Chapter 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The general chemicals used in the research work were procured from different companies such as Himedia (Mumbai, India); Merck (India); Qualigens Fine Chemicals Ltd. (India); S.D. Fine Chemicals (India). Also, chemicals of high purity and analytical grades were procured from Sigma Aldrich (St. Louis, USA).

3.1.2 Microorganisms

The following microbial cultures as listed in Table 3.1 were used throughout the studies. They were procured from various microbial culture collection centers.

Table 3.1 List of microbial cultures procured from different culture collections

S.No.	Name of microorganism	Culture identity	Purpose
1.	*Streptomyces roseosporus*	NBRC 12910 Biological Resource Center, NITE (NBRC),Chiba, Japan	Daptomycin Producing microorganism
2.	*Aspergillus niger*	NCIM 1025 National Chemical Laboratory, Pune	For biosynthesis of nanoparticles
3.	*Staphylococcus*	MTCC 7443	

aureus(MRSA strain)	Institute of Microbial Technology (IMTECH), Chandigarh	Test microorganism for topical gel assay

3.1.3 Media

The following media were frequently used throughout the studies. The compositions of the media used were as follows:

3.1.3.1 Medium and culture conditions for *Streptomyces roseosporus*

Daptomycin producing strain *Streptomyces roseosporus* NBRC 12910 was grown in MGYP medium which consists (g/l): malt extract 3.0, dextrose 10.0, yeast 3.0, and peptone 5.0; followed by incubation at 30 °C in an orbital shaker using and 200 rpm. Fermentation medium included (g/l) Dextrin 30, Glucose 10, Soyabean flour 20, Fe $(NH_4)_2$ SO_4 0.6, KH_2PO_4 0.2, and pH 7, incubated at 30 °C for 6 consecutive days. Cofactors were added in the medium nicotinic acid(4 mg/l), riboflavin(0.5 mg/l), heme(9 mg/l), thiamine(0.4 mg/l), biotin(0.1 mg/l), cyanocobalamin(0.04 mg/l), tetrahydrofolic acid(6 mg/l) and pyridoxal 5-phosphate(0.4 mg/l) [Yu *et al*., 2011].The pH was controlled at 7.0 using 8 M NaOH solution and the aeration rate of 1 vvm in case of airlift bioreactor. N-decanoic acid (fatty acid) at 0.2 g/l was introduced in the media at 48 h post inoculation.

3.1.3.2 Media and culture conditions for *Aspergillus niger*

Aspergillus niger strain NCIM 1025 was maintained in and Potato Dextrose Agar (PDA) media. Master plates were revived and sub-cultured for this mold strain. The plates and the slants were stored at 4°C in the refrigerator. The culture was revived on Czapek Yeast

Extract Broth and maintained in agar slants. The strain *Aspergillus niger* was grown aerobically in 250 ml flasks containing Czapek Dox growth medium. The culture was grown in an orbital shaker at 200 rpm for 72 hours maintaining at 28°C. The initial pH was kept at 7.0[Chakravarty *et al*., 2015].

3.1.3.3 Media and culture conditions for *Staphylococcus aureus*

Staphylococcus aureus was procured from Microbial Type Culture Collections (MTCC) (Institute of Microbial Technology, Chandigarh). The bacterial lawn was grown in nutrient agar medium and incubated overnight at 37°C [Mohapatra *et al*., 2011].

3.2 Analytical Method for Daptomycin

3.2.1 Determination of Daptomycin by HPLC

5 ml of crude antibiotic (supernatant of the samples taken) was mixed with 5 ml acetonitrile, incubated for 5 minutes and then centrifuged at 10 000 rpm for 10 mins. HPLC analysis of concentrations of Daptomycin in reaction samples was performed. 20 µL of supernatant (using 0.22 µm filter) was injected into the Waters HPLC system. The stationary phase was SunFireTM C18 5µm (4.8X250mm) Column (Waters Corporation, Milford, MA, USA). The mobile phase consisted 0.2 M phosphate buffer (pH 5.5) and acetonitrile at 70:30 ratio. In order to determine the concentration of Daptomycin, after optimization of the parameters of Daptomycin production, High-Pressure Liquid Chromatography (HPLC) was done using the C18 column at 218 nm using PDA detector. HPLC was carried out both for standard Daptomycin as well as for the Daptomycin producing fermentation broth. A standard curve was plotted between the area of the peak and varying concentration of the standard Daptomycin as shown in Figure 3.1. Retention time for Daptomycin was observed as 6.0

minutes. This analytical technique was carried out at the flow rate of 1ml/min for a large number of samples taken for various studies at fixed time intervals [Tobin *et al*., 2008; I-Son Ng *et al*., 2014].

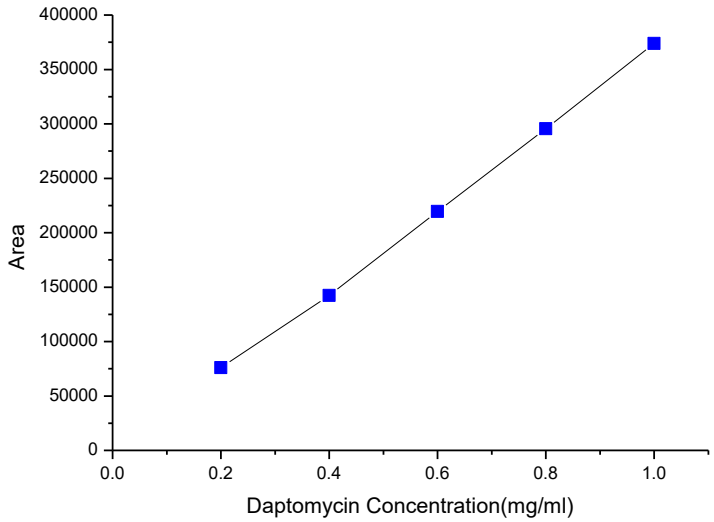

Figure 3.1 Standard curve for determination of Daptomycin by HPLC

Linear relationships of the peak area of the antibiotic and its standard form were evaluated. From the standard graph, concentrations of Daptomycin present in production culture were calculated by taking samples on each day for six days. HPLC was done for standard Daptomycin and Daptomycin in the sample as shown in Figure 3.2 and 3.3. The retention time of Daptomycin is 6.054 minutes and 6.104 minutes respectively which indicates the presence of Daptomycin.

