding Method
- Single and Combination of nanoparticles

Phase III

↓

Analysing and comparing of functional compounds for the antimicrobial and insect repellent property and their efficacy during their encapsulation on cotton and polyester fabric

↓

1. Surface analysis of the nanoparticle using SEM
2. EDX analysis of the finished fabrics
3. Determining the physicochemical stability of the nanoparticles over time and storage conditions
4. Assessment of Antibacterial activity of the finished cotton and polyester fabric by Parallel Streak Method (AATCC 147)
5. Determination of Antifungal activity: Mildew and Rot resistance (AATCC 30)
6. Insect repellent test (ISO 3998: 1997)

3.1 Studying the preservative practices followed at the museums and textile collectors

The researcher had visited various museums to study the preservative practices followed at the museums, including the Calico Museum in Ahmedabad, Raja Dinkar Kelkar Museum in Pune, National Museum in New Delhi, Crafts Museum in New Delhi, and the Textile Art Museum at the Department of Clothing and Textiles, The Maharaja Sayajirao University of Baroda, Vadodara, Baroda Museum and Picture Gallery, Vadodara, Anne Lambert Clothing and Textile collection at the University of Alberta, Edmonton, Canada. The data was collected through discussions with the museum curators and by observation method. The researcher also conducted personal interviews with six purposively selected textile collectors using an open-ended questionnaire to gain in-depth insights on their preservative practices. Additionally, details on the textiles preservation and conservation methods were acquired from secondary data sources.

3.2 Studying the micro flora of the preserved cellulosic and protein textiles.

To understand the type and species of microbes present on the deteriorated textiles that were stored in preserved conditions. The entire procedure of isolating and identifying the microbes was carried out at the Department of Microbiology, Faculty of Science, The Maharaja Sayajirao University of Baroda.

3.2.1 Selection of the raw materials

Fabrics of both cellulosic and protein in nature were selected for the study. Three fabrics such as cotton, silk, and wool that had undergone degradation after storing in a preserving condition were been chosen for the investigation to isolate and identify the bacteria and fungi that had grown on them. To isolate and identify the strains, sodium chloride was used as a medium to extract the microbes from the fabric. Nutrient agar was used as a medium to isolate all the bacteria while Potato Dextrose Agar medium was used to isolate fungi. Pseudomonas agar and MacConkey agar were used to further classify and isolate gram-negative bacteria. Mannitol Salt Broth (MSB) medium was used for the identification of *Staphylococcus* species. A kit containing crystal violet stain, iodine, ethanol, and safranin stain was used to perform a gram staining test. 3% Hydrogen peroxide and phenylenediamine solution were used to perform catalase and oxidase test.

3.2.2 Isolating and identifying the microorganisms present on the preserved cellulosic and protein fabrics undergone deterioration.

Three samples of 10X10 cm each from the cotton, wool, and silk fabric was cut from the three different areas of the fabric. Each sample was soaked in 6 ml of 0.9% NaCl saline solution in a flask and kept at shaking condition at 500 rpm for 30 minutes. The fabric was squeezed to remove the excess saline solution and then transferred into three different Eppendorf tubes. These tubes were centrifuged at 7,000 rpm for 5 minutes until the pellet formation at the bottom surface of the tubes. The surface suspension liquid was discarded and 100 µm of NaCl solution was added to the pellets to prevent overcrowding and the formation of confluent growth followed by mixing thoroughly. With the help of micropipette, 100 µm of suspension of the solution from each tube was then placed in the centre of the two nutrient agar plates for bacteria and on potato dextrose agar medium for fungi and spread over the surface of the using a sterile glass spreader. By spreading the suspension over the plate, an even layer of cells is established so that individual bacteria are separated from the other in the suspension and at deposited at a discrete location (Figure 3.1). For the bacteria to grow, the nutrient agar plates were placed in an incubator at 37ºC for 24 hours and then observed the colonies for its further identification.

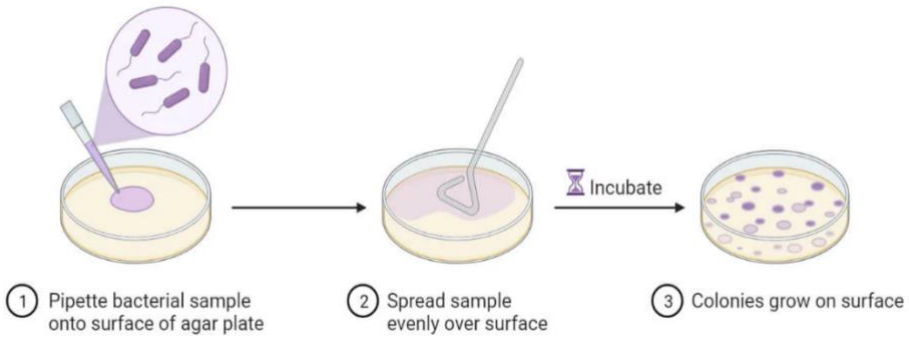

Fig. 3.1 : Spread plate method
Picture source: https://microbenotes.com/spread-plate-technique/

3.2.3 Characterization and identification of bacterial isolates:

3.2.3.1 Morphological characterization:

The identification of bacteria and fungi was based on how the organism grew on the media and it's colonial morphology. Gram staining results were observed under a compound microscope which further classified them into gram-positive and gram-negative bacteria. All the test procedures were conducted following the Bergey's Manual of Determinative Bacteriology (Bergey et al., 1974).

2.2.3.1.a Morphological characterization:

The morphological characterization of each bacterial isolate was performed according to colour, size, colony characterization (margin, form, and elevation) as seen in figure 3.2.

2.2.3.1.b Gram staining

Gram staining is a method used to differentiate bacterial species based on the characteristics of their cell walls, including their chemical and physical properties.

To conduct the test, a sample of bacteria was placed on a clean and oil-free slide; it was then left to air-dry and heat fixed. The slide was flooded with crystal violet dye for 30-60 seconds, then Lugol's iodine was added and left for an additional 60 seconds. After draining off the dye, the slide was washed with water and decolorized with ethanol for 10 seconds. Safranin was then applied to the slide for 60 seconds, and afterward washed and blotted dry. The samples were examined under a microscope with an oil immersion lens; this procedure was used to

differentiate between Gram-positive and Gram-negative bacteria, which showed up as blue-purple and pink-red, respectively, when stained with the Gram staining method as shown in figure 3.3.

