MATERIALS AND METHODS

3.1. Cleaning of Glasswares

The glassware used in the study were first soaked in cleaning solution (100 g of potassium dichromate dissolved in 100 ml distilled water followed by addition of 500 ml of concentrated sulphuric acid) for about 24 h and washed in tap water. Further, they were thoroughly rinsed with distilled water, air dried and stored in a sterile condition until further use.

3.2. Sterilization

The methods of sterilization in a hot air oven and autoclave were done as per standard techniques in microbiology (Collins and Taylor, 1967). The air dried glassware were sterilized at 180° C for 2 h in a hot air oven and all the microbiological media were sterilized in an autoclave at 15 lbs pressure for 20 min and used in the study.

3.3. Chemicals

All the chemicals used were of analytical reagent (AR) grade quality unless or otherwise stated and the double glass distilled water was used throughout the study.

3.4. pH adjustment

The pH of all the microbiological media were adjusted with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid.

3.5. Reference strains

The *Escherichia coli* (ATCC 11230), *Staphylococcus aureus* (ATCC 6538), *Aspergillus niger* (ATCC 6275), *Penicillium varians* (ATCC 28070), *Trichoderma viride* (ATCC 13233) strains obtained from American Type Culture Collection (ATCC) were used as standard for comparison. Periodical subculturing was done in a
month on sterile Nutrient agar slants and Potato Dextrose Agar and maintained at 4°C for further use.

3.6. Collection of mangrove soil samples

The mangrove soil samples were collected from Pitchavaram (South Pitchavaram, Pitchavaram - boat house), Mulukkuthuri, Kodyampilayam, Artificially grown mangrove forest, Tamil Nadu, India and designated accordingly. From the five places twenty one different soil samples were collected from the Mangrove sediments and rhizosphere region of mangrove plant. The soil samples were designated as PBSA, PBSB, PBSC, PBSD, PBSE, PBSF, PBSG, SPBSA, SPBSB, SPBSC, SPBSD, MBSA, MBSB, MBSC, MBSD, KBSA, KBSB, KBSC, ABSA, ABSB and ABSC (Table 6).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Location</th>
<th>Designation of the isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pitchavarm (Boat house)</td>
<td>PBSA, PBSB, PBSC, PBSD, PBSE, PBSF, PBSG</td>
</tr>
<tr>
<td>2</td>
<td>South Pitchavarm</td>
<td>SBSA, SBSB, SBSC, SBSD</td>
</tr>
<tr>
<td>3</td>
<td>Muzhukuthurai</td>
<td>MBSA, MBSB, MBSC, MBSD</td>
</tr>
<tr>
<td>4</td>
<td>Kodyampilayam</td>
<td>KBSA, KBSB, KBSC</td>
</tr>
<tr>
<td>5</td>
<td>Artificial Mangrove forest</td>
<td>ABSA, ABSB, ABSC</td>
</tr>
</tbody>
</table>

3.7. Isolation of total heterotrophic bacteria from soil

3.7.1. Enrichment of soil samples

One hundred gram of freshly collected soil samples were taken in a 250 ml Erlenmeyer flask. It was added with 10 ml of crude oil thrice (to selectively enrich the biosurfactant producers) and thoroughly mixed everytime and kept for incubation at room temperature (28 ± 2°C) for 30 days. The crude oil was obtained from Indian Oil Corporation, Narimanam, Tamil Nadu, India. Once in a week the samples were moistened with sterile distilled water to avoid desiccation if necessary.

3.7.2. Serial dilution plate technique (Allen, 1953)

A quantity of 1.0 g of representative and homogenized soil was suspended in 100 ml sterile water in a 250 ml Erlenmeyer flask. After thorough agitation over a rotary shaker
for 10 min, one ml of the supernatant was aseptically transferred to 9 ml sterile water blank in a test tube. Dilution process was continued till $10^{-6}$ dilution. One ml of $10^{-6}$ dilution aliquots was withdrawn and transferred to sterile Petri plates.

In the mean time, a selective medium nutrient agar for total aerobic heterotrophic bacteria was prepared as per the composition given in Appendix I and sterilized in an autoclave at 15 lbs for 15 min. The medium was cooled and poured on to Petri plates containing appropriate dilution of the samples and rotated in clockwise and anticlockwise directions for even spreading and allowed for solidification. The plates were inverted and incubated at room temperature (28 ± 2°C). When the bacterial colonies appeared on the plates, morphologically distinct colonies were picked up, purified and maintained on nutrient agar slants.

3.8. Isolation of biosurfactant producing bacteria - Glass plate assay (Jain et al., 1991)

Rapid screening test for biosurfactant production is tested based on Glass plate assay described by Jain et al. (1991). A grease free glass plate was taken (15×10 cm) wiped with alcohol to give a uniform coating of coconut oil using a absorbent cotton wool and air dried. Small drop of culture filtrate was placed on the glass plate using a sterile 2 ml syringe. If the glass plate was large enough, 15 - 20 drops of culture filtrate could be placed. A drop of distilled water served as a blank. The drops were observed carefully. Culture filtrates of the isolates which were capable of producing biosurfactant, would collapse and spread on the glass plate. The larger the collapse area, the greater is the activity of biosurfactant.

3.9. Characterization of biosurfactant producing bacterial isolates

The cultural, morphological and biochemical characters of all the isolates were studied by following Bergey’s Manual of Determinative Bacteriology (Buchanan et al.,
1974). The reagents, indicators and media used in the biochemical tests were given in Appendix (I - IV).

3.9.1. Cell morphology

The isolates of bacteria were grown in yeast extract glucose broth for 72 h in 100 ml Erlenmeyer flask over a shaker. The cell morphology of the isolates was done as per the method suggested by Gerhardt et al. (1981). The bacterial shape was observed under oil immersion objective of a research microscope (Olympus, Japan).

3.9.2. Gram’s staining

The Gram's reaction was performed as per the methods described by Bartholomew and Mittwer (1952).

3.9.3. Spore staining

The presence or absence of endospore was observed by spore staining as per the standard method (Harigan and Mc Cance, 1966).

3.10. Biochemical characteristics of the bacterial isolates

The biochemical tests were carried out as per the methods described by Seeley and Vandemark (1981).

3.10.1. Gelatin liquefaction

The ability of the bacterial isolates to produce gelatinase enzyme which liquefies gelatin was tested. The cultures were inoculated into nutrient gelatin deep tubes and incubated for 48 h at 28°C. Then the tubes were placed in a refrigerator at 4°C for 30 min and observed for gelatin liquefaction.

3.10.2. Sugar Utilization test

The bacterial isolates were inoculated in peptone broth with 1 per cent concentration of glucose, lactose, sucrose, mannitol, starch, maltose, trehalose, fructose, galactose, rhamnose, sorbitol, mannose, xylose, inositol were added to each set
respectively with phenol red as a indicator. Inverted Durham’s tube was dispensed without any air bubbles into each test tube and then kept for steam sterilization. Tubes were then inoculated and incubated at 37°C for 24 hours. After incubation, the presence or the absence of Gas in Durham’s tube and colour change from red to yellow were observed.

3.10.3. Catalase activity

Catalase test was performed by adding one ml of 0.5 per cent hydrogen peroxide to agar slant culture. Release of free oxygen gas bubbles indicated catalase positivity.

3.10.4. Starch hydrolysis

The bacterial isolates were streaked on nutrient agar plates containing 2 per cent starch and incubated at room temperature. Hydrolysis of starch was tested by flooding Gram’s iodine. Plates were observed for the presence of clear zones around the bacterial colonies (Dunican and Seeley, 1962).

3.10.5. Indole production

The isolates of bacteria were inoculated into glucose tryptone broth taken in test tubes. After 48 h of incubation, 0.3 ml of Kovac's reagent was added and mixed well. The reddening of the alcohol layer within a few min indicated indole production.

3.10.6. Methyl-red Test

The MR-VP (Methyl-red and Voges-Proskauer) broth was inoculated with the bacterial isolate and incubated for 48 h at 35°C. To the test tube, a few drops of an alcoholic solution of methyl red were added. The development of distinct red colour indicated of positive reaction.

3.10.7. Voges-Proskauer Test

The MR-VP broth was inoculated with the bacterial isolate and incubated for 48 h at 35°C. A quantity of 0.5 ml of n-Naphthol solution (5 per cent solution in 70 per
cent ethyl alcohol) was added and shaken gently for 15 min. The positive reaction of acetyl methyl carbinol production was indicated by the development of red colour.

3.10.8. Citrate Utilization Test

The isolates of bacteria were inoculated into test tubes having Simmon's citrate agar medium and incubated for 48 h at 35°C. Simmon's citrate agar contains citrate as its only carbon and energy source. The presence of growth and a change of colour from green to blue due to pH change is a positive test.

3.10.9. Hydrogen sulphide production

Sulphide Indole Motility (SIM) agar deep tubes were stab inoculated with the isolates of bacteria and incubated at 35°C for 48 h. Black colouration along the line of stab inoculation indicates H₂S production.

3.10.10. Casein hydrolysis

The casein hydrolysis test was performed by the supplementation of nutrient agar medium with skim milk powder. The formation of clear zones adjacent to the bacterial growth was considered positive for casein hydrolysis (Smibert and Krieg, 1981).

3.10.11. Urease test

Urease test was performed on urea broth containing phenol red as pH indicator (pH 6.8). The bacterial isolates were inoculated into the sterilized urea broth and incubated for 24 h. The development of red colour in the broth indicated the positive reaction for the test (Christensen, 1946).

3.10.12. Nitrate reduction test

Nitrate reduction by the culture was tested by growing in 0.1 per cent (W/V) nitrate broth for 48 h. Appearance of distinct red colour after adding a drop of sulfanilic acid (dissolve 0.8 g of sulfanilic acid in 150 ml of 5 N acetic acid) and a drop of
α-napthylamine reagent to each of the nitrate broth cultures indicated the test as positive (Neyra et al., 1977).