Empower 2 software

SAMPLE INFORMATION

Sample Name:	Dapto 1	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	1	Acq. Method Set:	Antibiotic
Injection #:	1	Processing Method:	dapt
Injection Volume:	20.00 ul	Proc. Chnl. Descr.:	PDA 218.0 nm
Run Time:	40.0 Minutes		
Date Acquired:	1/27/2016 5:17:21 PM IST		
Date Processed:	1/27/2016 5:47:05 PM IST		

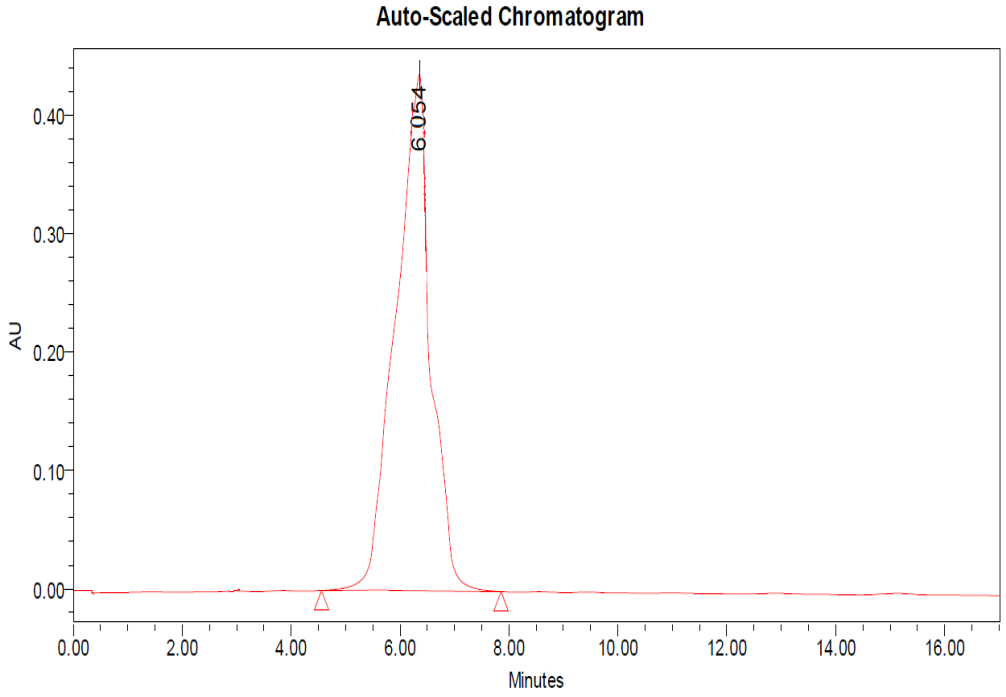

Figure 3.2 HPLC profile for Standard Daptomycin

Empower 2 software

SAMPLE INFORMATION

Sample Name:	Dapto 4	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	1	Acq. Method Set:	Antibiotic
Injection #:	5	Processing Method:	dapt
Injection Volume:	20.00 ul	Proc. Chnl. Descr.:	PDA 218.0 nm
Run Time:	40.0 Minutes		
Date Acquired:	1/27/2016 6:12:33 PM IST		
Date Processed:	1/27/2016 6:24:12 PM IST		

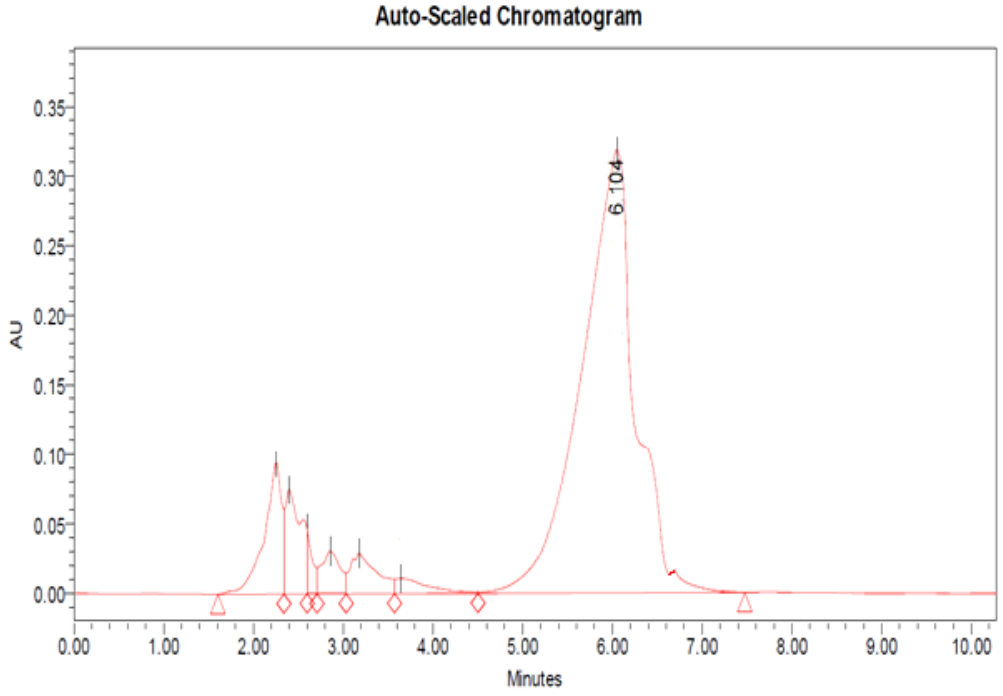

Figure 3.3 HPLC profile for Daptomycin in Sample

3.3 Optimization of process parameters enhanced Daptomycin production using OFAT (one-factor-at-a-time) method and Taguchi Method.

3.3.1 Effect of various factors by OFAT method

The effect of temperature, pH, inoculum age, inoculum volume, carbon sources, nitrogen sources and the precursor on Daptomycin production was evaluated through fermentation runs by changing one factor at a time. The studies were done in an orbital shaker using 500ml of shake flask containing a 100ml of fermentation media at 200rpm kept for six days. Each fermentation run was performed in triplicates. In order to optimize the temperature for maximum Daptomycin production, *S. roseosporus* was grown in a shake flask containing fermentation medium at different temperatures in the range of 20°C-40°C at 200 rpm. All other cultural conditions were kept identical. The influence of pH on Daptomycin production by *S. roseosporus* was also studied in a shake flask containing sterilized fermentation medium for a range of pH of the medium i.e. 5-8 at 30°C. To study the effect of inoculum age on the Daptomycin production, seed culture of different incubation time ranging from 12-72 h were prepared and added to the fermentation media at 30°C. To evaluate the effect of inoculum volume on the Daptomycin production, different inoculum sizes of the seed medium (2-7% v/v) were inoculated to the fermentation medium. All the other physical parameters were kept identical as mentioned earlier. The dextrin, glucose, sucrose, and starch were considered in the comparison of carbon source. The ammonium chloride, soyabean meal, tryptone, yeast extract, beef extract, sodium nitrite, and peptone were considered for the comparison of nitrogen sources. The carbon and nitrogen sources were taken at a concentration of 2%(w/v), and its role in the induction of Daptomycin production was determined. Decanoic acid, the precursor of Daptomycin was varied from 0 to 0.6 g/l and the

precursor addition time was varied from day 1 of fermentation to day 5(one day prior to the end of fermentation) of fermentation. The best parameters were considered and utilized for the subsequent studies. Daptomycin was estimated by the HPLC method.