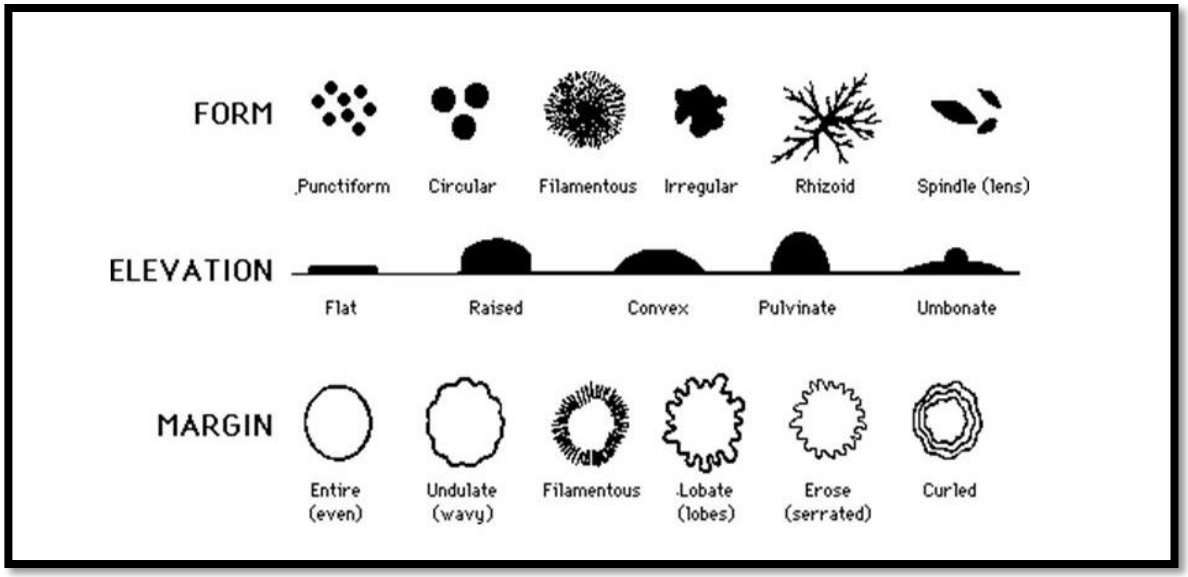

Fig. 3.2: Morphological characteristics of bacterial colonies
Picture source: https://microbeonline.com/colony-morphology-bacteria-describe-bacterial-colonies/

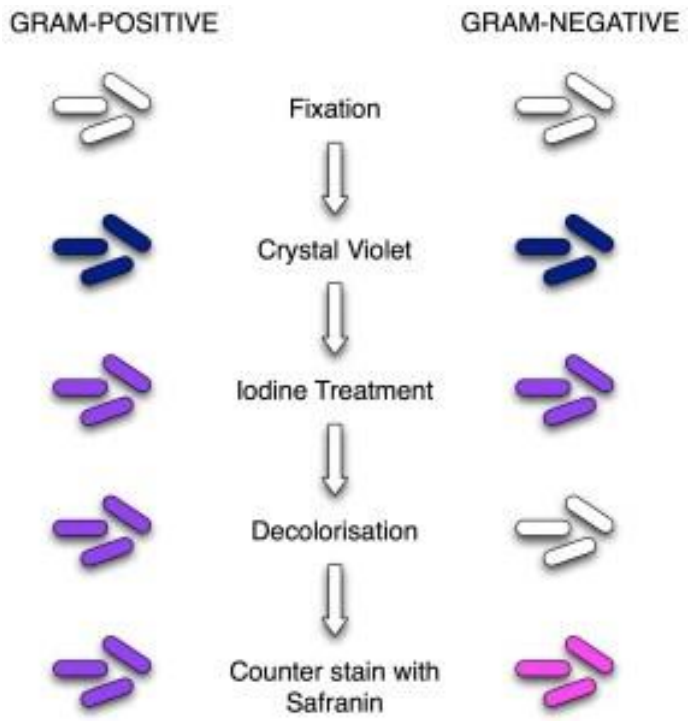

Fig 3.3: Steps for Gram staining test
Picture source: https://microbeonline.com/gram-staining-principle-procedure-results/

3.2.3.2 Biochemical characterization:

The bacterial isolates were further characterized using biochemical tests like Catalase, and Oxidase test, and Motility test as described in Bergey's Manual of Determinative Bacteriology (Bergey et al., 1974). Finally, the bacterial colonies were grown on selective media to confirm its identification. Certain types of media are designed to inhibit the growth of undesirable bacteria while promoting the growth of the desired microorganisms. Thereby, confirming their identification.

For the bacterial colonies that were unsure of its gram positive or gram negative nature after performing gram staining test, they were further grown on MacConkey agar to confirm their identification. Later, Pseudomonas Isolation Agar was used to confirm the Pseudomonas species and Mannitol Salt Agar (MSA) medium was used for the identification of *Staphylococcus* species.

3.2.3.2.a Catalase test

A positive reaction for the presence of catalase was tested by mixing a few drops of a 3% hydrogen peroxide solution with a loop full of colonies on a clean microscope slide. If bubbling or effervescence occurred, it indicated the presence of the enzyme catalase, which is found in obligate aerobes and most facultative anaerobes, and which breaks down hydrogen peroxide into water and oxygen gas.

3.2.3.2.b Oxidase test

This test is designed to detect the presence of cytochrome C oxidases, an enzyme found in obligate aerobic bacteria. To do this, a 1% aqueous solution of the oxidase reagent was prepared by dissolving 1g of N1 N-Dimethyl phenylenediamine Dihydrochloride. This solution was then used to soak a small piece of Whatman filter paper. With the assistance of a glass rod, a small amount of the bacterial colony was rubbed onto the filter paper. If the enzyme is present, it will catalyse the transport of electrons between the electron donors in the bacteria and the reagent, causing the reagent to be reduced to a purple-blue colour within 10 seconds, indicating a positive result.

3.2.3.2.c Motility test

The motility test is a biochemical test used to determine whether a bacterial species is motile or non-motile. Motility is the ability of bacteria to move towards or away from a particular stimulus or in a random pattern, and it is an important characteristic used to differentiate between different bacterial species. The test was performed using a microscope to observe the movement of bacterial cells directly. Saline solution was added in the centre of a sterilize slide and using a sterile wire loop, a small amount of bacterial culture was transferred to the drop of saline solution and mixed together thoroughly. A cover slip was placed carefully and observed under the microscope with the 10x or 20x objective lens and observed for any movement of the cells for a minimum of 10 minutes to ensure the presence or absence of motility. d. Record the observations and results. If the bacterial cells show movement like swimming, twitching, gliding, etc they are considered motile.

