Chapter 3: MATERIALS AND METHODS
3.1 Materials

Most of the chemicals and reagents used in the research were obtained from Hi Media Labs. Pvt. Ltd. Mumbai, Qualigenes Fine chemicals, Mumbai, E-Merck (India) Ltd. Mumbai or SRL (SISCO Research Laboratories, Pvt. Ltd.) Mumbai. Readymade broths and agars were obtained only from Hi Media labs Pvt. Ltd. Bombay (India)

3.1.1 Isolation and growth media

a) NY Agar- Nutrient Agar supplemented with 0.05% Yeast Extract.

b) LB acetate (pH 6.8, 0.25M Sodium acetate)- For making 100 ml media 1g Bactotryptone, 0.5g Yeast Extract and 1g NaCl were weighed and added to separately prepared 100ml of 0.25M CH₃COONa.3H₂O and the pH was adjusted to 6.8.

c) Luria Bertiani Broth- For 100ml media, 1g Bactotryptone, 0.5g Yeast Extract and 1g NaCl were weighed and the volume was made to 100ml in sterilized distilled water and autoclaved.

d) L-Agar—Luria Broth supplemented with 2% Agar Agar.

e) NYSM (Nutrient Broth Yeast Extract Salts/Sporulating Medium)- This was prepared by the method of Yousten et al, 1982. It contained Nutrient Broth supplemented with 0.05% YE; 5x10⁻⁵M MnCl₂; 7x10⁻⁴M CaCl₂ and 1x10⁻³M MgCl₂.

For preparing 100ml media 1.3gm of NB were supplemented with 0.05gm YE, the volume was made to nearly 95ml with sterilized distilled water and autoclaved. Then 1ml of the each stock solution of the salts was added. The stock solutions of MnCl₂ (9.895g in 100ml d.w.), MgCl₂ (2.033g in 100ml d.w.) and CaCl₂ (1.0304g in 100ml d.w.) were prepared and autoclaved separately.
3.1.2. Grams stain

Crysal Violet 1% in water

Grams Iodine (Weighed 1g Iodine & 2g KI and made the volume to 100ml in d.w).

Acetone. Ethanol 50:50 mixture

Saffranin 0.5% w/v

3.1.3 Endospore stain

Malachite green 5%

Saffranin 0.5%

3.1.4. Biochemical tests: Reagents and media

All media were made according to laid out procedures in the ‘Laboratory Methods in Microbiology’ (Harrigan & Margaret, 1966).

a) Starch hydrolysis-

Medium—Nutrient Agar supplemented with 0.2-1% starch.

Test Reagent—Grams Iodine (as used in Grams stain)

b) Methyl red test-

Medium—Glucose Phosphate Broth which contained

D-Glucose 0.5g

K₂HPO₄ 0.5g

Peptone 0.5g

d.w. 100ml

Test reagent—Methyl Red solution (0.5g Methyl Red in 300ml of 95% Ethanol and volume made to 500ml with d.w).
c) **Voges Proskauer test**

Medium- Glucose Phosphate Broth

Test Reagent – O’Meara’s solution (Creatine + 40% sol. of NaOH)

d) **Gelatin liquification**

Medium- Nutrient Agar + 0.4% gelatin (pH 7.2)

Test Reagent- Mercuric Chloride solution (HgCl₂, 15gm; conc.HCl 20ml and d.w. 100ml)

e) **Production of Indole**

Medium- Peptone Water (Tryptone 1-2%; NaCl 0.5%, final pH 7.2)

Test Reagent- Kovacs Indole Reagent containing

- Isoamyl alcohol 150ml
- Para-dimethylaminobenzaldehyde 10g
- Conc. HCl 50ml

f) **Hydrolysis of Casein**

Medium- Nutrient Agar + 5% Casein (fat free milk)

Test Reagent- Mercuric Chloride solution, 1%HCl

g) **Reduction of Nitrate**

Medium- Nitrate Peptone Water (peptone water with the addition of 0.02 - 0.2% Potassium Nitrate AR grade)