3.3.2 Optimization by L16-Orthogonal Array Method.

The influence of the different media components was evaluated for Daptomycin production using the orthogonal array methodology L16 (4^4) was selected [Fukushima *et al*., 1990]. Based on Taguchi statistical, orthogonal arrays were used to design a minimum number of experiments which could give the specifications of the variables affecting the process [Fukushima *et al*., 1990; Xu *et al*., 2003]. A modified method of Taguchi [Taguchi *et al*., 1986, Taguchi *et al*., 1980] could cope up the problems of conventional optimization strategies. The experiments were validated by running the design using Minitab Version 17. The media components/variables were arranged into an L16 orthogonal array shown in Table 3.2. Each column in the design correspond individual medium components and each row indicate individual levels. Four defined concentrations of each component were considered over which their effect on Daptomycin production could be determined. The independence of statistical arrays reveals that the effect of each factor can be distinguished from others. These results were obtained by interpreting Taguchi's signal-to-noise ratios for each medium component. The objective of medium optimization was to maximize the yield of Daptomycin. For this Taguchi designed the following signal-to-noise ratio function

$$S/N = -10\log\left[\frac{1}{n}\sum_{n=1}^{n}\left(\frac{1}{\mathrm{Yi}^2}\right)\right] \qquad (1)$$

In which S/N represents the signal-to-noise ratio, n indicates the number of trials for particular concentration, and Yi is the yield of the trials. For each component, the optimum conditions are the ones giving the largest S/N ratio. The polynomial regression from the S/N ratio for each variable was used to obtain curves for which the maximum corresponded the best production value. All experiments were performed in triplicates. Fermentation using *S.roseosporus* was carried out under the submerged condition for six days. The fermentation requirement of *S.roseosporus* was determined. To carry out the L16 (4^4) orthogonal matrix, five factors were selected and varied at four levels as shown in Table 3.2 along with the experimental conditions.

Table 3.2 Orthogonal project design for Daptomycin production

	Low →			High
MEDIA COMPONENT	**LEVEL 1 (g/l)**	**LEVEL 2 (g/l)**	**LEVEL 3 (g/l)**	**LEVEL 4 (g/l)**
Dextrin	10.0	15.0	20.0	30.0
Glucose	2.0	5.0	8.0	10.0
Soyabean flour	20.0	25.0	30.0	35.0
$Fe(NH_4)_2SO_4$	0.2	0.4	0.6	0.8
KH_2PO4	0.1	0.2	0.3	0.4

Conditions such as temperature (30^0C), agitation (200 rpm), incubation time (144 hours), initial pH (7.0), and media volume ratio were fixed. Initially, fermentation was carried out in medium containing (g/l) glucose 20, soyabean meal 20, Decanoic acid(0.2), $CaCO_3$ (1), NaCl(5), pH 7 and incubated at 30 °C for 6 days (Unoptimized Media). For optimization, the carbon and nitrogen sources were chosen by OFAT methodology and further optimized by a statistical method. Each component has a unique role in Daptomycin fermentation process.

Dextrin is a slowly utilized reducing sugar while glucose helps to stimulate Daptomycin production as a carbon source. Soyabean meal contains several amino acids required for the entire biosynthesis of the Daptomycin. Kynurenine is a product of kynurenine metabolic pathway for degradation of L-tryptophan. Tryptophan is cleaved by tryptophan-2, 3-dioxygenase (TDO) to yield N-formylkynurenine. Kynurenine is formed by the removal of the formyl group by N-formylkynurenine formamidase (KFA). Fe $(NH_4)_2SO_4$ is a cofactor for this enzyme. KH_2PO_4 contributes to the phosphate required for antibiotic production process [Kurnasov et al., 2003]. The optimized media was used for further evaluation of shake flask studies of Daptomycin production [Yu *et al*., 2011].

3.4 Rheological properties of the fermentation broth

3.4.1 Morphological changes of *S.roseosporus* during the course of fermentation

The morphological changes of *S.roseosporus* were detected using Olympus CKX53 inverted microscope at 40X magnification in due course of time of fermentation.

3.4.2 Evaluation of the Rheology of Cells

The consequence of the stress conditions subjected to the glass beads on the microbial cells was assessed by various rheological model plots. The rheological properties were measured using the Brookfield viscometer. The Non-Newtonian behavior of the microbial cell mass was predicted using the following fluid models.

1. **Newtonian Model: $\tau = n\,\gamma$**

As per the definition of a Newtonian fluid, the shear stress, and the shear rate are proportional to each other.

1. **Bingham:** $\tau - \tau_{y,B} = \mu_P \gamma$

Newtonian model and the Bingham plastic model can be described by straight lines in terms of shear rate and shear stress. The viscosity and yield stress can be found out by calculating the slope and the intercept.

3. Casson: $\tau^{0.5} = (\tau_{y,C})^{0.5} + \mu_{P,C} \gamma$ **0.5**

In Casson model, a straight line results when the square root of shear rate is plotted against the square root of shear stress.The Casson yield stress is calculated as the square of the intercept and the Casson plastic viscosity as the square of the slope.

4. Power law: $\tau = K \gamma^n$

Shear stress-shear rate plots of many fluids become linear when plotted on double logarithmic coordinates and the power law model describes the data of shear-thinning and shear thickening fluids**.**

In the equations,τ denotes the shear stress, γ the shear rate, $\tau_{y,B}$ the Bingham yield stress, μ_P the plastic viscosity, $\tau_{y,C}$ the Casson yield stress, $\mu_{P,C}$ the Casson viscosity, K the consistency index, *n* the flow behavior (power-law) index [Rao *et al*., 2014].