3.2.3.2.d Selective Media growth

The colonies which were identified as Gram-negative bacteria were further sub-cultured and grown on MacConkey agar using the streak plate method as shown in figure 3.4 for their conformation. The isolates that were unable to ferment lactose and so formed un-dyed colonies on the MacConkey medium pointed them towards *Pseudomonas species*. Further, these colonies were grown on Pseudomonas agar for their verification.

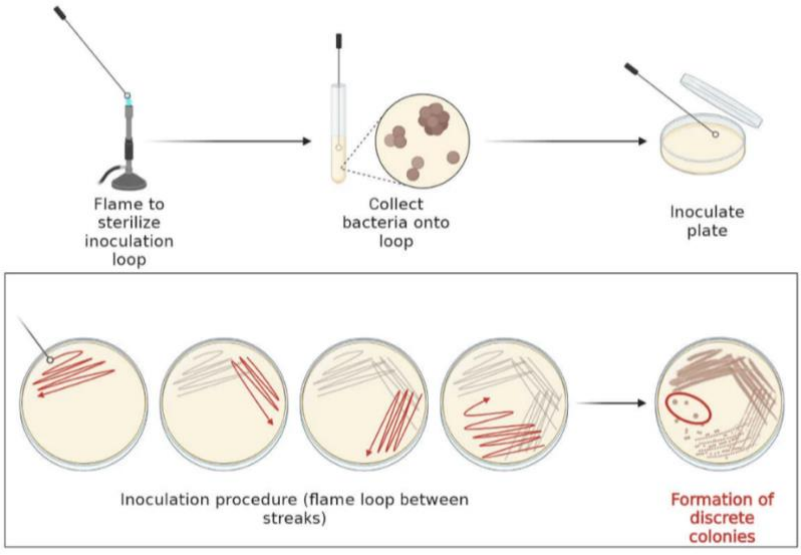

Fig. 3.4: Procedure for streak plate method
Picture source: https://microbenotes.com/streak-plate-method-principle-methods-significance-limitations/

3.2.3.3 Identification of fungal isolates:

Fungal isolates were grown at the room temperature on Potato dextrose agar medium for three to four days and then observed under a compound microscope. Fungi were identified on the basis of their spore and mycelial morphology.

3.3 Preparation of nanoparticles (NPs)

The preparation of the essential oil nanoparticles was done in order to repel the microbial colonies and the insects selected under the study. The preparation of NPs and its characterization was carried out at the Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda.

3.3.1 Selection of the raw materials

The selection of essential oils like neem, clove, cinnamon, and carom for the study was based on the interviews conducted with textile collectors, museum curators and conservators, as well as information from the literature review. These oils possessing antimicrobial and insect repellent property were used as a core material for the nanoparticle. Neem oil was purchased from Sigma Aldrich Co. Whereas, Clove and Cinnamon oil were sponsored by Jess Black Co, Alberta, Canada. Carom oil were purchased from local Ayurvedic store in Pune, Maharashtra. Chitosan well known for its use in encapsulating essential oils and its antimicrobial and mucoadhesive properties, was chosen as the wall material/shell due to its great matrix capabilities. A medium molecular weight chitosan of 84.8% degree of deacetylation purchased from HIMEDIA was selected for the study. Sodium tripolyphosphate (TPP), Tween 80, Poloxamer 188 were purchased from Sigma Aldrich Co. Glacial acetic acid and solvents such as Dichloromethane, Ethanol, Methanol was also purchased from Sigma Aldrich Co.

3.3.2 Preparation of essential oil chitosan nanoparticles (CNPs):

The preparation of the selected essential oil nanoparticles such as neem, clove, cinnamon, and carom using chitosan as a polymer was done by two methods: Emulsification followed by ionic gelation and Nano-emulsion followed by ionic gelation.

3.2.2.1 Oil in water (O/W) emulsion preparation + Ionic gelation process

Preparation of Essential oil (EO) loaded Chitosan nanoparticles (CNPs) was performed at two steps based on the method described by (Shetta, 2017) with slight modifications. The process of EO encapsulation in CS NPs relied on two steps as shown in figure 3.5. Different concentrations (w/v) of chitosan were prepared in 1% acetic acid by agitating the solution overnight at 25 °C. Following this, a surfactant was added to this solution and stirred for another two hours. To prepare the oil phase, the selected essential oil was dissolved separately in 10 ml of suitable solvent and added drop wise into the chitosan surfactant solution dropwise using a syringe and the solution was stirred for another 2 hours. Later, 3 ml of different concentrations of a negatively-charged cross-linker sodium tripolyphosphate (TPP) were added to the solution drop wise using a syringe to interconnect the positively charged chitosan polymer chains. The solution was further agitated for one hour at 25°C.

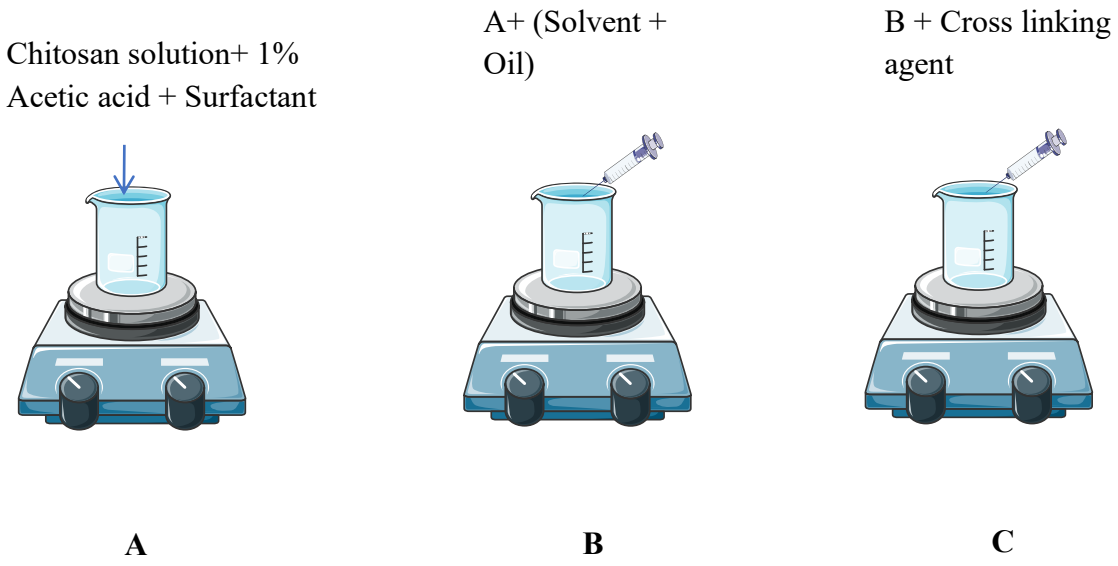

Fig. 3.5: Emulsification + Ionic gelation method of preparing EO CSNPs

3.2.2.2 Nano-emulsion + Ionic gelation process

Nanoparticles with essential oil core surrounded by a chitosan polymeric shell were prepared by two step method, i.e., microemulsion and ionic gelation method. Figure 3.6 represents the procedure followed. Different percentages of chitosan solutions were prepared by dissolving chitosan in 1% acetic acid (w/v) and stirred on a magnetic stirrer for one hour at 25°C or until the chitosan was dissolved completely. Subsequently, Tween 80 surfactant of different concentrations were added to it and was continued stirring for the next two hours.