Test Reagent- Griess Ilosvays reagent containing

i) 8gm Sulphanalic acid in 1000ml 5N Acetic acid

ii) 5gm α-Napthalamine in 1000ml of 5N Acetic acid
h) **Hydrolysis of Tween compounds**-

Medium – Tween agar containing

- Peptone 1gm
- NaCl 0.5gm
- CaCl₂ 0.1gm
- Tween 1ml
- Agar 1.5gm
- pH 7- 7.4

i) **Hydrolysis of Arginine**-

Medium-Thornley’s medium containing

- Peptone 1gm
- NaCl 0.5gm
- K₂HPO₄ 0.03gm
- Arginine monohydro chloride 1gm
- Phenol Red 0.001gm
- d.w 100ml
- Agar 0.3gm

j) **Fermentation of sugars**-

Medium – Peptone water, 20% stock solutions of sugars (Mannitol, Glucose, Maltose, Sucrose etc). prepared and sterilized separately.

Test Reagent-Phenol Red
k) **Catalase production** -

   Medium - Nutrient Agar
   Test Reagent - Hydrogen Peroxide

### 3.1.5 Growth conditions

a) **Anaerobic growth** -

   Medium - Nutrient Agar
   Other requirement - liquid paraffin

b) **Growth at 50°C & 65°C** -

   Medium - LB

c) **Growth in 7% NaCl** -

   Medium - Peptone water containing 7% NaCl

d) **Growth at pH 5.7** -

   Medium - Peptone water pH 5.7

### 3.1.6 Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

a) **Lysis solution** - contained 50 mM Glucose; 25 mM Tris Cl, pH 8.0; 10 mM EDTA.

   10ml of this solution was prepared and kept in the refrigerator.

b) **Lysozyme** - powder was obtained from Sigma Laboratories.

c) **Protein molecular weight markers** - Lower Range Markers of Mol. Wt. 14,000 to 100,000 and Higher Range Markers of Mol. Wt. 29,000 to 200,000 were obtained from Genei Labs. Pvt. Ltd. Bangalore.

d) **Extraction Buffer** - containing 1 mM Phenylmethylsulphonylflouride (PMSF), 10 mm EDTA and 2% SDS, about 50 ml was prepared and stored in the refrigerator.
e) **Dissociation buffer (Blue juice or sample buffer)**- 50 ml of it containing 0.625M Tris-Cl pH 6.8; 2% SDS; 5% β-mercaptoethanol; 10% glycerol and 0.005% Bromophenol blue was prepared and stored in the refrigerator.

f) **Monomer solution**- A stock solution containing 29% (w/v) Acrylamide and 1% (w/v) N-N’-Methylenebisacrylamide was prepared in warm distilled water (to assist the dissolution of Bisacrylamide). Its pH was checked (7 or less) and was stored at room temperature in a dark bottle.

g) **Sodium Dodecyl Sulphate**- 10% stock solution of SDS was prepared and stored at room temperature.

h) **Separating gel buffer / Running gel buffer (1.5M, pH 8.8)**- For 200ml soln. dissolved 36.3g of Tris-base in d.w. Made the volume to 200ml with d.w. and adjusted the pH with HCl.

i) **Initiator**- A very small amount of 10% (w/v) stock solution Ammonium persulphate (APS), which acts as an initiator for polymerizing the gel, was prepared fresh each time in d.w. for electrophoresis.

k) **Tank Buffer (0.025M Tris buffer, 0.192M Glycine and 0.1% SDS)** – For every litre of buffer weighed 3g Tris -base, 14.4g Glycine added 10ml 10% SDS and made the volume to 1litre with d.w.

l) **Staining solution**- Dissolved 0.1g Coomassie Brilliant Blue R-250 in 50% TCA in water (100ml) or Methanol:Acetic acid: distilled water (45:10:45).

m) **Destaining solution**- 7% Glacial Acetic Acid or Methanol: Acetic Acid: water prepared in a ratio of 45:10:45.
3.1.7 Scanning Electron Microscopy (SEM)

Fixative used was 2.5% glutaraldehyde in phosphate buffer. Phosphate buffer (pH 7.2) was prepared by mixing 0.2M Na₂HPO₄ (72ml) and 0.2M NaH₂PO₄ (28ml).