3.5 Fermentation Studies in Stirred tank fermenter

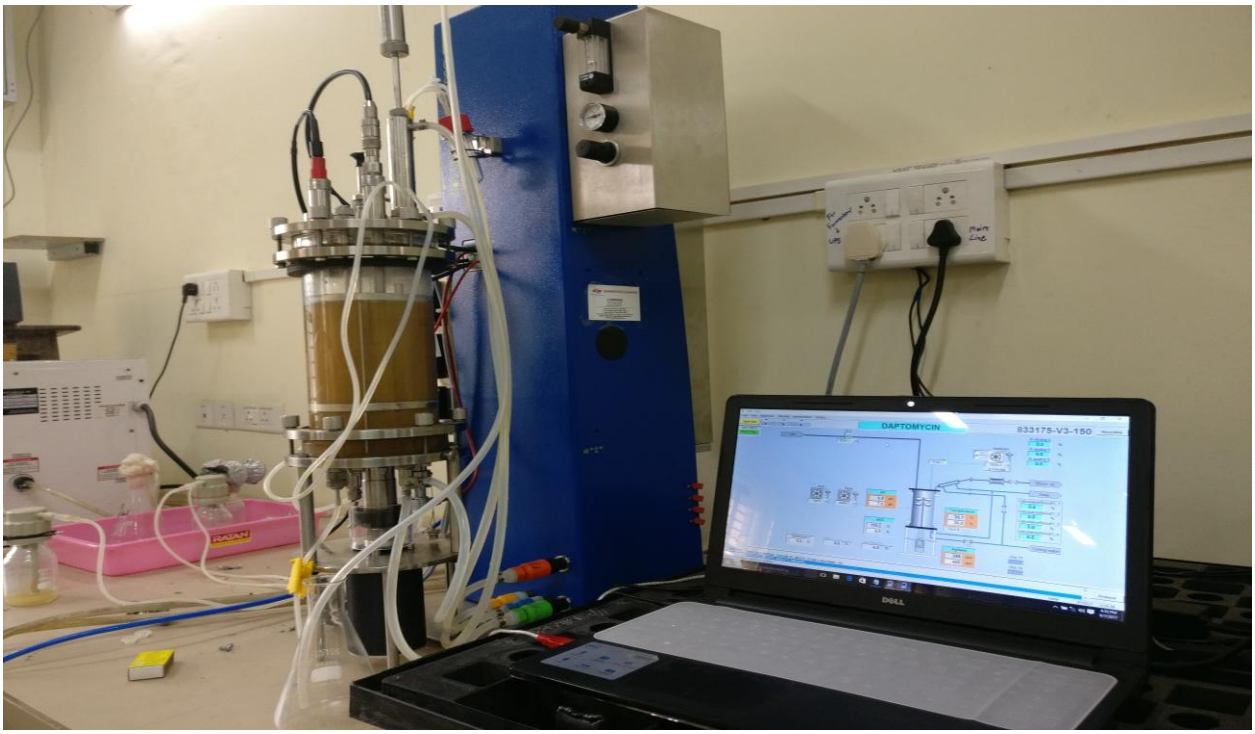

Figure 3.4 Daptomycin fermentation in a 3L stirred tank fermenter

Batch culture studies were conducted in a 3L-glass fermenter (Bioengineering AG, Switzerland) at 2L (minimum requisite by the company) working volume. The fermenter was equipped with monitoring and control facilities for pH, temperature, agitation, aeration and dissolved oxygen concentration. After running the automated sterilization (based on autoclaving principle) program, 5% v/v of inoculum was added to the media. The mixing was carried out at different agitator speeds and varying air flow rates. pH was controlled at 7.2 by intermittent addition of either 2N sodium hydroxide or 2N sulphuric acid. An aqueous emulsion of silicone oil was used as antifoam agent and added to the system to control foaming (whenever needed). Samples were drawn aseptically every 4 hours to estimate the

cell concentration, reducing sugar concentration and Daptomycin concentration. The silicone tubing used for the transfer of sterile medium, broth harvest and sampling were autoclaved. The setup of stirred tank fermentation is shown in Figure 3.4.

3.6 Continuous Production of Daptomycin in a Packed Bed Bioreactor using *Streptomyces roseosporus* by novel immobilization technique

3.6.1 Concentrated seed culture

An 8-days old slant grown culture was suspended in 5.0 ml sterile distilled water and then inoculated in seed medium. After 3 days of growth in an orbital shaker, cells were harvested by centrifugation at 10000 rpm for 15 minutes and washed with citrate buffer. This concentrated seed was used for various immobilization purposes.

3.6.2 Whole cell immobilization modes

Three different carrier modes were followed for immobilizing cells of *S. roseosporus* as depicted in Figure 3.5.

Figure 3.5 Whole cell immobilization support matrices in packed bed bioreactor

3.6.3 Calcium alginate bead formation

Sodium alginate (2.0g, Sigma) was dissolved in distilled water(90.0ml) and concentrated seed culture(10.0ml) was mixed with presterilized 90.0 ml of sodium alginate solution. The cell alginate solution was completely mixed and added through a needle(no. 18 gauge) to a stirred solution of 1.0M $CaCl_2.2H_2O$. The beads were cured for 1.5 h at room temperature and washed with sterile water. The bead diameter so obtained was 2.8±0.1mm. Different cells-to-carrier ratios were prepared. The immobilized sample was washed with citrate buffer (pH 4.8) until there was no further leakage of cells.

3.6.4 Immobilization in Natural Loofah sponges

Natural Loofah sponge (agro waste) procured locally was thoroughly washed, dried for 3 days. It was chopped into 5.0 cm pieces and sterilized. The concentrated seed culture was brought in contact with a packed loofah in the reactor in such a way that the cell loading was varied from 0.5 to 3.0 grams of cells per grams of Loofah sponge. The reactor was left to rest for 2-3 hours. The system so devised is autoregulatory and the equilibrium was maintained between the growing cells and those leaving the reactor. These operating conditions were satisfactory to start the reactor and the column was thus ready for operation. A continuous supply of sterile air was then used. The surface properties were determined by BET analysis [Brunauer *et al*., 1938] as shown in Figure 3.6.

3.6.5 Immobilization in silk sachets

Silk sachets (4 cm X 2 cm) were sterilized and concentrated seed culture was put into each silk sachet aseptically. These were loaded into the presterilized column.

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Sample: IPSITA-1

Cold Free Space: 50.8052 cm³

Low Pressure Dose: None

Analysis Adsorptive: N2

Analysis Bath Temp.: -195.850 °C

Thermal Correction: No

Warm Free Space: 17.1117 cm³ Measured

Equilibration Interval: 5 s

Automatic Degas: Yes

Carrier Name	Carrier diameter (cm)	Specific surface area(cm2/cm3)	Particle density(g/ml)	Porosity (%)
Natural Loofah Sponge	5 cm	0.96	0.02	85

Figure 3.6 Report of BET analysis for loofah sponge

3.6.6 Packed Bed Bioreactor

In an indigenously developed tubular glass bioreactor (70cm X 3cm), the immobilized microbial cells of *S.roseosporus* were packed as depicted schematically in Figure 3.7. The fermentation media was aseptically passed through the peristaltic pump at different flow rates and the samples were collected at the top of the column to evaluate antibiotic concentration. The sterilized air was injected at various points along the column. The packed bed column temperature was regulated using cool water passed through the jacket. The entire fermentation process was carried out in a continuous mode.