Then, different percent of essential oils batches were prepared separately in 10 ml of suitable solvent. This oil and solvent solution were added in the chitosan surfactant solution drop by drop using a syringe and stirred on a high-shear homogenizer at different stirring rates for 10 minutes. The solution was then moved on a magnetic stirrer and agitated at 1800 RPM for two hours until the solvent evaporated completely. Later, in the step 2 process of ionic gelation a negatively-charged cross- linker such as 3 ml of sodium tripolyphosphate (TPP) was added to the nano-emulsion in different concentrations drop wise using a syringe. The solution was further agitated for one hour at 25°C.

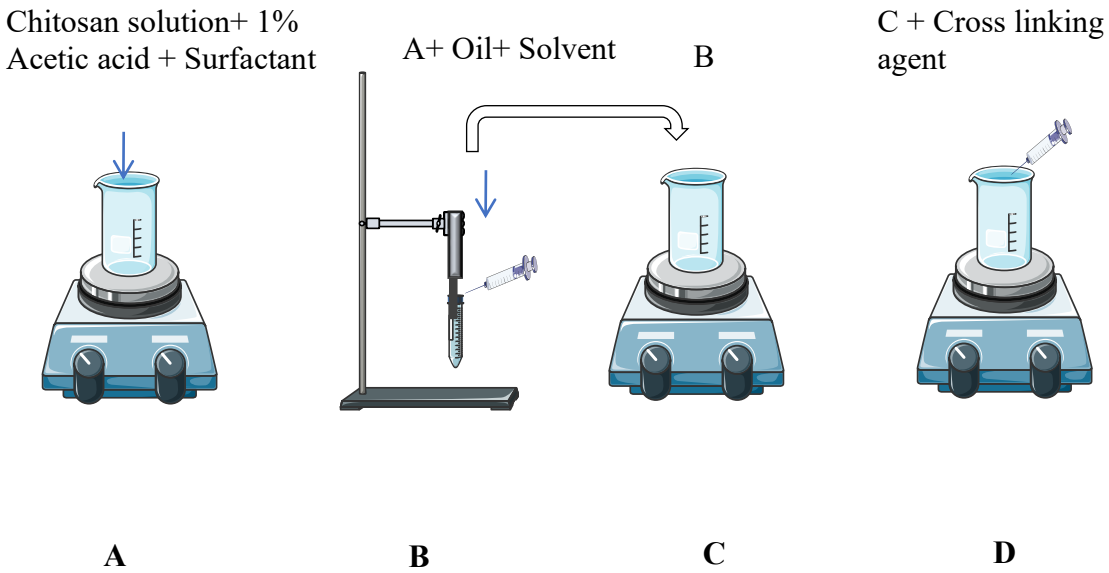

Fig 3.6 : Nano-emulsion + Ionic gelation method for preparing EO CSNPs

High-shear homogenizer:

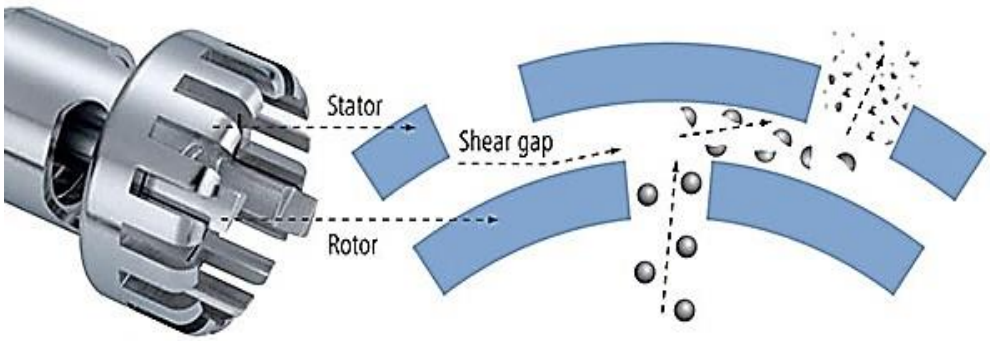

Fig 3.7 : High-shear homogenizer
Picture source: Shetta (2017)

A high-shear homogenizer is a mechanical homogenizer that utilizes cutting blades to mix materials. It consists of a rotor that spins at high speeds, creating a strong shear force in the gap between the rotor and stator. This force causes the material to be expelled from the gaps, leading to cavitation and turbulence. As a result, large materials are broken down into nano-sized particles quickly. This process is illustrated in figure 3.7. Rotor-stator generators are primarily used to create emulsions and dispersions in a nano-sized form.

3.4 Optimization of the NPs formulation and process parameters

All the formulation and the process parameters (Table 3.1) were optimized on the basis of highest entrapment efficiency obtained for each essential oil.

Table 3.1: Formulation and process parameters

Sr.No	Parameters	Values			
1	Chitosan (%)	0.5	1	1.5	2
2	Polymer: Oil	1:1	1:2	1:3	-
3	Surfactant (%)				
	Tween 80 (Poloxamer 188)	0.5	0.75	1	2
4	Sodium tripolyphosphate (TPP) %	1	2	3	-
5	Method of preparation	Emulsification + Ionic gelation		Nano-emulsion + Ionic gelation	
6	RPM (Emulsification+ Ionic gelation)	1200	1500	18800	-
7	RPM (Nano- emulsion+ Ionic Gelation)	10,000	13,000	16,000	-

3.4.1 Optimizing the Chitosan polymer percent.

Different percentages of chitosan solutions (0.5, 1, 1.5, and 2%) were prepared by dissolving chitosan in 1% of acetic acid (w/v).