3.1.8 Antibiotic sensitivity

NY Agar plates and antibiotic discs (Streptomycin & Chloramphenicol) from Hi Media.

3.1.9 Plasmid profiles

a) Luria Broth
b) Agarose -0.7% in TBE
c) Lysozyme (10mg/ml added to solution I)
d) Solution I - contained 50 mM glucose, 25m M Tris.Cl (pH 8) and EDTA (pH 8) about 100ml of the solution was prepared and autoclaved before storage at 4°C till use.
e) Solution II - contained 0.2N NaOH (freshly diluted from 10N stock) and 1%SDS.
f) Solution III - contained 5M Potassium Acetate (60ml), Glacial Acetic Acid (11.5ml) and H₂O (28.5ml).
g) Solution IV - contained 100mM NaOAc.2H₂O and Tris.HCl (pH8).
h) Ethanol chilled.
i) Gel Loading Buffer - contained Glycerol 50%, TBE 0.5X and Bromophenol Blue 1%.
j) Ethidium Bromide (stock 5mg/ml in distilled water) for staining the gel.
k) Tank Buffer-Tris –EDTA (TE).

3.1.10 Mosquito food

A mixture of dog biscuit and Yeast Extract in the ratio of 60:40 was used as larval food. Live chicken/fowl placed in a steel cage was introduced daily into the each cloth cage containing adult mosquitoes to provide blood meal to the females. In addition,
cotton soaked in 5% glucose solution with a few raisins was placed in a petriplate in mosquito cages for their feeding.

3.1.11 Mosquito cages

Adult mosquitoes were kept in cubical cages of 2 x 2 x 2ft dimension that were made of muslin nylon net and cotton/terricot cloth having an opening on one side, large enough for placing of fowl cage, transfer of mosquitoes, etc. This opening was kept tied to prevent the adults from escaping. Many such cages were used to rear three test mosquito species, *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* in the insectary.

3.1.12 Plastic trays and bowls for the rearing of mosquito immature stages

Enamel/Plastic trays measuring 15 x 10 x 3 inches and plastic bowls of 500ml capacity were used for larval rearing.

3.1.13 Aspirator/Suction tube

Aspirator/Suction tube used for capturing adult mosquitoes consisted of a plastic tube (app. 15 inches long) attached to flexible rubber tubing (about 20 inches long) and a small plastic or glass mouth-piece (Fig 3.1.13). A fine wire mesh/gauze separated the plastic and rubber tubes to prevent mosquitoes from entering into the mouth while collection.

![Fig 3.1.13: Sucking tube or Aspirator](image)
3.2. Methodology

3.2.1. Soil sampling

Soil has been found to be the important source of mosquito-pathogenic bacilli (Manonmani et al., 1987; Travers et al., 1987; Martin & Travers, 1989; Orduz et al., 1992; Hastowa et al., 1992 and Asimeng & Mutiga, 1992). The spores of these bacteria are known to settle rapidly and accumulate in the mud at the bottom of the pools, etc. (Mulligan et al., 1980; Ignoffo et al., 1981; Silapanuntakul et al., 1983; Davidson et al., 1984). Taking into consideration these facts, soil sampling was carried out from various mosquito-breeding habitats spread all over Goa (Fig. 4.1).

The samples were collected from 11 selected sites viz., Santa Cruz (paddy field), Pilar (stagnant pond), Pernem (paddy field), Valpoi (pond), Carambolim lake, Usgao (paddy field), Ponda (pond), Sanguem (pond), Quepem (paddy field), Cuncolim (stagnant pond) and Panaji (Mangrove soil). From each site, 5-10gm soil devoid of organic debris was collected from the upper most layer of the habitat with the help of sterile spatula/spoon into plastic bags or sterilized glass vials. The plastic bags/vials were sealed, labeled and brought to the laboratory. These were stored at room temperature till processed. Extreme care was taken to avoid any contamination during sampling and processing.