3.10.13. Litmus milk test

The cultures were inoculated into the test tube containing litmus milk medium (containing 10 per cent skim milk powder and litmus and incubated for 24 h). The colour change from lavender to purplish blue indicated alkalinity and the appearance of red colour indicated acidity (Smibert and Krieg, 1981).

3.10.14. Fluorescence

Petri plates streaked with the bacterial isolates were exposed to Ultra Violet rays (Phillips, Holland 2200°A) and observed for fluorescence, if any.

3.11. Screening of bacteria for higher biosurfactant production by different methods

The bacterial isolates selected for biosurfactant production was further subjected to various tests to know their biosurfactant production ability.

3.11.1. Blood agar lysis (Thavasi et al., 2011c)

Hemolytic assay was performed in 5 per cent sheep blood agar plates. Final pH was adjusted to 7.3 before autoclaving at 121°C for 15 min. 50μl of bacterial culture grown in Mineral Salt Medium (MSM) was pour plated on to blood agar plates and incubated for 48 h at 37°C. The plates were visually inspected for clear zone (hemolysis) around the colony. The diameter of the clear zone depends on the concentration of the biosurfactant (Mulligan et al., 1984). The zones of clearing were scored as follows: ‘−’, no hemolysis; ‘+’, hemolysis. Three replicates for each strain were inoculated and clear zones in each plate were analyzed.
3.11.2. Drop-collapse methodology

Drop collapse test was performed by following the procedure described by Jain et al. (1991) and Modified by Bodour and Miller-Maier (1998). The drop-collapse technique was performed in the polystyrene lid of a 96 well micro titre plate (12.7× 8.5 cm) (Tarson, India). The wells were rinsed with hot water, ethanol and distilled water followed by drying. Each well was coated with 2 µl of coconut oil which was spread as a thin coating over the bottom of the well. The lid was equilibrated for 24 h at room temperature to ensure a uniform oil coating and then 5 µl of the culture supernatant was added to the well using 25 ml glass syringe (Hamilton, Reno, NV, USA) by holding the syringe at an angle of 45 degree. The syringe was rinsed three times between each sample by addition of water followed by acetone. The drop results were determined visually after one minute. Biosurfactant producing cultures were giving flat drops scored as positive ‘+’. Those cultures that gave rounded drops were scored as negative ‘−’ indicative of the lack of biosurfactant production.

3.11.3. Oil spreading test

Oil spreading experiment was performed using the method described by Morikawa et al. (2000). In brief, 20 ml of distilled water was added to a plastic Petri dish followed by addition of 20 µl of crude oil to the surface of the water. 10 µl of cell free culture broth was then added to the oil surface. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surfactant activity, also called oil displacement activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and Triton X-100 was used as the positive control.
3.11.4. Bacterial adherence to hydrocarbons (BATH)

BATH assay was carried out as described by Rosenberg et al. (1980), based on the degree of cell adherence to liquid hydrocarbon following a brief period of mixing. Bacterial isolates were cultured in MSM medium with 2 per cent glucose (10 ml) as the carbon source and incubated at 37°C for 48 h under an Orbital shaker. After the incubation the bacterial cells (8 ml) were harvested, washed twice with 4 ml PUM buffer (pH 7.1) containing 16.9 g of K$_2$HPO$_4$, 7.3 g of KH$_2$PO$_4$, 1.8 g of urea and 0.2 g of MgSO$_4$.7H$_2$O dissolved in one litre distilled water. Then, the cells were again suspended in the same buffer (8 ml) prior to the measurement of the initial density of cell suspension spectrophotometrically (M/s Elico, India) at the wavelength of 610 nm. The 8 ml of bacterial cell suspension was then mixed with 2 ml of crude oil in tissue culture tubes and incubated at room temperature for 10 min prior to vigorous mixing by vortex for about 2 min. After shaking, crude oil and reactant phase was then measured at 610 nm in a spectrophotometer. From the OD values, percentage of cells attached to crude oil was calculated using the following formula:

\[
\text{Percentage of bacterial cell adherence} = \left[1 - \frac{\text{OD}_{\text{shaken with oil}}}{\text{OD}_{\text{original}}} \right] \times 100
\]

Where: \( \text{OD}_{\text{shaken with oil}} \) - OD of the mixture containing cells and crude oil

\( \text{OD}_{\text{original}} \) - OD of the cell suspension in the buffer solution (before mixing with crude oil) to differentiate intracellular reduction

OD- optical density

A few drops of 2-(4-Iodophenyl)-3-(4-Nitrophenyl)-5- Phenyltetrazolium chloride (INT) solution was added to the above BATH assay solution and observed under a light microscope. The INT turned red if it was reduced inside the cells, indicating the viability of the cells adhered to the crude oil droplets.
3.11.5. Cetyl Trimethyl Ammonium Bromide (CTAB) plate assay

CTAB plate assay was performed according to Siegmund and Wagner (1991). The cultures were grown in a Orbital rotary shaker using 500 ml shaker flasks at 34°C for 24 h with the Siegmund and Wagner Medium (SWM).

The SWM contained 20 g glucose, 0.7 g KH$_2$PO$_4$, 0.9 g Na$_2$HPO$_4$, 2 g NaNO$_3$, 0.4g MgSO$_4$H$_2$O, 0.1 g CaCl$_2$2H$_2$O, distilled water- 1000 ml and 2 ml of a trace element solution, which contained, per litre, 2 g FeSO$_4$.7H$_2$O, 1.5 g MnSO$_4$.H$_2$O and 0.6 g (NH$_4$)$_6$Mo7O$_{24}$.4H$_2$O.

The SWM agar plates were prepared by adding 0.2 g CTAB, 0.5µl methylene blue and 15 g agar to 1 litre of the above medium. Following the improved procedure of Gunther et al. (2005), shallow wells were cut on the agar plate surface using the gel puncher. Ten µl of the inoculum of different isolates were prepared already and added into each wells respectively. The plates were incubated for 48 h at 34°C. In control plates only medium without inoculum was added in the wells. Appearance of blue halos in these plates indicated the production of biosurfactant. The blue halo zones indicated positive; whereas, negative as no halos. Three replicates for each isolates were maintained and a clear zone in each plate was analyzed.

3.11.6. Surface tension measurement (Du Nouy methodology)

Surface tension was measured with a du Nouy Tensiometer (Kruss Digital-tensiometer 10, Hamburg, Germany) at room temperature (28 ± 2°C). Twenty ml volume of each cell free culture broth was placed into a clean 50 ml glass beaker and placed onto the tensiometer platform. Cell free culture broth was equilibrated for 15 min in small weighing dish prior to the surface tension determination. A platinum wire ring submerged into the solution was then slowly pulled through the liquid-air interface, to measure the surface tension (mN/m). Surface tension measurement values
were recorded and expressed as mN/m. Between each measurement, the platinum wire ring was rinsed three times with water, followed by acetone and was allowed to dry. The surface tension values shown are the average of three replicates from the same culture. Control consisted of a sterile culture medium plus inoculum. The experiments were carried out at 28 ± 2°C temperature. Distilled water and isopropanol were used as standards (Du-Nouy, 1919; McInerney et al., 1990).

3.11.7. Emulsification measurements

The emulsifying activity of the biosurfactant was determined by using the cell free culture broth (Cooper and Goldenberg 1987). Cell free culture broth was used as the biosurfactant source to check the emulsification of crude oil. 1 ml of cell free culture broth was added to 5 ml of 50 mM Tris buffer (pH 8.0) in a 30 ml screw-capped test tube. Five milligram of hydrocarbon was added to the above solution and vortex-shaken for one minute and the emulsion mixture was allowed to stand for 20 min. A negative control was maintained only with buffer solution and crude oil and Triton X-100 was used as the positive control. The equation used to determine the Emulsion Index $E_{24}$ (%) is as follows:

$$E_{24} (%) = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

3.12. 16S rRNA sequencing of the selected bacterial isolates.

The genomic DNA of the isolate PBSC1 and KBSB1 were extracted by salting out method (Ferrara et al., 2006). The polymerase chain reaction was carried out on a thermal cycler (MJ mini PTC-225 Peltier Thermal cycler, BIO-RAD) in a 50 µl reaction mix. The reaction mix contained 10X amplification buffer (5 ml), 1.5 mM MgCl2 (5µl), 1µl forward primer (10 mM), 1µl reverse primer (10 mM), 1 µl dNTP and 0.25 µl Taq polymerase. After an initial denaturation at 95°C for 1 min, amplification was performed with 35 cycles of 35 s at 94°C, 40 s at 55°C, 2 min at 72°C followed by a
final extension at 72°C for 8 min. The PCR product was purified by removing the unincorporated PCR primers and dNTPs from PCR products by using Montage PCR clean up kit (Millipore). The purified PCR products of approximately 1,400 bp were sequenced using 2 Universal 518F forward primer, 5'CCAGCAGCCGCGGTAATACG-3'; and 800R reverse primer, 5'TACCAGGGTATCTAATCC-3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were resolved in a Biosystem model 3730XL automated DNA sequencing system (Applied Biosystems, USA).

3.13. **Dry cell biomass estimation (DCBM)**

Dry cell biomass of a hydrocarbon-grown microorganism (water immiscible) was determined by centrifugation of 50 ml of culture broth at 19,300 \( \times \) g for 20 min. The sediment cells were then extracted with a mixture of acetone/hexane (3:1) to remove the adhering hydrocarbon. This was followed by centrifugation with hexane (10 ml) and drying at 80°C overnight in an oven to obtain a concordant value of dry biomass. When the culture was grown on a water-miscible substrate, DCBM was determined by centrifuging culture broth at 7,740 \( \times \) g for 15 min. The cell pellet obtained was dried overnight at 60°C and weighed. The dry cell biomass was expressed in g/l.