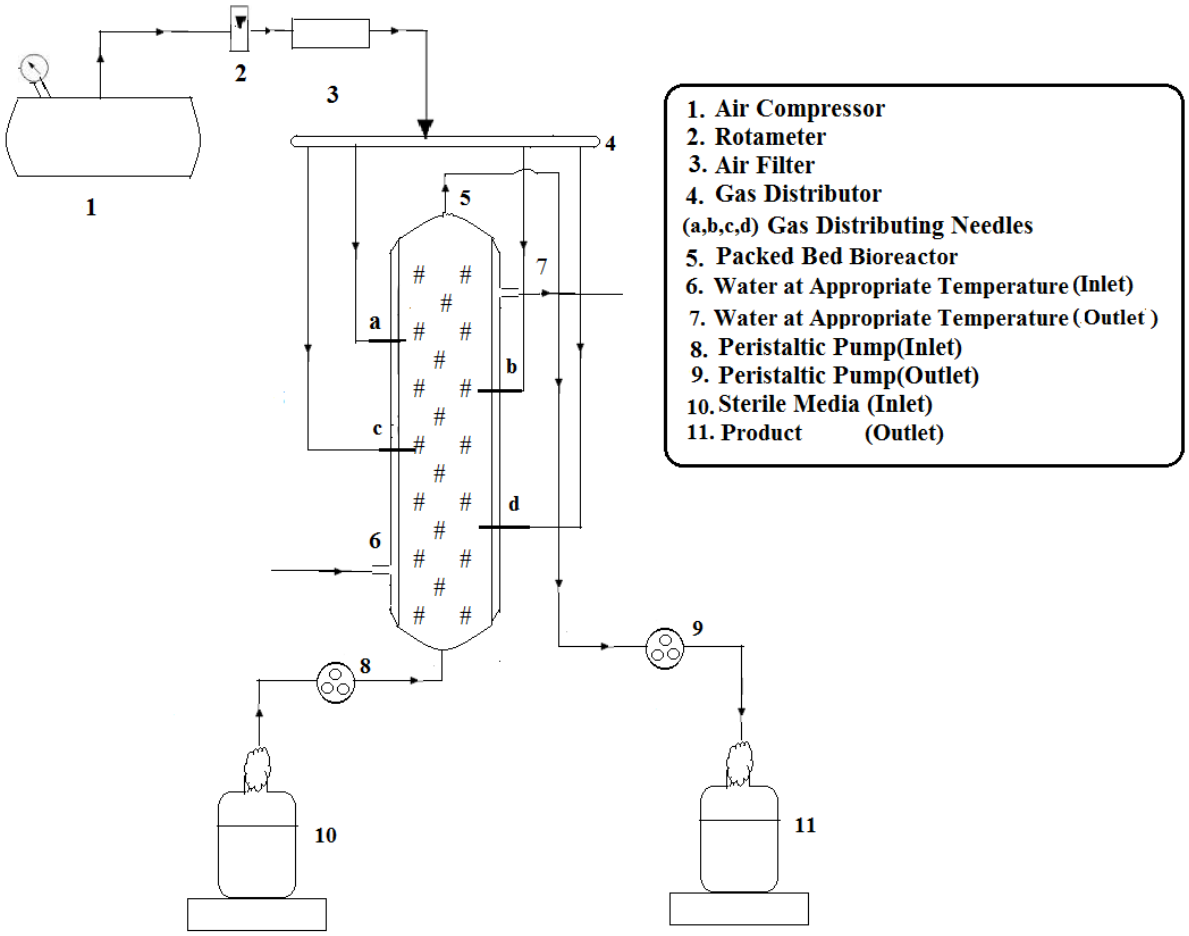

Figure 3.7 Schematic representation of packed bed bioreactor**.**

3.6.7 Cell Growth and Product Estimation

10 mL broth sample was centrifuged and the supernatant was discarded. Pellets were washed thrice with distilled water. The centrifuged biomass was transferred to a pre-weighed aluminium cup kept at 85 °C for 24 h until a constant weight was achieved. The dry cell weight was determined by subtracting the weight of aluminium cup from the previous weight. At specified intervals, Daptomycin was analyzed using disk diffusion method using *M. luteus* as the assay organism and confirmed by HPLC. Total residual reducing sugar in the sample filtrate was determined by the established DNS method [Miller, 1959].

3.6.8 Cell Leakage Estimation

The free cells and cells leaked from the support matrix were collected by centrifugation at 10,000 rpm for 20 min and dried at 90 °C. The initial weight of each support matrix was determined by drying specified quantity to a constant weight. The support matrices with cells were carefully washed with distilled water and dried again. The difference between the weights of the support matrices before and after cell adsorption is considered to be the weight of adsorbed cells [D'Souza *et al*., 1986].

3.6.9 SEM Analysis of Immobilized cells on matrices:

Scanning electron microscopy (SEM) was carried out for different supporting matrices containing *S.roseosporus* cells were observed by scanning electron microscopy. The specimens were chemically fixed and examined in Zeiss scanning electron microscope at 30kV. All images were digitally recorded.

3.7 Daptomycin production in an airlift bioreactor by morphologically modified and immobilized cells of *Streptomyces roseosporus*

3.7.1 Morphological variations of *S. roseosporus*

The growth conditions were altered for pelletization the cells. The morphological variation of *S. roseosporus* was carried out by changing the inoculum size, the nitrogen source and the aeration rate in the fermentation medium. Morphological changes during the fermentation were observed. Suspended pellets were imaged and pellet diameter was calculated, assuming that the pellets were perfectly spherical. Pellet size distribution was calculated by averaging 20–50 pellets. Both visual observations and image processing tools were taken into consideration.

3.7.2 Immobilization of *S. roseosporus* on refractory brick flakes and silk sachets

Refractory bricks were mechanically crushed and sieved uniformly to a particle size of 5 mm. These were pretreated by boiling in water for 20 min at 80 °C and washed with distilled water. They were placed in methanol for 3 h. Then, washed again with distilled water. For the immobilization of *S. roseosporus* on the support matrices, 2.0 g of the pretreated carrier was placed in a 250 ml Erlenmeyer flask containing seed medium. After sterilization (121 °C and 15 lbs/in2 pressure for 20 min), the flasks were inoculated with 0.5 ml of homogenized mycelia (0.15 dry cell weight of mycelia g/l) under sterile conditions. The surface properties were determined by BET analysis [Brunauer *et al*., 1938] as shown in Figure 3.8.