3.2.2 Screening of soil samples for mosquito-pathogenic bacilli

In the earlier stages of the work, attempts were made to isolate mosquito-pathogenic bacilli by plating the serial dilutions of the soil samples on nutrient agar plates and those colonies which had typical bacilli like morphologies were hand picked, purified and tested for pathogenicity. But soon it was realized that this process was too
laborious, time consuming and involved a lot of wastage of the expensive media. Moreover, it did not yield the desired results many a times. Therefore a new technique was developed for screening during the course of this research work.

3.2.2.1 New screening technique

Taking a clue from the acetate selection method of Travers et al, 1987 (modified by Carozzi et al, 1991) for the selective isolation of bacilli, we devised a new technique that would screen the soil samples and indicate the presence of mosquito-pathogenic bacilli in them (Protocol 3.2.2.1). This new method when introduced as first step before isolation made the process of isolation of desired bacilli quicker and cheaper with less wastage as we could identify positive soil samples for mosquito-pathogenic bacilli and reject negative those samples at the first stage itself.

3.2.3 Isolation

The isolation of the bacilli was done from the soil samples that were found to be positive for the mosquito-pathogenic bacilli following above screening technique. Only those samples (i.e. from Santa Cruz, Pilar and Usgao) that produced more than 50% mortality against Culex/Anopheles mosquito larvae were further processed. Since Mangrove/hydrophyte surfaces were earlier known to be promising for the search of Bacillus spp. (Manonmani et al, 1991), successful isolation of pathogenic bacteria was also carried out from the mangrove soils from Panaji. The isolation of bacilli was carried out by the usual acetate selection method of Travers et al, 1987 modified by Carrozi et al, 1991 (Protocol 3.2.3).
Protocol 3.2.2.1. New screening process/protocol developed

0.5gm soil sample

↓

10ml Luria Broth buffered with 0.25M Sodium Acetate (pH6.8) in 125ml flask

↓

Incubation on shaker (200RPM) for 4 hrs at 30°C

↓

1ml aliquot was heated to 65°C for 10min in pre warmed glass tube

↓

0.1ml of the sample put on 1ml L-medium for 24hrs

↓

+ 1ml NYSM (sporulating medium)

↓

Incubated on a shaker for 2 days

↓

0.1ml culture

↓

Preliminary toxicity testing against mosquito vector

(10ml larvae of vector species in 10ml sterile distilled water)
Protocol 3.2.3. Isolation of Bacilli

0.5gm soil sample

\[ \downarrow \]

10ml Luria Broth buffered with 0.25M Sodium Acetate (pH 6.8) in 125ml flask

\[ \downarrow \]

Incubation on the shaker at 200RPM for 4hrs at 30\(^{\circ}\)C

\[ \downarrow \]

1ml aliquot was heated to 65\(^{\circ}\)C for 10min in pre warmed 5ml glass tube

\[ \downarrow \]

0.1ml supernatant plated on L-Agar plates

\[ \downarrow \]

Incubated at 37\(^{\circ}\)C for 1-2 days

\[ \downarrow \]

Colonies picked up at random

\[ \downarrow \]

Purified by subculturing on NY-Agar plates

\[ \downarrow \]

Grown on NYSM for 3-5 days (till complete sporulation)

\[ \downarrow \]

Preliminary efficacy tests, microscopy, biochemical characterization, etc.
3.2.4 Preliminary toxicity testing of the isolates