3.14. **Extraction of biosurfactant**

One hundred ml of Mineral Salt Medium (MSM) with 2 per cent glucose (pH 7.0) was prepared in a 250 ml Erlenmeyer flask and sterilized in an autoclave. It was inoculated with 5.0 ml \( \times 10^9 \) cells per ml of culture broth of selected bacterial isolates with 1ml of cyclohexane and the flask were incubated at 35°C for 3 days in an orbital rotary shaker set at 120 rpm/min. After incubation, the culture broth was centrifuged at 12,000 \( \times \) g for 10 min at 4°C to remove the bacterial cells and the supernatant was
filtered through a 0.2 µm of Whatman filter paper. The filtered supernatant was employed for extraction of biosurfactant as described in Table 7. The resultant aliquot was concentrated in a rotary vacuum evaporator and tested for emulsification activity as described in the section 3.10.7.

Table - 7 Extraction of Biosurfactant from isolates PBSC1 and KBSB1

<table>
<thead>
<tr>
<th>S. No</th>
<th>Solvents</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetone</td>
<td>Equal volume&lt;br&gt;Acidification using 6N HCl to pH 2 and added with equal volume of Acetone</td>
</tr>
<tr>
<td>2</td>
<td>Ethyl Acetate</td>
<td>Equal volume&lt;br&gt;Acidification using 6N HCl to pH 2 and added with equal volume of ethyl acetate</td>
</tr>
<tr>
<td>3</td>
<td>Dichloromethane</td>
<td>Equal volume&lt;br&gt;Acidification using 6N HCl to pH 2 and added with equal volume of dichloromethane</td>
</tr>
<tr>
<td>4</td>
<td>Diethyl ether</td>
<td>Equal volume&lt;br&gt;Acidification using 6N HCl to pH 2 and added with equal volume of dichloromethane</td>
</tr>
<tr>
<td>5</td>
<td>Methanol: Chloroform (2:1 ratio)</td>
<td>Equal volume&lt;br&gt;Acidification using 6N HCl to pH 2 and added with equal volume of Methanol: Chloroform</td>
</tr>
</tbody>
</table>

3.15. Biosurfactant Characterization

3.15.1. Biochemical characterization

3.15.1.1. Estimation of protein

To estimate the protein content of surface active compound Bradford’s dye binding protein assay (Bradford, 1976) with Bovine Serum Albumin as the standard was followed. One ml of the cell free extract of *Pseudomonas aeruginosa* PBSC1 and the isolate, *Bacillus cereus* KBSB1 were separately added with 4 ml of dye concentrate (100 mg of coomassie brilliant blue G 250 dissolved in 50 ml of 95 per cent ethanol and
to this 100 ml of concentrated orthophosphoric acid was added. The solution was made up to 200 ml with distilled water and kept in amber colour bottles stored in refrigerator. The contents were mixed well and allowed for the development of colour for at least with in 30 min. The colour intensity was measured at 595 nm.

3.15.1.1. Standard curve preparation for protein estimation

Weighed accurately 50 mg of Bovine Serum Albumin and dissolved in distilled water and made up to 50 ml in standard flask as a stock solution. Then, diluted 10 ml of the stock solution to 50 ml with distilled water in a standard flask (working standard). From that pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml working standard into a series of test tubes and followed the above said procedure (Section 3.14.1.1.). One ml of the working standard contained 200 µg of protein. From that, standard graph was drawn and calculated the amount of protein in the sample.

3.15.1.2. Carbohydrate estimation

The presence of carbohydrates in the cell free extract was estimated by the method described by Chaplin and Kennedy (1994). To each hundred µl cell free extract of *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1 were mixed with 1 ml of 5 per cent phenol and 2.5 ml of 96 per cent of concentrated H$_2$SO$_4$ and it was mixed well and incubated for 15 min. Then the absorbance was recorded at 490 nm. The distilled water with all reagents served as control. The experiment was performed in five replicates.

3.15.1.3. Estimation of free fatty acids

The free fatty acid content in the cell free extract of *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1 were estimated by titrating the extract against 0.1 N KOH using the indicator phenolphthalein until a pink colour was obtained. The free fatty acid was attained by using the formula (Sadasivam and Manickam, 1991).
\[
\text{Acid value} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample}}
\]

The sterile distilled water with all reagents served as blank. The experiment was performed in five replicates.

3.15.2. **Rhamnose test for the biosurfactant**

The presence of carbohydrate group in the biosurfactant molecule was assayed by rhamnose test using the method of Dubois *et al.* (1956). About 0.5 ml of cell free supernatant from the isolates were mixed with 0.5 ml of 5 per cent phenol solution and 2.5 ml of sulphuric acid and incubated for 15 min before measuring absorbance at 490 nm in a spectrometer (M/s Elico, India). The absorbance maximum indicated the presence of carbohydrate groups.

3.15.3. **Activity Characterization**

3.15.3.1. **Foaming properties of the biosurfactant**

The foam was produced by hand shaking a 5 g/l of crude biosurfactant solution from the isolates of *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1 for several minutes. The stability of the foam was monitored by observing them for 2 h duration.

3.15.3.2. **Emulsifying properties of the biosurfactant**

The emulsifying activity of the biosurfactant from the selected isolates was determined as described in the Section 3.10.7.

3.15.3.3. **Critical Micelle Concentration (CMC) value of the biosurfactant**

The Critical Micelle Concentration (CMC) value was used to measure the surface tension of dilution of the cell free culture broth of PBSC1 and KBSB1 (Cooper *et al.*, 1981, Zajic and Seffens, 1984). This method involved the measurement of the surface tension of a series of dilutions. The CMC was determined from the break point of the surface tension versus dilution times curve. The dilution reduces the biosurfactant
levels below the CMC values at a point in which the surface tension of the media increase suddenly. The selected isolates were grown in the MSM containing glucose 2 per cent for 72 h in a Orbital shaker at 120 rpm. Then, the surface tension of the media was determined as described earlier (Section 3.10.6.) after making dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$. The dilution at which the surface tension suddenly increased was recorded as the break point known as Critical Micelle Concentration (CMC).

3.15.4. Stability characterization

3.15.4.1. Determination of the effect of temperature, pH and NaCl, on the activity of the biosurfactant

To determine the thermal stability of the biosurfactant, cell-free broth of the isolate PBSC1 and KBSB1 were maintained at a constant temperature range of 20 - 80°C for 15 min, followed by cooling at room temperature (28 ± 2°C). The effect of pH and salinity on stability of the biosurfactant was evaluated by altering the pH (2 - 12) prior to filter sterilization (Whatman qualitative filter paper 42, 125 mm diameter) and the concentration of NaCl (0 - 1 per cent, 5 per cent) of the cell free culture supernatant and measuring the surface tension and Emulsification index ($E_{24}$, %) as described above in Section 3.10.6 and 3.10.7 respectively.

3.15.5. Chemical characterization of the biosurfactant

3.15.5.1. Preparative column chromatography

For the purification of biosurfactant by column chromatography, A column of dimensions 30 ×4 cm (length ×diameter) was prepared with 60 g of activated silica gel slurry in enough chloroform. A portion (1 g) of the crude biosurfactant was dissolved in 5ml of chloroform, micro filtered (pore size 3 μm) and loaded on the silica gel column with the help of Pasteur pipette. The sample-loaded column was first washed with n-hexane (~ 200 ml), followed by chloroform (~ 300 ml) finally Chloroform: Methanol
mobile phases were then applied in a sequence: 1:1 v/v (200 ml) and 1:2 v/v (200 ml) at a flow rate of 1 ml/min. The biosurfactant fractions were collected separately, freeze-dried, weighed and characterized.

3.15.5.2. Thin layer chromatography

Each of the Column chromatography purified fraction was applied on preparative silica gel plates. Chromatograms were developed with the following saturation buffer and spraying agents (Table 8). The Rf value of each macromolecule was noted using the formula:

\[ R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}} \]

Table - 8 Saturation buffer and spraying agent for the macromolecules

<table>
<thead>
<tr>
<th>S. No</th>
<th>Macromolecule</th>
<th>Saturation buffer</th>
<th>Spraying agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carbohydrate</td>
<td>Chloroform:Acetic acid:Water (60:30:10)</td>
<td>50 per cent H₂SO₄</td>
</tr>
<tr>
<td>2</td>
<td>Lipids</td>
<td>Chloroform:Methanol:Water (65:25:4)</td>
<td>10 per cent H₂SO₄ in ethanol</td>
</tr>
<tr>
<td>3</td>
<td>Amino acids</td>
<td>96 per cent Ethanol: Water(7:3)</td>
<td>0.1 per cent Ninhydrin in acetone</td>
</tr>
<tr>
<td>4</td>
<td>Glycolipid</td>
<td>Chloroform:Methanol:7N NH₄OH</td>
<td>Anthrone reagent</td>
</tr>
</tbody>
</table>

The fraction were scrapped and eluted with chloroform: methanol (1:2, v/v) mixture. The solvent fraction was centrifuged for 10 min to remove the silica gel; the aliquots were micro-filtered and concentrated by air-drying. Emulsification activity was performed again for the confirmation of the biosurfactant production.

3.15.5.3. Fourier Transform Infrared (FT-IR) spectral analysis of biosurfactants from the isolates

The FT-IR spectra was recorded in a Thermo Nicolet, AVATAR 330 FT-IR system, Madison WI 53711-4495, in the spectral region of 4000-400 cm⁻¹ using potassium bromide (KBr) solid cells. The analysis was done in the Department of Chemistry, Annamalai University, India. The air dried biosurfactant sample was
ground with a purified potassium bromide salt to remove scattering effects from large crystals. This powdered mixture was then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can be passed. The spectrum was recorded and analyzed using the standard methods described by the previous authors (Pornsunthorntawee et al., 2009).

3.16. Kinetics of biosurfactant producing isolate

The kinetics studies were carried out in batch culture for biosurfactant production from a pure substrate by both the isolate *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1. One hundred ml of MSM broth (pH 7.0) with 2 percent glucose was prepared in different 250 ml Erlenmeyer flasks and sterilized in an autoclave. After autoclaving, each flask was added with one ml of cyclohexane aseptically. Then 2.5 ml of inoculum of *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1 were added individually to respective flasks and incubated for 3 days at room temperature over an orbital rotary shaker set at 120 rpm/min. In these experiments, variations in biomass, substrate and product concentrations were recorded over a period of 20 h with an interval of every 2 h starting from the zero hour. Five batches were maintained in order to take samples at an appropriate hour and that does not affect the statistical and experimental significance. Kinetic study was performed according to the Monad model. The experiment was designed in such a way to calculate, specific growth rate (µ).