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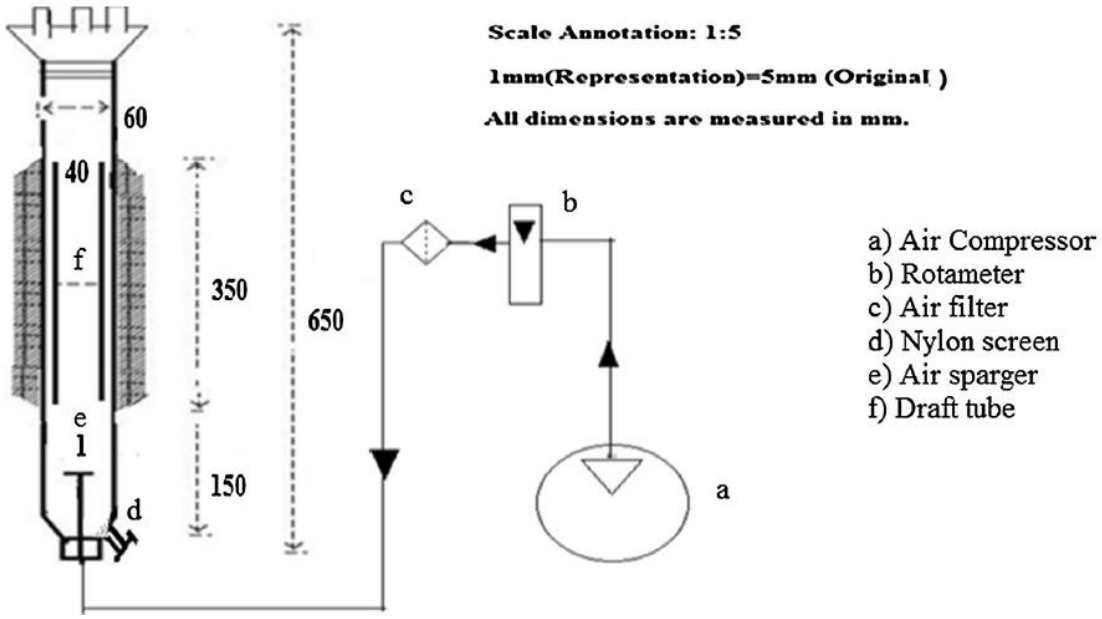
Sample: IPSITA-2

Cold Free Space: 50.8052 cm³
Low Pressure Dose: None
Analysis Adsorptive: N2
Analysis Bath Temp.: -195.850 °C
Thermal Correction: No
Warm Free Space: 17.1117 cm³ Measured
Equilibration Interval: 5 s
Automatic Degas: Yes

Carrier Name	Carrier diameter (mm)	Specific surface area(cm2/cm3)	Particle density(g/ml)	Porosity (%)
Refractory brick	5mm	0.93	0.032	75

Figure 3.8 Report of BET analysis for refractory brick

An equal weight of silk sachets (4 cm × 2 cm) was sterilized, and a concentrated seed culture was put into each silk sachet aseptically. *S. roseosporus* is found to grow on the carrier after 5 days of incubation. The growth medium was then removed and the immobilized matrices were thoroughly washed with distilled water under sterile conditions. Experiments were carried out for Daptomycin production by transferring the immobilized matrices into the production medium under sterile conditions. They were loaded into an indigenously developed, pre sterilized Airlift Bioreactor containing fermentation media as depicted in Figure 3.9.



Parameter	Volumetric Capacity of Reactor	Column Diameter	Column Height	Ungassed Liquid Height	Draft Tube Height	Draft Tube Diameter	Height of Draft Tube above the Fermenter Base	Sparger Hole Dia.
Dimension	2.0 L	60 mm	650 mm	150mm	350 mm	40 mm	80 mm	1mm

Figure 3.9 Schematic representation of airlift bioreactor and its dimensional specifications.

3.7.3 Cell growth and product estimation

10 mL broth sample was centrifuged and the supernatant was discarded. Pellets were washed thrice with distilled water. The centrifuged biomass was transferred to a pre-weighed aluminium cup kept at 85 °C for 24 h until a constant weight was achieved. The dry cell

weight was determined by subtracting the weight of aluminium cup from the previous weight. At specified intervals, Daptomycin was analyzed using disk diffusion method using *M. luteus* as the assay organism and confirmed by HPLC.

3.7.4 Cell leakage estimation

The free cells and cells leaked from the support matrix were collected by centrifugation at 10,000 rpm for 20 min and dried at 90 °C. The initial weight of each support matrix was determined by drying specified quantity to a constant weight. The support matrices with cells were carefully washed with distilled water and dried again. The difference between the weights of the support matrices before and after cell adsorption is considered to be the weight of adsorbed cells [D'souza *et al*., 1986].

3.7.5 Evaluation of the rheology and hydrodynamics of fermentation broth

The consequences of the stress conditions subjected in case of different morphological forms were assessed by the rheological properties which were measured using the Brookfield viscometer at different time intervals. The volumetric oxygen transfer coefficient was estimated by the dynamic gassing-out technique. Air flow rate was maintained at 1 vvm [Ruchti *et al*., 1981].

3.7.6 Reusability of immobilized and pelletized cells

The cell pellets and the immobilized cells were collected at the 50-micron nylon mesh screen at the sampling port and replenished with fresh fermentation medium for further batches.

3.7.7 Microscopic analysis of the pelletized and the immobilized cells

Scanning electron microscopy (SEM) was carried out for immobilized cells on refractory bricks to determine the morphological alterations and distribution of cells in the matrices during immobilization. The specimens were chemically fixed and examined in Zeiss scanning electron microscope at 20 kV. All images were digitally recorded. Pelletized cells and silk sachets containing *S. roseosporus* were observed by electron microscopy.

3.8 Bubble Characteristics and Mass Transfer in an Airlift Bioreactor

A single hole plate type sparger and a multiple hole (N=5) plate type spargers of the same diameter were taken as depicted in Figure 3.10. For each of the spargers, the superficial gas velocity was varied from 0.001 Usg (m/s) to 0.005 Usg (m/s).

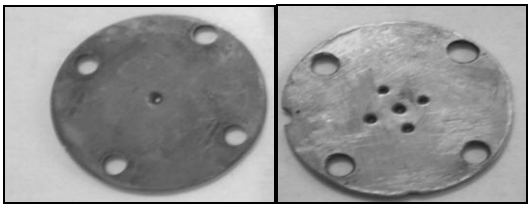


Figure 3.10 1mm Single Hole and 1mm Multiple Hole Plate type Sparger

A photographic method was applied to measure the bubble size and bubble numbers using high-speed DSLR camera. MATLAB Image Processing Tools using MATLAB 8.6/R2015 b was used to process images at a scan rate of 1000 frames per second to get the histogram for bubble diameter versus bubble numbers as shown in Figure 3.11.