The preliminary toxicity testing of the bacterial colonies isolated by the modified acetate selection method described above was carried out to select for the working isolates. For this the individual colonies purified by subculturing 2-3 times on NY plates, were grown on NYSM for 3-5 days till complete sporulation occurred. For each test 10 larvae were added to 10 ml distilled water in small petriplates/glass beakers and 0.1 ml of each sporulating culture was added to them. The negative control constituted 10 larvae in 10 ml distilled water only. The concurrent positive controls constituted 10 larvae in 10 ml d.w. each containing 0.1 ml of the reference strains *Bacillus sphaericus* (H5a5b) strain 101 and *B. thuringiensis* (H-14) strain 164 isolated from the commercial powder formulations Bacticide and Spherix respectively, grown under similar conditions. These effective powder formulations (used earlier in the field trials by Kumar *et al*, 1994; 1995 & 1996), were manufactured by the Berdsk Plant of Biological Preparations, Russia and supplied through the Ministry of Health & Family Welfare, New Delhi (India).

3.2.5 Storage of the isolates

The 8 working isolates were code named KSD-1 to KSD-8 and maintained on NY plates and slants which were kept in refrigerator at 4°C and sub cultured periodically. A pure sample of each isolate was kept safe at –20°C in 20% (v/v) glycerol (Carboulec & Priest, 1989). For this the cultures were first grown on NYSM for 3-5 days for complete sporulation and then an equal amount of sterilized stock of glycerol (40% v/v) was added.
3.2.6 Identification of the isolates

Prior to the identification of the isolates, they were purified by subculturing on NY plates. The identification was done as follows,

3.2.6.1. Colony and cell morphology: The cultures were grown on NY plates for 12-16hrs and their colony morphologies were observed. Vegetative cell shapes and sizes were observed under microscope after Gram staining of 12-16hrs old culture (Protocol 3.2.6.1A).

<table>
<thead>
<tr>
<th>3.2.6.1A Hans Christian gram staining protocol, 1884</th>
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<tbody>
<tr>
<td>Prepared a smear of the culture</td>
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<tr>
<td>on the clean glass slide</td>
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<tr>
<td>Heat fixed the smear</td>
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<tr>
<td>Applied Crystal Violet dye for 1min</td>
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<tr>
<td>Grams Iodine for 30 sec</td>
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<tr>
<td>Washed off the excess stain</td>
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<tr>
<td>Applied organic solvent (Acetone : Alcohol 50:50 ) for 20 sec</td>
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<tr>
<td>Washed the slide under slow running water</td>
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<tr>
<td>Applied Saffranin for 2-3 min</td>
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<tr>
<td>↓</td>
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<tr>
<td>Wash and blot dried the slide before microscopy</td>
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</table>
The endospore staining was performed with 48-72 hr old culture as follows (Protocol 3.2.6.1B) and spore shapes and sizes were noted.

3.2.6.1B Endospore staining (after Schaefer & Fulton's method)

Prepared smear of the culture

↓

Heat fixed the smear

↓

Stained with Malachite green soln. 7-8 min,
warmed the slide occasionally

↓

Washed in cold running water (10 min.)

↓

Counter stained with 0.5% Saffranin

↓

Washed the slide and blot dried

All the morphological characteristics of the new isolates were recorded and compared with the two reference strains for their basic identification.
3.2.6.2. Biochemical tests: Various biochemical tests for the bacilli identification were carried out as per Bergey's manual. These tests were performed following the techniques described in the Laboratory Methods in Microbiology (W.F. Harrigan & Margaret E. McCance, 1966).

i) Catalase- Bacterial colony was taken on a slide with the help of a Nichrome loop and on this a drop of H₂O₂ was added. Effervescence caused by the liberation of free oxygen as gas bubbles was noted which indicated the presence of catalase in the culture.

ii) Starch hydrolysis- The poured dried plates of the medium (Nutrient Agar + 0.2-1% soluble starch) were inoculated by streaking once across the surface and incubated at 37°C overnight. For testing, the plates were flooded with 5-10ml Gram's Iodine and clear zones formed due to starch hydrolysis were noted and width of the clear zone from the edge of the colony to the limit of clearance, if any was recorded.