The specific growth rates (µ) of culture was then calculated respectively, based on the plot of In (cell dry weight at log phase) versus time (hour). Values are recorded as mean from five batches. Substrate consumption (Y_{p/x}), product formation related to dry cell biomass (Y_{p/x}), bacterial growth related to substrate consumption (Y_{x/s}), specific substrate utilization rate (q_{s}) and specific product yield (q_{p}). Biomass were measured by
dry weight method as described in Section 3.7 and residual sugar and consumed sugar in the fermentation broth was estimated by Dinitrosalicylic acid method (Miller, 1972) and expressed in g/l. Crude biosurfactant was recovered from the cell free broth as described in Section 3.10 and expressed as g/l. Simultaneously surface tension reduction of the medium was recorded as described in the Section 3.10.7.

3.17. Factors influencing the production and activity of the biosurfactant

The selected bacterial isolates namely, PBSC1 and KBSB1 were studied for the biosurfactant production under the influence of certain physical and chemical factors. All the experiments were carried with five replicates. Biosurfactant production was determined by estimating the surface tension (ST) reduction and the emulsification assay ($E_{24\%}$) and the dry cell biomass (DCBM).

3.17.1. Effect of carbon sources on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) with five different carbon sources viz., glucose, glycerol, fructose, sodium citrate, mannitol and starch were prepared in a 250 ml Erlenmeyer flask and sterilized in an autoclave. It was added with 1.0 ml of crude oil. To that 5.0 ml of inoculum of different bacterial isolates were added and incubated at $28 \pm 2^\circ C$ for 5 days over a Orbital shaker set at 120 strokes/min. Extraction was done following the procedure in Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.17.2. Effect of nitrogen sources on the production and activity of the biosurfactant

One hundred ml of MSM broth (pH 7.0) with five different nitrogen sources like Yeast extract, Peptone, Ammonium chloride, Ammonium nitrate and Sodium nitratewere dispensed in 250 ml Erlenmeyer flasks and sterilized in an autoclave. A quantity of one ml of crude oil and 5.0 ml of culture inoculum were added. The flasks were
incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes/min. Extraction was done following the procedure in Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.17.3. Effect of pH on the production and activity of the biosurfactant

One hundred ml of MSM broth was prepared of varying pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 and sterilized. It was added with one ml of crude oil. To that 5.0 ml of bacterial inoculation of different isolates were added and incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes/min. Extraction was done following the procedure in Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.17.4. Effect of temperature on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) was prepared and sterilized. It was added with 1.0 ml of crude oil and different bacterial cultures were inoculated. The flasks were incubated at varying temperatures of 25, 30, 35, 40, 45 and 50°C in an incubator over shaker. Extraction was done following the procedure in Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.17.5. Effect of hydrocarbons on the production and activity of the biosurfactant

One hundred ml of MSM broth (pH 7.0) was taken in a clean 250 ml Erlenmeyer flask and sterilized. One ml of different heavy hydrocarbons viz., n-hexadecane, heptane, xylene, kerosene, petrol, diesel and crude motor oil were added in different flasks. To that 5.0 ml of inoculum of different bacterial isolates were added and incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes/min. Extraction was done following the procedure in
Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.17.6. Effect of trace elements on the production and activity of the biosurfactant

One hundred ml of MSM broth with 2 per cent glucose for B. cereus, glycerol for P. aeruginosa and pH 7.0 was prepared as follows; (a) without MgSO₄ (b) without MnSO₄, (c) without FeSO₄, (d) without FeSO₄ and MnSO₄, (e) without MgSO₄ and FeSO₄ (f) without MgSO₄, MnSO₄ and FeSO₄. One ml crude oil was added aseptically and to that 5 ml of inoculums from selected bacterial isolates was added and kept at room temperature 28 ± 2°C for 5 days over a shaker set at 120 strokes min⁻¹. Extraction was done following the procedure in Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.17.7. Effect of inoculum load on the production and activity of the biosurfactant

One hundred ml of MSM broth with 2 per cent glucose for B. cereus, glycerol for P. aeruginosa and pH 7.0 was prepared in seven 250 ml Erlenmeyer flask respectively. One ml crude oil was added aseptically and to that 0.5, 1, 1.5, 2, 2.5, 3 ml of inoculum and a control (without inoculums) from selected bacterial isolates was added to the respective flasks and kept at room temperature 28 ± 2°C for 5 days over a shaker set at 120 strokes/min. Extraction was done following the procedure in Section 3.13. The emulsification assay (Section 3.10.7), surface tension (Section 3.10.6) and dry cell biomass (Section 3.12) were also analyzed.

3.18. Studies on the constituents of Cashew Apple Juice (CAJ)

Cashew apple juice was extracted by compressing the cashew apple (Anacardium occidentale L.,) after compressing, the substrate was centrifuged at 3500 rpm for 20 min (REMI, India), filtered using a Whatman qualitative filter paper
(42, 125) and diluted with water (1 per cent v/v). Afterwards, pH was adjusted to 7.0 and was sterilized by filtering through a 0.45 µm Millipore membrane. Total reducing sugars were determined calorimetrically by dinitrosalicylic acid (DNS) method (Miller, 1972). 1.4 ml of DNA was added to 1.4 ml of sample. The mixture was heated in a water bath at 100°C for 5 min and potassium sodium tartarate was added subsequently. Afterwards, the solution was cooled to room temperature (28 ± 2°C) and the absorbance was measured at 540 nm. The calibration curve used to convert absorbance to concentration.

Total carbohydrates were determined according to the methods described by Somogyi (1952) and Dubois et al. (1956). Calcium was estimated using Triacid extract (Tandon, 1995). Frrrous-Atomic absorption spectrometer (M/s Elico, India) (Lindsay and Norvell, 1978) and total phosphorous by Vanadomolybdic method (Jackson, 1973).

The ascorbic acid was determined by Calorimetric analysis (Sadasivam and Manickam, 1992). Briefly, 10 - 100 µg standard dehydroascorbic solution was taken into a series of tubes. The cashew apple juice was brominated by adding bromine water until the sample turns yellow (10 ml approximately), further the samples were made up to 50 ml using 4 per cent oxalic acid. Similarly, 10 ml ascorbic acid stock solution was converted to dehydroform by bromination. Further, the samples were made up to the volume in each tube to 3 ml by adding distilled water. Added one ml of 2 per cent 2,4-dinitrophenyl Hydrazine reagent followed by 1 - 2 drops of thiourea to each tube. Water was used as blank to mix the contents of the tubes thoroughly and incubated at 37°C for 3 h. After incubation dissolved the orange-red osazone crystals formed by the addition of 7 ml of 80 per cent sulphuric acid and the absorbance was measured at 540 nm. Plotting standard ascorbic acid concentration, the sample concentration as determined.


3.19. Effect of cashew apple juice on biosurfactant production

Two hundred ml of MSM broth (pH 7.0) was taken in a clean 500 ml Erlenmeyer flask and sterilized in an autoclave at 121°C for 15 min. The sterilized MSM broth was added with various concentrations of cashew apple juice (2, 4, 6 and 8 per cent). To prove the effect of cashew apple juice on the biosurfactant production, cashew apple juice was used as such without inorganic mineral salts. For comparison, defined medium (MSM with 2 per cent glucose) was included in the study. To that, 5.0 ml, inoculum of *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1 were inoculated to the respective flasks and incubated at room temperature (28 ± 2°C) for 3 days over an Orbital rotary shaker set at 120 rpm/min.

To find out the variation in the biosurfactant production, the reduction in surface tension was made using 20 ml of cell free culture broth recorded at zero and at 72 h in a duNouy Tensiometer (Kruss Digital-Tensiometer, Germany) at room temperature (28 ± 2°C), as described in the section 3.10.6. An abiotic control flask was also incubated, provided with the same nutritional conditions, but without bacteria and the surface tension of the medium was determined. Dry cell biomass (DCBM) was determined on the zero hour as well as 72 h by centrifuging the culture broth at 7,740 × g for 15 min. The cell pellet obtained was dried overnight at 60°C and weighed. The dry cell biomass were expressed in g/l. Change in pH of the medium was recorded for all the treatments both initially and finally (72 h) using a pH meter (M/s Elico, India).

The biosurfactant was extracted from the various treatments as described earlier in section 3.13 at zero and at 72 h. The crude biosurfactant was stored at 5°C in the refrigerator. The concentration of biosurfactant was expressed in g/l. FT-IR analysis was performed for the best concentration of cashew apple juice as described in 3.5.14.3.
All the experiment was performed with five replicates. The average mean values were recorded.

3.20. Emulsification activity of biosurfactant derived from cashew apple juice

The biosurfactant derived from cashew apple juice of both *P. aeruginosa* PBSC1 and *B. cereus* KBSB1 were subjected to emulsification activity using hexane, heptanes, olive oil and kerosene. The emulsification activity was performed as the procedure described in section 3.10.7.