3.4.2 Optimization of the Chitosan polymer: core/ oil ratio

Different percent of both the polymer: oil ratios- 1:1, 1:2, and 1:3 batches were prepared separately in 10 ml of solvent suitable for the significant essential oil.

3.4.3 Optimizing the surfactant percent

Two different types of surfactants, Tween 80 and Poloxamer, were tested in various concentrations (0.5, 0.75, 1, and 2%) for the preparation of nanoparticles using by both the methods. The surfactant providing the highest entrapment efficiency at its best concentration was chosen for the particular oil.

3.4.4 Optimizing the TPP percent

Different concentrations of Sodium Tripolyphosphate (TPP) (1%, 2%, and 3%) were utilized to fabricate nanoparticles, with the aim of optimizing the concentration with the maximum entrapment efficiency of the essential oil.

3.4.5 Optimizing the stirring rate

For the first technique of emulsification and ionic gelation, the chitosan and oil/solvent mixture was stirred on a magnetic stirrer at speeds of 1200, 1500, and 1800 RPM. The second technique of microemulsion and ionic gelation was done with a high shear homogenizer at speeds of 10000, 13000, and 16000 RPM for 10 minutes. After that, the solution was blended on a magnetic stirrer at 1800 RPM for two hours. The chosen stirring times in both the techniques were based on established reviews and served as critical parameters for achieving optimal emulsification.

3.5 Characterization of the prepared NPs

3.5.1 Determining the calibration curve of the essential oils using Ultraviolet/Visible spectroscopy (UV-Vis spectroscopy)

To determine the entrapment efficiency of the essential oils in the nanoparticles, a calibration curve was created using a series of reference standards of known concentrations of the oils. 100 µL of the oils were dissolved in 10 mL of the appropriate solvent in individual tubes, then sonicated for three cycles and the volume was brought up to 100 mL with the suitable solvent. Aliquots of the stock solution were taken and diluted to the desired concentrations for six different concentrations. These solutions with different concentration where then analyzed by a UV spectrophotometer at the maximum wavelength (λ max). All the measurements were done in triplicate and a regression coefficient (R2) was obtained for each oil.

The principle and the mechanism of the Ultraviolet/Visible spectroscopy (UV-Vis spectroscopy) is as follows:

UV-Vis spectroscopy is a method that is employed to determine both the qualitative and quantitative composition of a given compound. It is based on the absorption of UV and visible radiations from the electromagnetic spectrum, with energies that vary from 36 to 143 kilocalories per mole. This energy is sufficient to cause electrons to jump from the ground state to an excited state. The energy of these UV and visible light waves can be calculated by the equation.

$$E= h. \nu.$$

Where E is the energy of the radiation, h is Planck's constant and ν is the frequency of the radiation. The wavelength at which maximum absorption occurs, also known as λmax, is unique to different chemical species, making UV-Vis spectroscopy a reliable way to identify compounds qualitatively. Additionally, UV/Vis spectroscopy can be used to measure the concentration of a given compound through the use of the Beer-Lambert law, shown in the equation.

$$A = \varepsilon . b . c.$$

The absorbance (A) of a sample is proportional to its absorptivity coefficient (ε), path length (b) of radiation passing through a cuvette containing the sample, and its concentration (c). Thus, by measuring the absorbance of the sample with an instrument, its concentration can be determined.

In the UV/Vis spectrophotometer depicted in figure 3.8, there is a radiation source, which could be either a deuterium lamp (for UV radiation within the 200-400 nm range) or a tungsten-halogen lamp (for visible radiation within the 400-800 nm range). It also includes a monochromator that selects a specific portion of the electromagnetic radiation to pass through the sample, either a grating or a prism. The sample holder can be either plastic cuvettes (for visible radiation) or quartz cuvettes (for UV and visible radiation). Finally, the detector, which could be a photomultiplier tube detector or a photodiode, transforms the transmitted light beam into an electrical signal (Shetta, 2017).

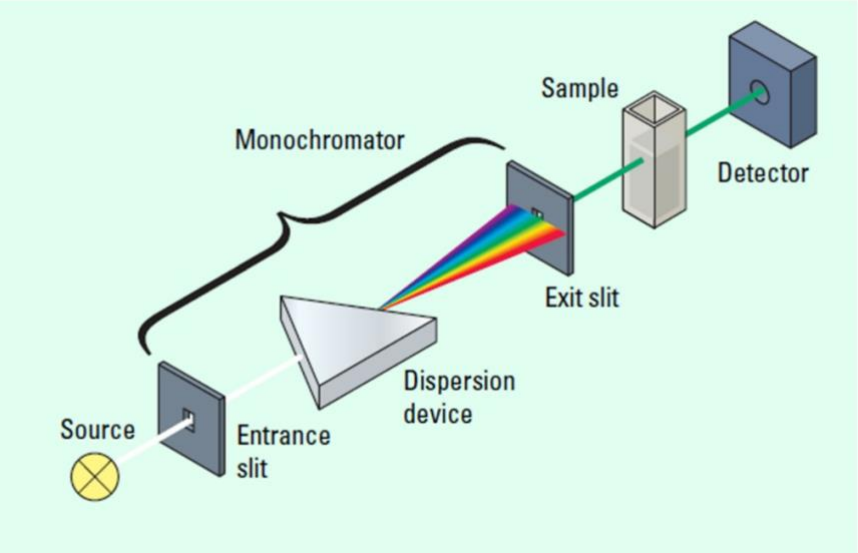

Fig 3.8: UV- Vis spectrophotometer
Picture source: Shetta (2017)

3.5.2 Determination of encapsulation efficiency (EE%) and loading capacity (LC%)

Entrapment efficiency is a measure of how much of the loaded substance remains in the nanoparticle after it has been loaded while loading efficiency is a measure of how much of a desired substance, such as an essential oil, can be loaded into a nanoparticle.