iii) Gelatin liquification- The poured dried plates of the medium (Nutrient Agar + 0.4% Gelatin) were inoculated by streaking once across the surface of the plates and incubated at 37°C overnight. For testing, the plates were flooded with 8-10ml of the test reagent. The opaque precipitate formed due to the presence of unhydrolysed gelatin or the clear zone due to hydrolysed gelatin was noted. The width of the clear zone in mm from the edge of the colony, if any was recorded.

iv) Methyl Red test- The test tubes containing media were inoculated and incubated. For recording of the results, a few drops of the indicator were added and the colour change was observed. The colour change to red was recorded as a positive test.
v) **Voges Proskauer test** - Broth culture was inoculated and incubated for 2-7 days. For recording of the results the reagent was added and the colour change was noted after shaking the tubes. The development of the red colour was recorded as a positive test.

vi) **Indole Production** - The sterilized broth medium in the test tubes was inoculated and incubated at 37°C for 2-7 days after which Kovac’s reagent was added, shaken and allowed to stand. If a deep red colour developed due to the presence of indole that separates out in the alcohol layer, it was recorded as a positive test.

vii) **Reduction of nitrate** - The medium was distributed in the test tubes each containing an inverted Durham’s tube and autoclaved for 15min. The broth was inoculated and incubated for 2-7 days at growth temperature. For recording the results 1ml of the reagent was added to test culture and the control. The colour change and the gas evolved which collects in the Durham’s tube were noted.

viii) **Casein Hydrolysis** - Poured dried plates of the medium were inoculated by streaking once across the surface and incubated at 37°C for 5 days. To record the results, the clear zone was noted after incubation. These plates were flooded with the test reagent following which the width of the clear zone if any was once again recorded.

ix) **Hydrolysis of Tween compounds** - Poured dried plates of the medium containing tween were inoculated by streaking once across the surface and were incubated for 24hrs. The presence of any clear zone surrounding the bacterial growth due to the hydrolysis was noted.

x) **Hydrolysis of Arginine** - Tubes containing medium were stab inoculated in duplicates with each culture and then one each of the two tubes was covered with liquid paraffin. The colour change to red due to the hydrolysis of Arginine, if any was noted.
xi) **Fermentation of sugars**- 5ml media was dispersed in each test tube containing an inverted Durham’s tube. These were sterilized, cooled and then 30μl of the 20% stock of each sugar (sterilized separately) and a few drops of the indicator were added to them. These were inoculated with the test cultures and incubated for 3-5 days. The change in the colour was noted for results.

3.2.6.3. **Antibiotic sensitivity**-The isolates were spread on LB Agar plates and the antibiotic discs were placed on them with the help of a sterilized spatula. Then the plates were incubated at 37°C overnight before observing the antibiotic sensitivity zones.

3.2.6.4. **Growth conditions**

i) **Temperature**: Luria Broth (about 10ml) was inoculated in 50ml conical flasks with each culture and incubated on the environmental shakers for 24-48hrs. One set of the cultures was incubated at 50°C and the other at 65°C. The bacilli growth in these two sets of culture was observed.

ii). **Growth in 7% NaCl**-Each culture was inoculated to 10ml of Peptone water containing 7% NaCl on a rotary shaker at room temperature. Growth was noted after 24-48hrs.

iii) **Growth at pH 5.7**-Each culture was inoculated to 10ml of Luria Broth (pH 5.7) and placed on the rotary shaker at room temperature. Growth was checked after 24-48hrs.