3.21. Response Surface Methodology (RSM) for the optimization of biosurfactant production from *P. aeruginosa* PBSC1 using Cashew Apple Juice

Based on the literature the isolate *Pseudomonas aeruginosa* PBSC1 was selected for enhancing the biosurfactant production using the cashew apple juice medium. The isolate *P. aeruginosa* PBSC1 recorded its better biosurfactant production in the media containing glycerol as carbon source, sodium nitrate as nitrogen source, pH 7.0 and temperature at 30°C. To examine the interaction effect of different selected parameters (glycerol, sodium nitrate, pH and temperature) on biosurfactant production by *P. aeruginosa* PBSC1, the Central Composite Design (CCD), with 30 experiments were performed in duplicate. The reduction in surface tension of the medium is the direct measure of the biosurfactant production. Hence, the value of the dependent response surface tension reduction was the mean of two replications. The reduction in surface tension was determined as per the methods described in the Section 3.10.7. The second-order polynomial coefficients were calculated and analyzed using Design Expert software (version 8.0.7.1, Stat-Ease Inc., USA), Central composite design was conducted in the optimum vicinity to locate the true optimum conditions of Glycerol (A), sodium nitrate (B), pH (C), Temperature (D) for the production of biosurfactant. For the four factors, this trial was a $2^{3-1}$ factorial design augmented by six axial points.
(or called star points) coded $\alpha \pm 2$ and two replicates of center points (all factors at level 0), resulting in a total number of 30 experiments (Box and Wilson, 1951). The coded values of the process parameters were determined by the following equation.

$$x_i = \frac{x_i - X_0}{\Delta x}$$

where $x_i$-coded value of the $i^{th}$ variable, $X_i$-uncoded value of the $i^{th}$ test variable and $X_0$-uncoded value of the $i^{th}$ test variable at centre point. The range and levels of individual variables are given in Table 9. The experimental design is given in the Table 10. In the study, surface tension reduction was processed for Eq. (2) including ANOVA to obtain the interaction between the process variables and the responses. The regression analysis was performed to estimate the response function as a second order polynomial

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,j<i}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

where $Y$ is the predicted response, $\beta_i, \beta_j, \beta_{ij}$ are coefficients estimated from regression. They represent the linear, quadratic and cross products of $x_1, x_2, x_3$ on response (Umesh et al., 2008). The experiment performed in batch fermentation using cashew apple juice in a one litre Erlenmeyer flask with thirty different trials as given below (Table 10). A statistical program package Design Expert 8.0.7.1 was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation. The equation was validated by the statistical tests called ANOVA analysis. The significance of each term in the equation is to estimate the goodness of fit in each case.

Response surfaces were drawn to determine the individual and interactive effects of the test variable on the reduction in the surface tension of the medium. The optimal values of the test variables were first obtained in coded units and then converted to the uncoded units. The quality fit of the model equation was expressed with the coefficient of determination $R^2$ and its statistical significance was determined.
by an F-test. The significance of the regression coefficients was tested by a t-test (Maddox and Richert, 1977; Montgomery, 1991).

Table - 9 Level of different process variables in coded and un-coded form for the reduction of surface tension (*P. aeruginosa* PBSC1)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Codes</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol (g/l)</td>
<td>A</td>
<td>-2</td>
</tr>
<tr>
<td>Sodium Nitrate (g/l)</td>
<td>B</td>
<td>-1</td>
</tr>
<tr>
<td>pH</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>Temperature (ºC)</td>
<td>D</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+2</td>
</tr>
</tbody>
</table>

Table - 10 Experimental conditions of $2^4$ central composition designs

<table>
<thead>
<tr>
<th>Std</th>
<th>Run Order</th>
<th>Glycerol</th>
<th>Sodium Nitrate</th>
<th>pH</th>
<th>Temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>27</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>22</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>20</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>17</td>
<td>11</td>
<td>-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>-2</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>15</td>
<td>0</td>
<td>-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>25</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>26</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>20</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>29</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>22</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>23</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>26</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>28</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
</tbody>
</table>
3.22. Studies on the constituents of Cassava waste water

Cassava effluent was obtained from Alif trading company manufacturer and exported of starch, Melur, Madurai, District of Tamil Nadu, India and stored at 4°C until needed. The medium was prepared by heating the waste until boiling to facilitate the removal of insoluble solid material. After cooling, the substrate was centrifuges at 8000× g for 20 min (REMI, India). The supernatant was distributed in flasks and sterilized in an autoclaved at 121°C for 15 min. natural pH of the medium was 5.9 and was adjusted to neutral pH 7.0 (using 1N KOH). The same cassava water was characterized by the following methods.

2. Total potassium-Neutralization triacid with ammonia and reading in Flame photometer (M/s Elico, India) (Jackson, 1973).
5. Iron and sodium-atomic absorption spectrometer (M/s Elico, India) (Lindsay and Norvell, 1978).
6. Reducing sugars and total carbohydrates (Somogyi, 1952; Dubois et al., 1956; Miller, 1972).
7. The mineral fraction (S, Al, B, Cu, Mn, Zn, Cd, Cr, Ni and Pb) - Atomic absorption spectrometer (M/s Elico, India) expressed as mg/l [all the minerals fractions were estimated by adopting EPA-US Environmental Protection Agency(1994) and Linsay and Norvell (1978) protocols. The ammonia content based on Bremner and Keeney, 1965].
3.23. Effect of Cassava waste water on biosurfactant production

Two hundred ml of MSM broth (pH 7.0) was taken in a clean 500 ml Erlenmeyer flask and sterilized in an autoclave at 121°C for 15 min. The sterilized MSM broth was added with various concentrations of cassava waste water that is 2, 4, 6 and 8 per cent. To prove the effect of cassava waste water on the biosurfactant production, cassava waste water was used as such without inorganic mineral salts. For comparison, defined medium (MSM with 2 per cent glucose) was included in the study. To that 5.0 ml inoculum of *Pseudomonas aeruginosa* PBSC1 and *Bacillus cereus* KBSB1 were inoculated to the respective flasks and incubated at room temperature (28 ± 2°C) for 3 days over an Orbital rotary shaker set at 120 rpm/min.

To find out the variation in the biosurfactant production, the reduction in surface tension was made using 20 ml of cell free culture broth recorded at zero and at 72 h in a duNouy Tensiometer (Kruss Digital-Tensiometer, Germany) at room temperature (28 ± 2°C), as described in the section 3.10.6. An abiotic control flask was also incubated, provided with the same nutritional conditions, but without bacteria and the surface tension of the medium was determined. Dry cell biomass (DCBM) was determined on the zero hour as well as 72 h by centrifuging the culture broth at 7,740 × g for 15 min. The cell pellet obtained was dried overnight at 60º C and weighed. The dry cell biomass were expressed in g/l. Change in pH of the medium was recorded for all the treatments both initially and finally (72 h) using a pH meter (M/s Elico, India).

The biosurfactant was extracted from the various treatments as described earlier in section 3.13 at zero and at 72 h. The crude biosurfactant was stored at 5ºC in the refrigerator. The concentration of biosurfactant was expressed in g/l. FT-IR analysis was performed for the best concentration of cassava waste water as described in
3.5.14.3. All the experiment was performed with five replicates. The average mean values were recorded.

3.24. Emulsification activity of biosurfactant derived from cassava waste water

The biosurfactant derived from cassava waste water of both *P. aeruginosa* PBSC1 and *B. cereus* KBSB1 were subjected to emulsification activity using hexane, heptanes, soy bean oil and kerosene. The emulsification activity was performed as the procedure described in section 3.10.7.

3.25. Response Surface Methodology (RSM) for the optimization of biosurfactant production from *B. cereus* KBSB1 using Cassava Waste Water

The isolate *B. cereus* KBSB1 isolate was selected for enhancing the biosurfactant production using the cassava waste water medium. The isolate recorded its better biosurfactant production in the media containing glucose as carbon source, ammonium nitrate as nitrogen, pH 7.0 and temperature at 30°C. To examine the interaction effect of different selected parameters (glucose, ammonium nitrate, pH and temperature) on biosurfactant production by *B. cereus* KBSB1, the Central Composite Design (CCD), with 30 experiments were performed in duplicate. The reduction in surface tension of the medium is the direct measure of the biosurfactant production. Hence, the value of the dependent response surface tension reduction was the mean of two replications. The reduction in surface tension was determined as per the methods described in the Section 3.10.7.

The second-order polynomial coefficients were calculated and analyzed using Design Expert software (version 8.0.7.1, Stat-Ease Inc., USA), Central composite design was conducted in the optimum vicinity to locate the true optimum conditions of glucose (A), ammonium nitrate (B), pH (C), Temperature (D) for the production of biosurfactant. For the four factors, this trial was a $2^{4-1}$ factorial design augmented by six
axial points (or called star points) coded $\alpha \pm 2$ and two replicates of center points (all factors at level 0), resulting in a total number of 30 experiments (Box and Wilson, 1951).

Table - 11 Level of different process variables in coded and un-coded form for the reduction of surface tension ($B. cereus$ KBSB1)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Codes</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (g/l)</td>
<td>A</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Ammonium Nitrate (g/l)</td>
<td>B</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>pH</td>
<td>C</td>
<td>6</td>
<td>6.5</td>
<td>7</td>
<td>7.5</td>
<td>8</td>
</tr>
<tr>
<td>Temperature (ºC)</td>
<td>D</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>40</td>
</tr>
</tbody>
</table>

Table - 12 Experimental conditions of $2^4$ central composition designs

<table>
<thead>
<tr>
<th>Std</th>
<th>Run Order</th>
<th>Glucose</th>
<th>Ammonium nitrate</th>
<th>pH</th>
<th>Temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>-2</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>27</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>11</td>
<td>-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>29</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>19</td>
<td>20</td>
<td>0</td>
<td>-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>28</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>22</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>26</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>27</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>26</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>29</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
</tbody>
</table>
The coded values of the process parameters and the regression analysis were determined as described in Section 3.18. Coefficient of determination $R^2$ and its statistical significance was determined by F-test. The significance of the regression coefficients was tested by a t-test was determined. The range and levels of individual variables are given in Table 11. The experimental design is given in the Table 12.

3.26. Production of silver nanoparticles from biosurfactant (Xie et al., 2006)

The synthesis of silver nanoparticles in situ in the water-in-oil microemulsion phase was performed by the addition of synthesized two reverse micelles in the presence NaBH$_4$ as reducing agent. The synthesis involves mixing up to 0.5 ml of 0.05 Mol/l aqueous AgNO$_3$ solution, 3.0 g biosurfactant, 1.5 g n-butanol and 0.5 g n-heptane together and stirred vigorously at room temperature until homogeneous reverse micelles were formed. Next, reverse micelles were also synthesized using 0.5 ml of 0.1 mol/l aqueous NaBH$_4$ solution instead of aqueous AgNO$_3$ solution. Then, the two types of reverse micelles were mixed and stirred for 60 min at 10,000 $\times$ g. Further, the reverse micelles were broken by adding ethanol (0.5 ml ethanol for 1 ml reverse micelles). When it was broken, a particle tends to precipitate from the solution. The precipitated silver particles were isolated by centrifugation at 15,000 $\times$ g. This procedure results in the production of silver nano sized particles. The particles were then sonicated in 10 ml n-heptane solution and stored for further studies.