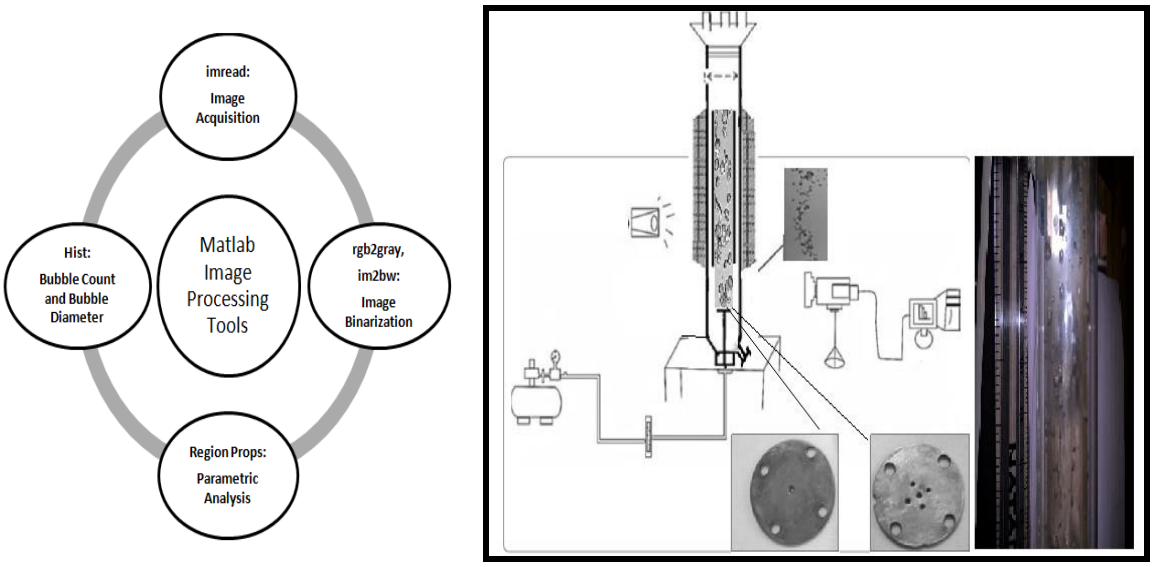


Figure 3.11 Scheme of Bubble Size Distribution Analysis

Between 1000 and 5000 bubbles were counted for determination of the size distribution, using 30 photographs. The Sauter mean bubble diameter was calculated.

3.8.1 Gas holdup

The gas holdup was determined experimentally by the volume expansion method with respect to different superficial gas velocity for two different spargers and also from the data obtained from bubble size distribution.

$$\varepsilon = \frac{VG}{VL+VG} \qquad (1)$$

Where V_G is the volume of gas bubbles in the bioreactor in m^3 and V_L is the volume of liquid media in the bioreactor in m^3. The volume of gas is actually the total volume of bubble multiplied by a number of bubbles of a particular size. The volume of liquid is the volume of liquid media taken i.e. 1 liter.

3.8.2 Volumetric mass transfer coefficient

The interfacial area was calculated from the gas hold up values obtained from the bubble size distribution

$$a = \frac{6\varepsilon}{dB(1-\varepsilon)} \qquad (2)$$

d is the Sauter mean bubble diameter.

The trend consistency of interfacial area of the gas bubbles for each sparger was studied with the volumetric mass transfer coefficient, K_La, measured by the dynamic gassing out method with respect to the superficial gas velocities [Tung *et al*., 1998]

3.9 Synergistic Oligo-dynamic Effect of Daptomycin with Mycogenic Gold, Silver and Bimetallic Nanoparticles against MRSA (Methicillin-resistant *Staphylococcus aureus*)

Topical antibacterial preparations have applicability in prevention and treatment of burn and wound infections. Several antimicrobial agents are formulated to prevent skin infections while others are designed to kill microbial flora that is proliferating within the burn affected region [Gunasekaran *et al.*, 2011]. Also, it has an important aspect that is selectivity towards pathogens. Gel-based formulations are greaseless, softening, soothing, spreadable, thixotropic and miscible. To channelize the synergism, both nanoparticles and Daptomycin can be delivered in a single package for effective topical antimicrobial activity against MRSA strain(s) which are mainly responsible for skin infections and sepsis. Evidence of few

side-effects of Daptomycin intravenous administration has encouraged the use of topical Daptomycin [Gonzalez-Ruiz A *et al.,* 2016].

Gold nanoparticles, silver nanoparticles and bimetallic silver and gold nanoparticles were biosynthesized using *Aspergillus niger* NCIM 1025 for further application [Jain *et al.,* 2009].

3.9.1 Growth of Microorganism

The fungal strain, *Aspergillus niger* NCIM 1025, was obtained from National Chemical Laboratory, Pune, India. The culture was revived on Czapek Yeast Extract Broth and maintained in agar slants. The strain *Aspergillus niger* was grown aerobically in 250 ml flasks containing Czapek Dox growth medium. The culture was grown in an incubator shaker at 200 rpm and at 28^{o}C for 72 hours, and initial pH was maintained at 7.0 [Chakravarty *et al.,*2015; Codner *et al.,*1963].

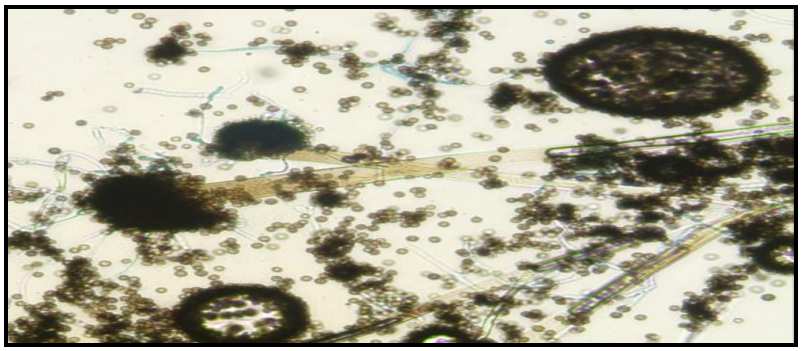


Figure 3.12 Microscopic view of *Aspergillus niger*

3.9.2 Production of Monometallic and Bimetallic (silver and gold) Nanoparticles by Co-reduction

Under aseptic conditions, sufficient cell mass of *Aspergillus niger* was separated and washed thrice with distilled water. 20 grams of wet biomass was taken and suspended in a 500 ml Erlenmeyer flask containing 100 ml of each 0.2mM $HAuCl_4$, 0.2mM $AgNO_3$ and 1:1 $HAuCl_4$ and an $AgNO_3$ solution prepared in deionized water. Above culture was incubated at 28°C at 200 rpm for 2 days. For control, fungal biomass was suspended in deionized water and incubated at 28°C at 200 rpm to observe color changes[Yoko *et al.*,2001].