In 100 µL of freshly prepared essential oil loaded chitosan nanoparticles, 900 µL of the solvent suitable for the essential oil was added to make up 1 ml. This solution was then centrifuged at 18000 rpm for 30 min. The supernatant was separated to estimate drug loading efficiency. Further, the free amount of oil was calculated by measuring the absorbance of all the essential oils at its significant wavelength (nm). This was then compared with their standard curve. The drug encapsulation efficiency and drug loading capacity were calculated using the formula below:

$$Entrapment\ efficiency\ (EE) = \frac{Total\ oil - Free\ oil}{Total\ oil}\ X\ 100$$

$$Loading\ capacity\ (LC) = \frac{Total\ oil - Free\ oil}{Weight\ of\ the\ solid\ content}\ X\ 100$$

3.5.3 Determining the particle size and polydispersity index (PDI) of the preparade NPs

The size and the PDI of the nanoparticles were analysed using Dynamic light scattering (DLS, Malvern Zeta sizer). The principle and mechanism of the Dynamic Light Scattering (DLS) is mentioned below:

Dynamic Light Scattering is a technique that utilizes a laser beam to shine on suspended particles, and then it analyzes the intensity variations of the scattered light. It usually contains six components as seen in Figure 3.9a, a laser light source, a sample in a plastic cuvette, two avalanche photodiode detectors at certain angles, an attenuator to adjust the scattering level, a collector, and finally a display software. The DLS system measures particle size by measuring the Brownian motion (random collision) between nanoparticles and solvent molecules using the Stokes-Einstein equation.

$$D(H)= kT/3\pi\eta D$$

Using the Stokes-Einstein equation, the hydrodynamic radius (d(H)) of a nanoparticle can be determined by measuring the velocity of its Brownian motion. This is because the hydrodynamic size is linked to a thin layer of liquid medium that envelops the outer surface of the nanoparticle and affects its Brownian motion. Small particles experience more random collisions with the solvent molecules than larger particles, resulting in the faster rate of scattered light fluctuation for smaller particles. This phenomenon can be observed in Figure 3.9b. DLS is a non-invasive technique that requires minimal sample preparation and doesn't require any calibration. Additionally, DLS instruments are compact, affordable and user friendly, allowing users to perform comprehensive data analysis (Shetta, A.)

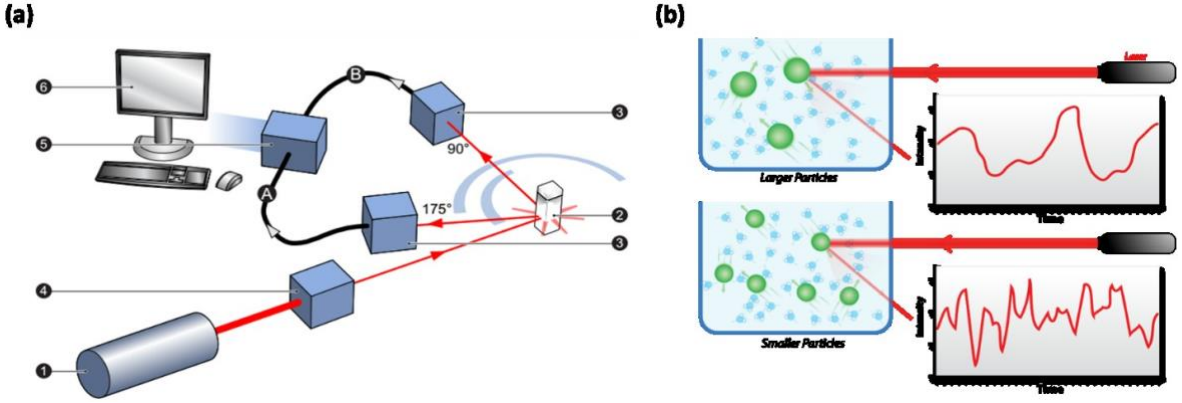

Fig: 3.9a A typical Dynamic light scattering system **(3.9b)** DLS principle
Picture source: Shetta (2017)

To study the nanoparticle hydrodynamic size and PDI, 100 µL of freshly prepared nanoparticles was centrifuged at 9000 rpm for 15 minutes. The supernatant was diluted with distilled water to a concentration of (1 mg/mL), and viewed under a Zeta sizer by Malvern in order to assess the particle size through dynamic light scattering (DLS). The size and the PDI of the nanoparticles were then calculated using the solution in a disposable cuvette with a 2.0 mm measurement position and 6 attenuators. Three samples of every combination of parameters performed were examined and the average result was reported. The samples showing highest entrapment efficiency for each oil were then proceeded for SEM analysis.

3.5.4 Determining the minimum inhibitory concentrations (MIC) of the NPs

The researcher selected colonies identified predominantly from degraded cotton, silk, and wool fabrics. *Bacillus cereus*, *Staphylococcus*, and *Pseudomonas* were selected to examine the repelling properties of the developed nanoparticles under the study. Literature review indicated that *Escherichia coli* was the most common form of bacteria present and hence it was included in the study too.

The MICs of the synthesized clove, neem, cinnamon and carom essential oil nanoparticles were examined in regards to these four bacterial colonies using the microdilution method as followed by (Quan et al., 2021). A series of dilutions of the NPs (Neem oil- 1476 ppm, Cinnamon oil- 1412 ppm, and Clove oil- 1436 ppm) were prepared in a 96-well plate using Mueller Hinton broth (MHB). The dilution series of 1, 2, 4, 6, 8, 16, 31, 64, and 128 used in MIC testing is a logarithmic dilution series. The purpose of the dilution series is to identify the minimum concentration at which the antimicrobial agent inhibits the visible growth of the microorganism, which is known as the MIC. In this case, the initial concentration was the undiluted concentration of the essential oil nanoparticles. The dilution series follows a logarithmic progression. Each subsequent dilution is a fraction of the previous one. For example: Dilution 1: Represents the undiluted concentration, Dilution 2: Represents a 1:2 dilution (half of the concentration of Dilution 1) and dilution 4: Represents a 1:2 dilution of Dilution 2.

Bacterial suspensions were created and diluted in MHB until a turbidity of 0.5 McFarland was achieved, then added to each well. The plates were then incubated at 37 °C for 24 hours. Later, the MIC of the combination of clove and neem, and clove and cinnamon were also performed as neem, clove and cinnamon gave better MIC values as compared to carom oil. The MIC was

recorded as the lowest concentration that prevented visible growth of the test strains. The modal MIC values were selected after conducting three separate experiments.

3.5.5 Surface analysis of the prepared NPs using Scanning electron microscope (SEM)

To study the surface morphology, the optimized and the final samples of nanoparticles loaded with clove, neem and cinnamon at its highest entrapment efficiency were centrifuged at 18000 rpm for 30 min and the pallets were lyophilized at -25C for 50 hours and then sent for the scanning electron microscope (SEM) analysis at 5000X, 10,000X and 20,000X magnification at IIT, Delhi.

The principle and mechanism of lyophilizing (freeze drying) the nanoparticles are mentioned below:

Lyophilization, also known as freeze-drying, is a widely used technique for drying colloidal carriers, such as liposomes, nano-emulsions and nanoparticles, to improve their stability. A lyophilizer consists of a drying chamber with temperature-controlled shelves linked to a condenser chamber and vacuum pumps as seen in Figure 3.10a. The lyophilized product retains its original characteristics, such as nanoparticle size distribution, intact drug activity and long shelf life. The lyophilization cycle is divided into three stages: solidification, ice sublimation and desorption of unfrozen water (Figure 3.10b).