3.27. Optical characterization

3.27.1. UV-Visible Spectroscopy

The optical characterizations of the synthesized silver nanoparticles were analyzed through absorption spectra measured in room temperature in a UV Visible absorption spectrometer (ELICO SL 244) at the wavelength of 200 to 800 nm under dispersion mode.
3.28. Physical characterization

3.28.1. Dynamic Light Scattering (DLS) (El-Shanshoury et al., 2011)

Dynamic Light Scattering (DLS) is a well established technique used for the study of average particle size (few nanometers to few microns) and Zeta potential. In the present study, prepared silver nanoparticles was taken in disposable sizing cuvette, at 25°C temperature, 109.7 kcps Count Rate and 4.65 mm Measurement Position to measure the average particle size and Zeta potential using computer controlled particle size analyser Zetasizer Ver. 6.01 (Malvern Instruments Ltd.,). The dispersant used for dispersing the nanoparticles was double distilled water.

3.28.2. Scanning Electron Microscopy

Scanning Electron Microscopy characterization, nanoparticles solution should be first converted into a dry powder, which was then mounted on a sample holder followed by coating with a conductive metal, such as gold, using a sputter coater. The sample was then scanned with a focused fine beam of electrons. The surface characteristics of the sample were obtained from the secondary electrons emitted from the sample surface. The nanoparticles must be able to withstand vacuum and the electron beam can damage the polymer. The mean size obtained by SEM was comparable with results obtained by dynamic light scattering. The morphological features of the nanoparticle such as particle size, shape and topography were observed and results were recorded using Scanning Electron Microscope (JSM-6390, USA) at Karunya University, Coimbatore.

3.28.3. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is an imaging technique whereby a beam of electrons is focused onto a specimen causing an enlarged version to appear on a fluorescent screen or layer of photographic film, or to be detected by a CCD camera.
The microstructure studied by use of the image mode. The sample preparation and analysis was done at PSG College of Technology, Coimbatore. In our study, the synthesized silver nanoparticles were lyophilized and dispersed in 100 per cent absolute ethanol. The ethanol dispersed particles were then sonicated to deposit on a copper grid. That was analysed in Transmission Electron Microscope (JEM-2100F LaB6, USA) under 10,000X magnification.

3.28.4. X-rays Diffractometry (XRD) (El-Shanshoury et al., 2011)

X-ray diffraction (XRD) is one of the most important non-destructive tools to analyse all kinds of matter e.g fluids, powders and crystals. In our study the phase and structural analysis of the prepared silver nanoparticles were analysed using X-ray Diffraction technique. The X-ray diffractometer used in our study was SHIMADZU-MODEL XRD 6000. Fine lyophilized silver nano particle samples were spread onto a glass slide using double sided sticky tape and analysed in an X-ray diffractometer. The X-ray generator in the diffractometer was of Cu, Cr type which has a maximum output, maximum tube voltage and maximum tube current of 3 kW, ± 0.01 per cent, 60 kV and 80 mA respectively. The goniometer was a vertical type that had a scanning radius of 185 mm, operating at continuous mode, with an angle reproducibility of ± 0.001° (2θ), scanning angle range from -6° to 163° (2θ), -180 to +180 (0) and scanning speed of 0.1° to 50°/min (2θ), 0.05° to 25° /min (θ).

In our study, Cu K α of wave length 1.514 Å with nickel as filter was used and the scanning rate was fixed as 0.2°/min with scanning range fixed as 20 - 80°. The resultant diffractograms were estimated for their the crystallite size \( T, \text{nm} \) using the Scherrer equation (equation 1.0) (Scherrer, 1918)

\[
T = \frac{0.9\lambda}{\beta \cos\theta} \quad \text{(1.0)}
\]
Where \( \lambda \) is the X-ray wavelength (1.54 Å), \( \theta \) is the Bragg angle of the peak of interest and \( \beta \) is the line broadening measured from the increased peak width at half height through a Gaussian fit obtained from Origin plotting software (Origin pro 8.5 version, Origin Pro Corporation Ltd., USA).

3.29. Stability studies of synthesized silver nanoparticles

To monitor the stability of the prepared silver particles, the absorption spectra of the solution on different days were analyzed according to the procedure described in Section 3.26.1. On increasing the time from 1, 30 to 60 days the Plasmon absorption bands of the prepared silver nanoparticles were tested. During the entire chemistry process, no passivator was added into the system.

3.30. Antimicrobial characterization (Wliilaime et al., 2006)

The antibacterial activity of silver nanoparticles was preliminarily assessed by disc diffusion test to find out Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

3.30.1. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC), defined as the lowest concentration of material that inhibits the growth of an organism (Qi et al., 2004), was determined based on batch cultures containing varying concentration of metal oxide nanoparticles in suspension (0.39 - 100 µg/l). Sterile Erlenmeyer flasks (500 ml), each containing 100 ml nutrient broth were sonicated for 10 min. Subsequently, the flasks were inoculated with 1 ml of the freshly prepared bacterial suspension (\(10^3 \) - \(10^4\) CFU/ml) and then incubated in an Orbital shaker at 200 rpm, 30°C.

The high rotary shaking speed was selected to minimize aggregation and settlement of the nanoparticles over the incubation period. Bacterial growth was measured by observing the absorbance at 600 nm using a spectrophotometer.
(UV Thermo Spectronic, Helios Epsilon, USA). The experiments also included a positive control (flask containing nanoparticles and nutrient media, devoid of inoculum) and a negative control (flask containing inoculum and nutrient media, devoid of nanoparticles).

The negative controls indicated the microbial growth profile in the absence of nanoparticles. The absorbance values for positive controls were subtracted from the experimental values (flasks containing nutrient media, inoculum and nanoparticles). The MIC of the prepared silver nanoparticles was tested against *Escherichia coli* (ATCC 11230) and *Staphylococcus aureus* (ATCC 6538). All the experiments were carried out in triplicate. The minimum concentration which inhibits the visible growth of the microorganism after 48h of incubation was recorded as the MIC value for that particular nanoparticle.

### 3.30.2. Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC), the lowest concentration of nanoparticles that kills 99.9 per cent of the bacteria was determined. For growth inhibitory concentration the presence of viable colonies were counted and the lowest concentration causing bactericidal effect was reported as MBC.

To test for bactericidal effect, a loopful of inoculum (×10^6) from each flask was inoculated on nutrient agar and incubated at 35°C for 24 h. The nanoparticles in suspension concentration of 0.39 - 100 µg/l studied for the bactericidal effect. The absences of colonies on the agar plate against the concentration were recorded. The MBC of the silver nanoparticles were also performed against tested against *Escherichia coli* (ATCC 11230) and *Staphylococcus aureus* (ATCC 6538). All the experiments were carried out in triplicate.
3.31. Application of silver nanoparticles in antimicrobial fabrics

3.31.1 Selection of fabrics and fabric pretreatment:

The 100 per cent bleached woven cotton fabric (Coimbatore Murugan Mills (NTC), Coimbatore) was used for the entire study the technical specification of the fabrics was given in the Table 13.

**Table - 13 Specifications of the cotton fabric used for treatment**

<table>
<thead>
<tr>
<th>Type</th>
<th>Pre Finishing</th>
<th>Warp Count</th>
<th>Weft Count</th>
<th>Ends per Inch</th>
<th>Picks</th>
<th>Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 per cent Woven Cotton Fabric [Coimbatore Murugan Mills (NTC), Coimbatore]</td>
<td>Bleached</td>
<td>20’sK</td>
<td>20’sK</td>
<td>54</td>
<td>40</td>
<td>122 cm</td>
</tr>
</tbody>
</table>

The commercially procured cotton fabrics contain sizing material (cellulose) and have low absorbancy. Hence, as an attempt to remove the sizing material (desizing) and, in turn, to improve the absorbency, the procured cotton fabrics were treated with cellulase enzyme along with the surfactant (Tween-80). The procedure for the enzymatic desizing was as follows. The cotton fabrics were immersed in a hot water solution, containing 2 per cent (w/v) of the commercial cellulase enzyme (Texacryl, S&D Impacts Pvt. Ltd.,) and 1 per cent (w/v) surfactant (Tween-80), for 20 min at 70°C. After 20 min the fabrics were taken out, immersed in boiling hot water (100°C) and subsequently with cold water to stop the enzyme activity. The fabrics were then rinsed in distilled water three times, finally air-dried. These pre-treated samples were used for nanoparticle finishing and further analysis.

3.31.2. Finishing of Fabrics:

The silver nanoparticles synthesized were applied onto cotton fabrics by three different methods namely
1. Dip dry method
2. Exhaustion method
3. Pad-dry cure method

The antimicrobial efficiency of the silver nanoparticles coated fabrics was assessed qualitatively by agar diffusion method (SN 195920) and quantitatively by Percentage reduction test (AATCC 100) against the test organism *E.coli* (ATCC 11230) and *S. aureus* (ATCC 6275) as described in Section 3.32.1.2 and 3.32.2.1 respectively. The durability of the fabrics coated with silver nanoparticles by different methods was evaluated by wash durability analysis as described in Section 3.33.

3.31.2.1. Dip dry method

This method is one of the simplest methods of coating the antimicrobial substances onto the fabrics and it does not require any special equipment for finishing. In this method, the fabrics were first dipped in the 1 per cent silver nanoparticle solution (6.24 µg/ml) to wetness and the fabrics were squeezed and dried at 80°C in the oven for 5 min and then cured at 120°C for 2 min.