3.9.3 Characterizations

Nanoparticles formation was studied both visually, by observing the color change, and with the help of UV visible spectroscopy where the characteristic of peak formed between specific nanometers ranges revealed formations of nanoparticles. Absorbance was measured using UV-Vis, Shimadzu UV-1800 in the range of 200-800 nm wavelengths. The nanoparticles were dislodged from the cell biomass using sonication and filtration. The fabrication of nanoparticles was analyzed by various analytical techniques. Samples were centrifuged at 10,000 rpm for 15 minutes respectively. The obtained biomass was freeze-dried at -54°C, followed by lyophilization. For XRD analysis, the samples were cast onto the glass slide and measurements were carried out using Rigaku Ultima IV instrument at the two-theta angle in the range of 20° to 80°[Barua *et al.*,2015, Chakravarty *et al.*,2015]. The exact shape and size of the nanoparticles were characterized using particularly the High-Resolution Transmission Electron Microscopy (HR-TEM) analysis which was carried out on TECNAI 20 G2-electron microscope which was operated at accelerating voltage 200 Kv [Karmakar *et al.*,2010].

3.9.4 Gel formulation

The simple gel was prepared according to the formula reported in the literature (Indian Pharmacopeia) using carbopol 934, propylene glycol and triethanolamine [Patel *et al*., 2011]. We also used the synthesized gold, silver, and bimetallic nanoparticles along with Daptomycin in the gel formulation as shown in Figure 3.13.

1. 1% Carbopol 934 was soaked in 25ml of distilled water for 24 hours.
2. The solution was vigorously stirred until the solution was free from lumps.
3. The powdered nanoparticles and the antibiotic were added to the solution while continuously stirring the solution.
4. After 30 minutes, a drop of both triethanolamine and propylene glycol were added.
5. The formed gels were packed and stored at 4 ^{o}C for topical usage.

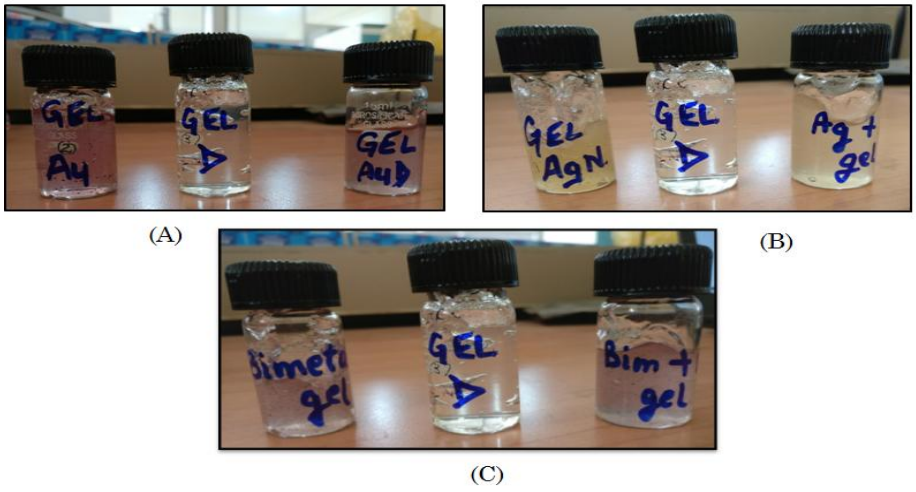


Figure 3.13 Topical gels containing antibiotic, nanoparticles and mixture of both of them, viz., (A) Gold nanoparticles, (B)Silver Nanoparticles,(C) Bimetallic Nanoparticles.

3.9.5 Evaluation of Properties of Anti-MRSA Topical Gel

For the determination of physical properties of the topical gel, some tests were performed and the results obtained were tabulated [Das *et al*., 2011].

Colour

We have assessed the color of the formulation by mere observation and also in comparison with simple topical gel.

pH

The pH of gel formulations was determined by using the digital pH meter, by dissolving the gel in distilled water. The measurements were conducted thrice and the average was considered.

Physical Stability

The gels formulated were packed in tubes and were kept for two weeks at standard conditions.

Solubility

This was assessed by dissolving the calculated amount of gel in distilled water.

Viscosity

The viscosity of different gels was measured using Brookfield's digital viscometer.

The gels were rotated at variable speeds. And the evaluation was conducted in triplicate.

Spreadability

A sample of gel formulated was pressed between two glass slides and left for about 5 minutes. The increase in the diameter due to spreadability of the gel was noted. The values obtained for various properties of the topical gel are depicted in Table 3.3.

Table 3.3 Characteristics of Topical Gel with antibiotic and noble metal nanoparticles

S.no.	Formulation	Colour	pH	Physical Stability	Solubility	Viscosity (cp)	Spreadability (mm)
1	Gel with Antibiotics (Daptomycin)	Transparent	6.68±0.01	No change in color and consistency	Yes	2809.42±0.33	4.8±0.10
2	Gel With Gold Nanoparticle	Purple	7.09±0.02	No change in color and consistency	Yes	3653.67±1.23	3.6±0.05
3	Gel with Gold Nanoparticle and Antibiotics	Purple	6.97±0.02	No change in color and consistency	Yes	3124.33±2.51	4.4±0.05
4	Gel with Silver Nanoparticle	Yellow	7.06±0.01	No change in color and consistency	Yes	3687.33±0.40	3.7±0.10
5	Gel with	Yellow	7.16±0.0	No change	Yes	3217.43±0.	4.5±0.11

	Silver Nanoparticle and Antibiotics		15	in color and consistency		53	
6	Gel with Bimetallic Nanoparticle	Lavender	6.77±0.01	No change in color and consistency	Yes	4208.35±0.85	4.0±0.10
7	Gel with Bimetallic Nanoparticle and Antibiotics	Lavender	6.21±0.03	No change in color and consistency	Yes	3245.31±1.47	4.5±0.15

3.9.6 Antimicrobial Assay

To determine the antibacterial activity of synthesized gold nanoparticles, standard disk diffusion method was carried out against Multi-Drug Resistant Strain (MRSA) *Staphylococcus aureus*. The agar plates having suitable nutrient media (LB media) was prepared, sterilized and allowed to solidify [Tassou *et al.,* 2000]. After solidification, the agar plates were inoculated with bacterial cultures. 6mm disks were impregnated with a required quantity of antibiotic and nanoparticles in Petri-plates containing suitable nutrient agar medium seeded with 120 µL of 36h of each pathogen. 20µg/ml (Minimum Inhibitory

Concentration (MIC) of Daptomycin antibiotic, 20µg/ml (equal amount as that of antibiotic) of nanoparticle and a mixture of both Daptomycin and lyophilized nanoparticle was formulated into topical gel[Bauer *et al.,*1959]. 150 µl of each of these was impregnated on the corresponding disks and incubated at 28°C for two days[Brown *et al*., 2012; Allahverdiyev *et al.,* 2011]. The zone of inhibition was calculated and recorded for each formulation and expressed in millimeter (mm). All the above experiments were carried out in triplicate.