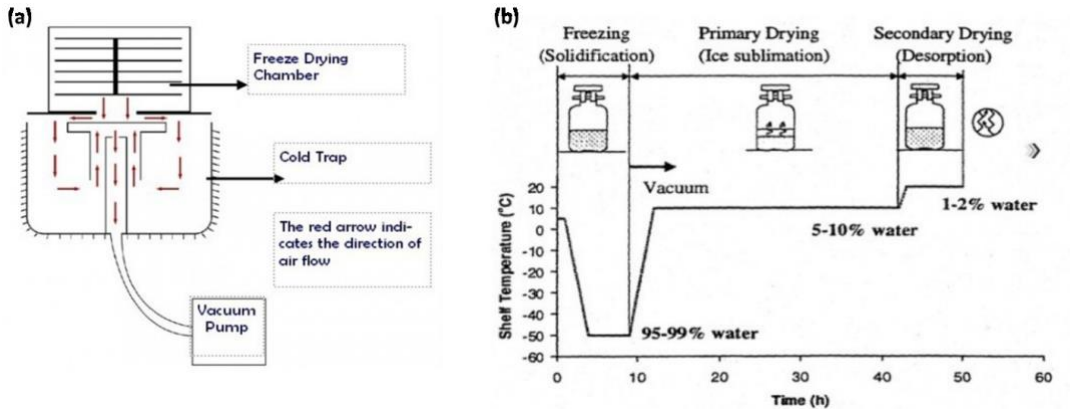

Fig 3.10 a) Benchtop freeze dryer, **3.10 b)** Lyophilization cycle
Picture source: Shetta (2017)

Initially, pure water present in a colloidal suspension is transformed into ice crystals during the solidification phase, leading to a rise in the concentration and viscosity of the suspension, which restricts further crystallization. Second, by lowering the boiling point through a vacuum,

an ice sublimation stage is induced. This step results in the formation of a porous pellet as the ice crystals sublime. Eventually, unfrozen water desorbs, and any water remaining after the earlier stages is removed. However, lyophilization can cause freezing and dehydration stresses, as well as phase separation during freezing, which can destabilize and permanently fuse the nanoparticles present in the colloidal suspension. As a result, cryoprotectants such as trehalose, sucrose, glucose, and mannitol must be used to shield the nanoparticles during the freezing phase by entrapping them in a glassy matrix.

3.6 Application of the optimized essential nanoparticles on the substrates

3.6.1 Selection of the substrate

For the study, two substrates were chosen to coat the finish on: cotton and polyester fabric. Cotton is a lightweight, breathable, strong, and durable natural-fiber fabric, making it an excellent choice for protecting delicate items. Additionally, it is acid- and lignin-free, making it a safe option for archival and preservation purposes, often seen in museum wrapping. The rationale behind using polyester fabric was that it is a synthetic material, which may hinder or reduce the growth of microbes in comparison to natural fabrics due to the absence of cellulose or protein.

3.6.1.1 Preliminary data of the fabric

Preliminary data of the fabrics was determined as per standard procedure given below:

3.6.1.1.a Determination of thread count and weave of the fabric.

Fabric count was calculated by counting the number of threads per square centimetre in warp and weft direction, with help of pick glass. Five reading were taken from the different part in each fabric and an average was taken as the final reading. For the determination of the weave of the fabric pick glass was used.

3.6.1.1.b Determination of fabric weight per unit area (GSM)

For the GSM reading specimen were cut into a size of 12.5×12.5 cm. The fabrics before cutting the sample were well ironed to make it free from any kind of crease or wrinkles. The specimens were conditioned in saturated salt solution in the desiccator for 24 hours. Each specimen then was weighed accurately using an Electronic Weighing Balance. Three reading from the fabric

were taken and an average was calculated for the final reading. Weight and final reading were calculated by using the following formula:

$$\text{GSM of the given fabric (gms/square meter)} = \frac{\text{Weight} \times 100 \times 100}{12.5 \times 12.5}$$

3.6.1.1.c Determination of thickness of the fabric

For testing the thickness of the fabric's specimen conditioned in desiccator for 24 hours. Compress-o-meter was used to determine the thickness of the fabric. For the test the fabric samples were placed between the anvil and pressure foot and the thickness indicated by the needle on the dial of the gauge was recorded. Five reading from each fabric were calculated and average of the five readings was recorded as the final reading.

3.6.1.1.d Determination of fabric weave

The weave of the fabric was observed under a pick glass to analyse its weave.

3.6.2 Application of the single NPs and in combination NPs

The nanoparticles that were made from neem, clove, and cinnamon essential oil at their optimized conditions were used for the application of the substrates at the concentrations that were achieved from the MIC results. The combination of the clove and cinnamon was also selected for the application process as it gave better MIC results when compared to neem and clove combination. This application of the finish was done by pad dry method. Citric acid was used as a binder in addition to the chitosan polymer which is well known for its mucoadhesive property. The selected cotton and polyester fabric samples were dipped in the nanoparticle finish along with 10% citric acid binder for 15 minutes, ensuring all the area is emerged completely, and then passed through padding mangle at a low pressure of 2.5 Kg, embedding the nanoparticles into the fabric and removing the excess solution. The fabric samples were then cured at 35 C for five minutes in order to make sure that the essential oils do not evaporate following by flat air drying at 25C for 10 minutes until completely dried.

3.7 Assessment of the coated fabrics

3.7.1 Assessment of Antibacterial activity of the finished cotton and polyester fabric using standard AATCC 147

The Parallel Streak Method (AATCC 147) was used to evaluate the antibacterial activity of untreated and NP coated fabrics. This qualitative method determines the effectiveness of diffusible antimicrobial agents applied to textiles. Specimens of 20X20 mm of the untreated and treated fabric samples containing clove, neem, cinnamon and a combination of clove and cinnamon were placed in contact with an agar surface in separate petri dishes. Before placing the fabrics, these agar surfaces of these plates were inoculated with *Bacillus cereus, Staphylococcus, Pseudomonas,* and *Escherichia coli.* The plates were then incubated at 37C for 24 hours. The specimens were then evaluated for interruption of growth along the streaks of the inoculum and beneath the fabric for a clear zone of inhibition which was calculated using the formula:

$$\mathrm{W} = \frac{T - D}{2}$$

Where, W is width of clear zone of inhibition, T is total diameter of test specimen and clear zone in mm, and D is diameter of the test specimen.