3.31.2.2. Exhaustion method (*Khalil-Abad et al., 2009*)

Antimicrobial finishing was performed to the fabric using exhaustion method with the following procedure. The fabrics were finished by immersing in the 1 per cent silver nanoparticle solution (6.25 µg/ml) for 30 min at 50°C in water bath. After finishing, the fabric was removed, squeezed and dried at 80°C in the oven for 5 min and then cured at 120°C for 2 min.

**Composition of nanoparticle solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nanoparticle solution</td>
<td>1 per cent</td>
</tr>
<tr>
<td>(Nanoparticles in distilled water 1 per cent w/v)</td>
<td></td>
</tr>
<tr>
<td>MLR (Material-to-liquor ratio)</td>
<td>1:20</td>
</tr>
</tbody>
</table>
Temperature : 50°C
Time : 30 min

3.3.1.2.3. Pad-dry cure method: (Rajendran et al., 2010)

The silver nanoparticles were finished onto the cotton fabrics by pad-dry-cure method using a laboratory scale padding mangle (R.B. Electronic and Engineering, Mumbai), running at a speed of 15 m/min with a pressure of 2 kgf/cm² to remove excess solution. A 100 per cent wet pick-up was maintained for all of the treatments. After padding, the fabric was air-dried and then cured for 3 min at 140°C and immersed for 5 min in 2 g/l of Sodium Lauryl Sulfate to remove unbound nanoparticles and rinsed to remove the soap solution followed by air-drying.

3.3.2. Antimicrobial characterization of silver nanoparticle finished fabrics

Pad dry cured silver nanoparticles finished fabrics were further analysed for the assessment of antimicrobial activity by following test methods.

3.3.2.1. Antibacterial tests (Qualitative test-screening or presumptive test)

3.3.2.1.1. Parallel streak method -AATCC 147 (Hebeish et al., 2011)

The Parallel Streak Method is relatively quick and easily executed qualitative method to determine antibacterial activity of diffusible antimicrobial agents on treated textile materials. Committee RA31 has come out with the procedure for this test and it is reproducible by various laboratories working with same materials. The objective of this test is to detect bacteriostatic activity on textile materials. The bacteriostatic activity tested against Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC 11230). AATCC bacteriostasis agar medium (Hi-media, India) was used as a growth medium for evaluation (Appendix I). The test specimens (non-sterile) were cut by a scissor. Rectangular specimens of convenient size (25 × 50 mm) were cut. A 50 mm
length permitted the specimen to lie across 5 parallel inoculum streaks each of diminishing width from about 8 mm to 4 mm wide.

A sample of the same material without the antimicrobial finish was used as a control. 15 ± 2 ml of Sterilized bacteriostasis agar medium (Hi-media, India) was prepared cooled and poured into each of standard (15 × 100 ml) Petri dishes. The agar was allowed to gel firmly before inoculation. The inoculum was prepared by transferring one ml of a 24 h broth culture into 9 ml of sterile distilled water contained in a test tube. The contents were mixed well using appropriate agitation. A loopful of the diluted inoculum was transferred to the surface of sterile agar plates by making 5 streaks approximately 60 mm in length, spaced 10 mm apart covering the central area of a standard Petri dish without refilling the loop. The test specimen was gently pressed transversely across the five inoculum streaks to ensure intimate contact with the agar surface. This was accomplished more easily by pressing the specimen to the agar surface with a sterilized forceps. If the specimen curled and prevented intimate contact with the inoculated surface, a sterile glass slide was placed on the ends of the specimen to hold it in place. The plates were incubated at 37°C for 18 - 24 hours. The incubated plates were examined for the interruption of growth along the streaks of inoculum beneath the specimen and for a clear zone of inhibition beyond its edge. The average width of zone of inhibition along a streak on either side of the test specimen was calculated using the following equation,

\[ W = \frac{(T-D)}{2} \]

Where

W - Width of the clear zone of inhibition in mm.

T - Total diameter of the test specimen and clear zone in mm.

D - Diameter of the test specimen in mm.
3.32.1.2. Agar diffusion method-SN 195920 (Mucha et al., 2002)

The testing of the antibacterial effect of silver nanoparticles finished textiles was carried out according to Swiss standard-agar diffusion tests. AATCC bacteriostatic agar medium (Hi-media, India) was used as a growth medium for evaluation (Appendix I). Test specimens and the untreated fabric samples (control) were taken and were cut into round shape pieces of 20 mm radius.

The appropriate bacteriostasis agar medium (Hi-Media, India) was sterilized and inoculated with 24 h culture (slant cultures) of Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC 11230). The sterile molten media were poured to Petri dishes and allowed to harden. The textile test specimen was placed on the solid agar and attached to it. For conditioning, the test dish was stored for 24 h at 5°C and then placed in an incubator. If the fabric curled preventing intimate contact with the inoculated surface, small sterile glass plates were placed on the ends of the fabric to hold it in place. The plates were then incubated at 37°C for 18 - 24 h. The evaluation was made on the basis of absence or presence of a zone of inhibition around the test specimen and the zone of bacteriostasis were measured in mm.

3.32.2. Antibacterial test (Quantitative test)

3.32.2.1. Percentage Reduction Test-AATCC 100 (Nithya et al., 2012)

Swatches of silver nanoparticles treated and control textile materials already qualitatively tested for antibacterial activity were evaluated quantitatively. Test and control swatches were inoculated with the test organisms (S. aureus ATCC 6538 and E. coli (ATCC 11230). Culture medium used was AATCC broth (Appendix I). A 24 hour culture of the test organism was shaken and allowed to stand for 15 - 20 min before preparing the inoculum. The swatches were placed in a sterile Petri dish. Using a micropipette, inoculation was done making sure that there was even distribution of the
inoculum. The swatches were then transferred aseptically to the jar. The jar tops were closed tightly to prevent evaporation. Immediately after inoculation about 100 ml of neutralizing solution (sterile distilled water) was added to each of the jars containing the inoculated untreated control swatches, the inoculated treated test swatches.

The jars were shaken vigorously for one minute. The serial dilutions were made with water and plated (in duplicate) on nutrient agar medium. Additional jars containing inoculated untreated control swatches and jars containing inoculated treated test swatches were incubated at 37 ± 2 °C for 18 - 24 h. Similar jars were incubated for 1 - 6 hours to provide information about the bactericidal activity of the treatment over such periods. After incubation, about 100 ml of neutralizing solution (sterile distilled water) was added to jars containing untreated control swatches and jars containing treated test swatches. The jars were vigorously shaken for one minute. Serial dilutions were made and plated (in duplicate) on nutrient agar. All the plates were incubated for 48 h at 37 ± 2°C. The bacterial counts were reported as the number of bacteria per sample (swatches in jar). The percentage reduction of bacteria by the treated specimens were calculated using following formulae,

\[ 100 \frac{(B-A)}{B} = R \]

Where,

- **R** - per cent reduction.
- **A** - The number of bacteria recovered from the inoculated treated test specimen swatches in the jar incubated over the desired contact period.
- **B** - The number of bacteria recovered from the inoculated treated test specimen swatches in the jar immediately after inoculation (at ‘0’ contact time).

The percentage reduction of bacteria by the treated specimen against each test organism was reported.
3.32.3. Antifungal Tests (AATCC test method 30)

The purpose of this test method was to determine the susceptibility of textile materials to mildews, rots and to evaluate the efficacy of fungicides on textile materials. There are three tests namely Soil Burial test, Agar Plate test and Humidity Jar- Mixed Spore Suspension. These tests can be used, singly or in combination, depending on the type of exposure to which the textile material will be subjected. The specific purpose of the tests and their methodology were as follows.

3.32.3.1. Antifungal assessment by Soil Burial test-AATCC 30 (Chattopadhyay and Patel, 2010)

This procedure was generally considered to be the most severe test for textile products. The procedure was carried according to the method described by AATCC Technical Manual, 2001. The fabric specimens were prepared using sample cutter. The specimens with dimensions 15.0 ± 1.0 × 4.0 ± 0.5 cm (6.0 ± 0.4 × 1.5 ± 0.2 in.), having the long dimension parallel to the warp and unravelling to 2.5 ± 0.1 cm width (1.0 ± 0.04 in.), or, in the case of fabric with less than 20 threads per 2.5 cm (1.0 in.) to a predetermined number of threads to give a specimen 2.5 ± 1.0 cm in width (1.0 ± 0.4 in.). The number of specimens was five for each treatment, control and reference fabric.

Soil Bed: The air-dry test soils in trays were placed to a depth of 13.0 ± 1.0 and were bring to optimum moisture content by gradual addition of double sterile water accompanied by mixing to avoid paddling. It was allowed to stand for 24 h, sieved it through a 6.4 mm (0.25 in.) mesh screen. Uniform moisture content was maintained by covering the soil container with a suitable lid. The moisture content of the soil during the test period was maintained between 25 ± 5 per cent (based on dry weight). If the surrounding air was maintained at higher than 83 ± 3 per cent relative humidity, the loss of moisture was negligible. The viability control was tested by exposing untreated
cotton cloth in the soil bed for seven days during the test period to verify fungal activity.

The soil bed was considered as satisfactory if the viability control fabric loses 90 per cent breaking strength after seven days exposure. The specimens was buried horizontally on 10.0 ± 1.0 cm (3.9 ± 0.4 in.) of soil, spaced at least 2.5 cm (1.0 in.) apart and then covered with 2.5 ± 0.5 cm (1.0 ± 0.2 in.) of test soil. The setup was incubated for 2 weeks at 28±1°C. The result of this test was determined by Strength loss determination. Remove specimens after the incubation gently wash with water, dry at room temperature for 22 ± 4 h and then condition in an atmosphere of 64 ± 2 per cent humidity and a temperature of 24 ± 3°C for 24 h. Determination of breaking strength by ASTM D 5035, Standard Test method (A test for breaking force and elongation of textile fabrics) (Strip Force). The tear strength loss was conducted with help of South Indian Textile Research Association (SITRA), Coimbatore. Further to ascertain the strength loss microscopic observations were also made.