3.2.7 **Scanning Electron Microscopy (SEM)**

The bacterial cultures were scanned after fixation, using the SEM models JSM 6100, JEOL at Central Instrumentation Lab. (RSIC), Panjab University Chandigarh and JEOL-5800 LV at National Institute of Oceanography (CSIR), Dona Paula, Goa (India).
a) **Fixation**- A bacterial colony was suspended in a microfuge tube containing 2.5% gluteraldehyde (in Phosphate Buffer pH 7.2) with the help of a Nichrome loop and was kept in the refrigerator at 4°C overnight for fixation.

b) **Washing**- Glutaraldehyde fixed cells/spores were washed twice with Phosphate buffer and once with triple distilled water.

c) **Mounting** - The fixed and washed samples were applied on the stubs with the help of double adhesive tape and were gently spread. Care was taken to apply very little sample for good results.

d) **Air-drying and gold sputtering**- The samples were air-dried for about half an hour and then the gold sputtering was done.

e) **Scanning**- The samples were removed from the gold sputtering unit and loaded into the evacuated chamber of the SEM. All the areas of the stubs were scanned carefully for the vegetative cells, spores and the toxin crystals.

f) **Photography**- The required areas of the stubs were focussed properly at about 10,000x and photographed with the help of a camera attached to the microscope.

### 3.2.8 Characterization using Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE with all the isolates and the two reference strains was carried out by the procedure described by Sambrook *et al.*, 1989 on mini gel apparatus (Mini Dual Mode Cat # 05-03: Genei Pvt. Ltd. Bangalore, India) and also on an LKB 2001 Vertical Electrophoresis unit for the bigger gels.

a) **Sample preparation**- The samples were prepared by the method of Murphy & Stevens, 1992 with slight modifications (Protocol 3.2.8).
Protocol 3.2.8 Protein sample preparation for SDS-PAGE

10ml culture grown on NYSM

↓

Centrifuged at 8,500RPM for 15min

↓

Supernatant (discarded) →

Pellet

↓

1ml Lysis solution (vortexed)

↓

50mg Lysozyme

↓

Vortexed and incubated for 10min

↓

Centrifuged at 8,500RPM for 15min

↓

Washed with Lysis solution

↓

500µl Extraction Buffer

↓

500µl Dissociation Buffer or Sample Buffer (Blue Juice)

(kept in boiling water for 5min)

↓

Loaded 50µl each time in mini gel wells or

70-80µl in big gel wells
b) **Gel pouring and setting**: Glass plates and the spacers of the mini gel apparatus were assembled and the two plates were joined at the base with an adhesive tape. The sides and the base of the two plates were sealed with 1% agar so that no leakage occurred while pouring the gel solution. The 10% resolving gel solution was prepared and poured in between the two plates till only 2-3 cm space was left from the top, for the stacking gel. Then up to 1cm water/butanol was poured on top of the resolving gel quickly as air inhibits polymerization. It was allowed to set for 5-6hrs after which the water/butanol was removed from the top of polymerized resolving gel by inverting the plates and the upper surface of the resolving gel was wiped dry with an absorbent paper. The 5% stacking gel solution mix was then poured on top of the stacking gel, with a Teflon comb inserted in between the two plates. Care was taken to prevent any air bubble formation in the gel while setting. The apparatus was left undisturbed for about an hour.

c) **Loading of the samples**: Comb was removed after the gel got set and the wells were washed with distilled water using a syringe. The whole apparatus was then set and the buffer was poured in the upper and the lower tanks. The samples were loaded in the wells with the help of a sample-loading syringe. The samples sank to the bottom of the wells immediately being heavier due to the presence of glycerol.

d) **Separation**: After loading samples in the wells, the whole apparatus was connected to the power pack and the electrophoresis was carried out at the required voltage. Voltage was kept low (corresponding to 15mA current) till the dye reached the junction of the two gels, then the voltage was increased (corresponding to 25mA current). The current was
passed till dye moved to the end of the resolving gel. Then the power supply was switched off.

e) Fixation and staining of the gel- The gel was removed by separating two glass plates carefully and placed in the staining solution containing 0.25% Coommasie Brilliant Blue R250 in 50% TCA in water for 2-3hrs or 0.25% Coommasie Brilliant Blue R250 in methanol: acetic acid: water (45:10:45) for 16- 24hrs, with occasional shaking in both the cases.

f) Destaining- The gels were destained by keeping in 7% glacial acetic acid on the shaker at 42°C and 150RPM for 6-8hrs or in methanol: acetic acid: water (45: 10: 45) solution till it was completely destained and the bands became very clear.

f) Photography and gel drying- The gels were photographed by keeping them on the LKB 2017 Macroread Light Table with the help of CANON 500NE camera. The gels were dried for storage in the lab using LKB 2003 Slab gel drier Unit.