Higher the zone of inhibition, greater was the repellency or the activity of the nanoparticles against the bacteria.

3.7.2 Determination of Antifungal test using standard AATCC 30: Antifungal Assessment and Mildew Resistance Test

The AATCC 30 method is used to assess the vulnerability of textile materials to mildew and fungi, as well as the effectiveness of fungicides. It involves growing the specified fungus, *Aspergillus fumigatus,* on a solid medium, producing a spore suspension, and then inoculating an agar medium in a petri dish with the spore suspension. The test sample (20mmX20 mm) was placed on top of the inoculated agar medium, and then the top of the sample is also inoculated with the spore suspension. The petri dishes were sealed and incubated at 30^{o}C ± 2^{o}C for 14 days. After 14 days, the inoculated test samples were evaluated and rated based on the presence of macroscopic (visible to the naked eye), microscopic, or no growth.

The samples of cotton and polyester fabric that had been treated with the optimized finishes including clove, neem, cinnamon and a combination of clove and cinnamon were exposed to the fungal spores to test the effectiveness of the finish. Three samples of each category were tested to get an average result.

3.7.3 Surface analysis of the uncoated and coated fabric using Energy dispersive X-ray spectroscopy (EDX)

EDX (Energy Dispersive X-ray Spectroscopy) analysis is a technique used to measure the elemental composition of a sample by measuring the energy of the X-rays emitted from the sample. The energy of the X-rays is related to the atomic number of the elements in the sample, allowing for the identification of the elements present.

As shown in figure 3.11, when an incident electron collides with the inner electron shell of an atom, it causes an electron to be knocked out. In order to fill the empty slot, an electron from an outer shell then moves to the inner shell, emitting a photon with an x-ray wavelength that is specific to the element. By analyzing the energy of the X-rays, the relative amounts of the elements present are determined, providing information about the composition of the nanoparticle-coated fabric in this case.

By using EDX microscopy, triplicate samples of the cotton and polyester fabric samples that had been treated with optimized chitosan nanoparticles containing neem, clove, cinnamon, and a combination of clove and cinnamon were assessed. This examination was used to establish the presence of the coating on the fabric's surface by recognizing the atoms from the finish. As a result, it would demonstrate whether the finish had been absorbed or not by both the cotton and polyester fabric samples. The test was conducted at the NanoFab Lab of University of Alberta.

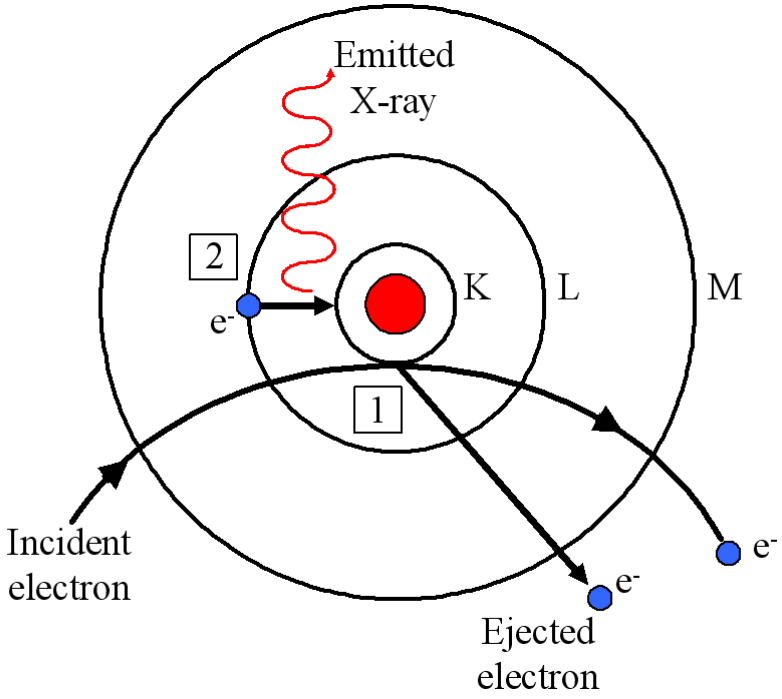

Fig 3.11: Illustration of the principle of energy dispersive x-ray analysis (EDX)
Picture source: https://www.researchgate.net/figure/Illustration-of-the-principle-of-energy-dispersive-x-ray-analysis-EDX-1-An_fig30_263555725

3.7.4 Determining the physicochemical stability of the nanoparticles over time and storage conditions.

This experiment was conducted to assess the changes in the amount of oil trapped in nanoparticles over a period of two months, under different storage conditions. Considering the fabric's intended use for preserving textiles in museums or for textile collectors at home, it is anticipated that it will be stored under conditions approximating room temperature (25°C ± 2) and a relative humidity of 65 ± 2%, either within an enclosed chamber, such as a drawer, or, in an alternative scenario, directly exposed to an open environment. For this purpose, 2 cm x 2 cm samples of the finished nanoparticle cotton fabrics were evaluated for 1 and 2 months. One set of samples was kept in a closed environment in a large petri dish, while the other set was exposed to an open environment. The amount of oil released from the nanoparticle-coated samples was determined by soaking them in a suitable solvent for 24 hours, and the absorbance was calculated using a UV spectrophotometer and compared to that of the initial sample.

3.7.5 Determination of resistance to insects using standard ISO 3998:1977

The polyester fabric did not demonstrate any repellency to bacterial or fungal species, indicating that the finish had not been absorbed into the fabric; this is likely due to its hydrophobic nature. EDX analysis further confirmed this, as no presence of nanoparticles was observed on the coated polyester fabric. As a result, the fabric was eliminated from the further test for insect repellency. Therefore, only treated cotton fabric samples that had been optimized with neem, clove, cinnamon, and a combination of clove and cinnamon essential oil chitosan nanoparticles were tested for its repellency against the cigarette beetles.

The test involves placing the treated and control/ untreated fabric specimens of known mass in contact with started 25 larvae for 14 days in a closed clear container or a jar. The lid or the cover of the container were perforated with tiny holes with the purpose of aeration for the larvae's. The test recommends incubating the insects in the box at 25± 2 °C, for 14 days and assessing the samples for its resistance towards the insects by calculating the weight loss of both the treated and untreated fabric. The fabric is also visually assessed for any damages, holes or death of larvae in them. However, it is recommended to periodically inspect the reaction of the insects to the treated samples, to see if they are repelled by it, comfortable with it, or if the treatment is causing any harm or death.

The test was conducted at the Department of Agricultural and Ecological Entomology, University of Alberta.