3.32.3.2. Antifungal assessment by Agar Plate method- AATCC 30 (AATCC Technical Manual, 2001)

Certain fungi were able to grow on textile products without causing measurable breaking strength loss within a laboratory experimental time frame. This procedure was used to evaluate those types of fungi. Fabric samples were cut into 3.8 ± 0.8 cm in diameter in duplicate and used. The test organism used was Aspergillus niger (ATCC 6275).

An inoculum of 1.0 ± 0.1 ml was evenly distributed over the surface of the agar. The discs of the silver nano particle treated fabrics were pre-wetted (not rubbed or squeezed) in water containing 0.05 per cent of a non-ionic wetting agent (Triton X-100) and placed on the agar surface. The inoculum of 0.2 ± 0.01 ml was distributed evenly over each
disc by means of a sterile pipette. All the specimens were incubated at a temperature of 28 ± 1°C for seven days.

3.32.3.3. Antifungal assessment Humidity Jar test-AATCC 30 (Mixed Spore Suspension Test)

This test method was designed to determine the fungistatic effectiveness treatment to control mildew and non-pathogenic fungal growth on waterproofed articles or surfaces composed of textile materials intended for outdoor and above ground use. The specimens were prepared by cutting 2.5 ± 0.5 cm × 7.5 ± 0.5 cm strips from sample weighing 170.0 ± 34.0 g/m². At least four specimens of each treated and untreated fabric were used. Untreated fabric strips, identical in all other respects to the treated specimens under test, were required to establish the test validity. Conidial suspensions of fungal organisms were prepared by adding 10 ml of a sterile 0.5 per cent saline solution containing 0.05 per cent of a non-fungicidal wetting Agent to a 7-10 day agar culture.

To ensure luxuriant growth, both the test and control strips were saturated with a sterilized glycerol nutrient solution of the following composition: 97.6 per cent distilled water, 2.0 per cent glycerol, 0.1 per cent K₂HPO₄, 0.1 per cent NH₄NO₃, 0.05 per cent MgSO₄·7H₂O, 0.1 per cent yeast extract and 0.05 per cent of a nonionic wetting agent. Adjust the pH to 6.3 ± 0.1. Sufficient nutrient solution was prepared to saturate all the specimens used in a single test. Each strip was soaked in nutrient for three min or until saturated. Excess liquid was squeezed and fabric strips were allowed to air dry before proceeding with application against test fungi. Pre-mixed equal volumes of well agitated conidial suspensions of Aspergillus niger (ATCC 6275), Trichoderma viride (ATCC13233) and Penicillium varians (ATCC28070). The above suspension (1.0 ± 0.1 ml) was evenly distributed onto both sides of each specimen either by spraying or by means of a pipette. Fabric strips was suspended using plastic paper clips or nylon thread from
the caps of individual jars containing 90 ± 3 ml of water each. Hook position was adjusted so that the bottom ends of attached strips were all at a uniform height above the water level. The caps were tightened and then backed off one-eighth turn to allow for some ventilation and incubated at 28 ± 1°C for 14 days.

3.33. Wash durability of finished fabrics

The wash durability was performed for the finished fabrics according to the procedure described by Sarkar et al. (2003). Representative specimen fabrics of 92 × 92 cm in size cut parallel to the fabric length and width were prepared. Each specimen contained different groups of lengthwise and width wise yarns. This wash method performed by soaking finished fabric in 1:30 (fabric:liquid). The liquid is normally a 0.2 per cent detergent (neutral soap).

The status of the washing machine was as follows: water level: 18 ± 1 gal, agitator speed: 179 ± 2 rpm, wash time: 12 min, spin speed: 645 ± 15 rpm, final spin cycle: 5-6 min. One laundering cycle included subsequent steps of 5 min of laundering and 2 min of rinsing, followed by another 2 min of rinsing and tumble drying. For tumble-drying, the washed and rinsed specimens were placed in the tumble dryer and the temperature was set below 60°C for 10 min. The dryer was operated until the total load was dry. The load was removed once the machine is stopped to avoid over drying. Selected washing and drying cycles were repeated up to 50. One wash cycle was considered equivalent to 5 washes in domestic practice.

3.34. Physical characterization of nanoparticle treated fabrics

The physical properties such as air permeability test, Tensile strength and stiffness tests of the silver nanoparticle treated fabrics and untreated (control) fabrics were analyzed by standard methods as described below.
3.34.1. Air Permeability-ASTM D737-96

This test method covers the measurement of the air permeability; the rate of air flow passing perpendicularly through a known area under a prescribed air pressure is calculated. Construction factors and finishing techniques can have an effect upon air permeability by causing a change in the length of airflow paths through a fabric. This test was done with an air permeability testing apparatus. Pressure gauge and flow meter were used to measure the air permeability. The samples were tested at a relative humidity 65 per cent and at a temperature of 21°C.

3.34.2. Tensile Strength

The breaking strength and elongation of wrap and weft yarns at fracture were tested. Tensile strength is the measure of the resistance of the fabric tensile load or stress in either warp or weft direction. It is the strength shown by a specimen subjected to tension as distinct from torsion, compression, or shear. Elongation defines the length to which a fiber may stretch before breaking. A sample of 12” × 2” was taken for the test. The tensile strength of the fabric was determined by cloth tensile strength tester using cut strip method.

3.34.3. Stiffness -ASTM D6828 – 02:2007

This test method covers the determination of the stiffness of fabrics by measuring the force required to push a specimen into a slot of predetermined width with a metal blade working at a predetermined capacity.

3.35. Characterization of other functional properties nanoparticle treated fabrics

3.35.1. UV repellent properties-AATCC Test Method 183

UV repellent properties were tested using AATCC Test method 183. This test is very common to determine the ultraviolet radiation blocked or transmitted by textile fabrics intended to be used for UV protection. The transmission of ultraviolet radiation (UV-R)
through a specimen was measured in a spectrophotometer at a known wavelength intervals. The ultraviolet protection factor (UPF) was computed as the ratio of the erythemally weighted ultraviolet radiation (UV-R) irradiance of the specimen at the detector with no specimen to the erythemally weighted UV-R irradiance at the detector (Schindler and Hauser, 2004; Khalilabad et al., 2013). Two specimens from each sample for the wet and the dry testing were cut with each specimen having at least 50 × 50 mm (2.0 × 2.0 in.) or 50 mm (2.0 in.) in diameter. Distortion of the specimens was avoided while preparation and handling. Placed the specimen flush against the sample transmission port opening in the sphere. Made one UV transmission measurement with the specimen oriented in one direction, a second measurement at 0.79 rad (45°) to the first and a third at 0.79 rad (45°) to the second. The transmission values were recorded for the respective specimens.

The Ultraviolet Protection Factor (UPF) of each specimen was calculated using the following Equation

$$ \text{UPF} = \frac{\sum_{\lambda=290}^{\lambda=400} E(\lambda) \varepsilon(\lambda) \Delta\lambda}{\sum_{\lambda=290}^{\lambda=400} E(\lambda) T(\lambda) \varepsilon(\lambda) \Delta\lambda} $$

$E\lambda = \text{relative erythemal spectral effectiveness}$

$S\lambda = \text{solar spectral irradiance}$

$T\lambda = \text{average spectral transmittance of the specimen}$

$\Delta\lambda = \text{measured wavelength interval (nm)}$

From the UPF values obtained, the UPF rating was assigned accordingly and the protection category (Good, Very good and excellent) of the fabrics were determined using the Table 14.
Table - 14 UV Protection and classification according to AS/NZS 4399:1996

<table>
<thead>
<tr>
<th>UPF Range</th>
<th>Protection Category</th>
<th>Effective UVR Transmission (per cent)</th>
<th>UPF Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>non-rateable</td>
<td>&gt; 6.7 per cent</td>
<td>0, 5, 10</td>
</tr>
<tr>
<td>15-24</td>
<td>Good</td>
<td>6.7-4.2</td>
<td>15, 20</td>
</tr>
<tr>
<td>25-39</td>
<td>Very Good</td>
<td>4.1-2.6</td>
<td>25, 30, 35</td>
</tr>
<tr>
<td>40-50, &gt;50</td>
<td>Excellent</td>
<td>Less than 2.5</td>
<td>40, 45, 50, &gt;50</td>
</tr>
</tbody>
</table>

3.35.2. Antistatic properties - AATCC Test Method 76-2005

The antistatic behaviour of the metal oxide nanoparticles treated fabrics were evaluated by measuring the electrical surface resistivity of fabrics by AATCC Test Method 76-2005 (Xue et al., 2012). The purpose of this test method was to determine the electrical surface resistivity of fabrics. The surface resistivity is calculated by using the measured electrical resistance between superficially positioned parallel plates and their spacing and the surface is calculated resistivity as ohms per square.

Placed test specimens in firm contact with the electrodes. Prepared 2 sets of 3 test specimens each, 1 set each so that the direction of testing is parallel to the yarns in the length direction of the fabric and 1 set with the direction of testing parallel to the width direction of the fabric. Care was taken so that the width of the specimen must not exceed the width of the electrodes. The contact with the electrodes was such that, when additional pressure was applied between the fabric and the electrodes, the test results were not affected. Measured the electrical resistance of the test specimen, width of specimen and distance between electrodes and calculated to the nearest ohms per square as follows:
\[ R = O \times \frac{W}{D} \]

\( R \) = resistivity in ohms per square

\( O \) = measured resistance in ohms

\( W \) = width of specimen

\( D \) = distance between electrodes

3.36. Statistical analysis

The experimental results were statistically analyzed using SPSS software (Version 19.). The optimization studies were analyzed using Duncan’s Multiple Range Test (DMRT) after analysis of variance. In the Response Surface Methodology (RSM) the second-order polynomial coefficients, the coded values of the process parameters and the regression analysis, Coefficient of determination \( R^2 \) and its statistical significance was determined by F-test. The significance of the regression coefficients was tested by a t-test. All these tests were analyzed using Design Expert software (version 8.0.7.1, Stat-Ease Inc., USA).