3.2.9 Plasmid analysis

3.2.9.1. Small scale preparation of plasmid DNA- This was carried out by the method of Sambrook et al, 1989 with some modifications. For this the isolates were grown on LB at 37°C overnight and with vigorous shaking (Protocol 3.2.9).
Protocol 3.2.9. Small scale preparation of Plasmid DNA

1.5ml liquid culture
↓
centrifuged at 12,000g at 4°C
↓
cell pellet
↓
100μl Solution I (with 10mg/ml lysozyme)
vortexed lightly
incubated for 45min
↓
added 200μl solution
mixed by inverting 5-10 min
↓
150μl sol III
mixed cells by inverting, microfuge & kept for 5 min
↓
supernatant
↓
added equal volume of chilled ethanol & mixed well by inverting
↓
kept at room temperature for 5mts & centrifuged for 10min
↓
discarded the supernatant and all traces of liquid removed
dried the precipitate at 37°C
↓
added 200μl solution IV
reprecipitated with 400μl chilled ethanol
↓
centrifuged for 10min
resuspended the pellet in 20μl TE buffer
↓
stored at 4°C or on ice

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3.2.9.2. Pouring and setting the gel- The gel casting apparatus was assembled and 0.7% agarose gel (prepared in 1x TBE buffer) was poured and allowed to set along with a comb.

3.2.9.3. Gel loading- The set gel was placed in the tank containing TE buffer. 10μl of the sample and 5μl dye were loaded in each well after mixing. To one of the wells 5μl marker and 10μl dye were loaded.

3.2.9.4. Separation- The apparatus was connected to the power pack and electrophoresis was carried out at 100V. The current was switched off when the dye reached the other end of the gel.

3.2.9.5. Staining and visualization- The gel was removed, stained with EtBr solution and shifted onto the platform of UV light apparatus (LKB transilluminator). The plasmid bands were visualized under UV light and were photographed using a red filter.

3.2.10 Primary powder preparation

The primary powders of the new isolates were obtained by lyophilization and by Lactose- Acetone Coprecipitation method of Dulmage et al., 1970.

3.2.10.1. Lyophilization- For this the cultures were grown on NYSM for 3-5 days till complete sporulation occurred and centrifuged. The pellet was washed twice with distilled water, resuspended in the minimum quantity of water and frozen at −20°C. The frozen samples were lyophilized using the Lyophilizer SAVANT model No. RT 100A220. The viability and pathogenicity of the samples was then tested. The lyophilized samples were stored in sealed glass vials.

3.2.10.2 Lactose-Acetone Coprecipitation- The spore crystal complexes of the two isolates KSD-4 & KSD-7 were recovered by the method of Dulmage et al. (1970). For this
each time 1 litre of the culture was grown on NYSM for 3-5 days, till complete sporulation occurred. The samples were monitored microscopically and further processed (Protocol 3.2.10.2). This method yielded a primary powder for the bioassays of the mosquito-pathogenic bacilli using Lactose- Acetone Co-precipitation as technique.

3.2.11 Dry weight determination

The dry weight experiments were performed with various concentrations (O.D.) of the cultures for the conversion of O.D.’s into dry weights so that the LC values could be expressed in terms of dry weights of the cultures. Each culture was grown on NYSM for 3-5 days, till complete sporulation occurred and the pellet was obtained after centrifuging the sporulating culture. The pellet was re-suspended in sterilized distilled water and the various concentrations of the culture were prepared by dilution and recording corresponding O.D.’s. The clean new eppendorfs were dried overnight in the oven and weighed. 1ml of each culture was added to the weighed dried eppendorfs and this culture was dried overnight in the oven. While transferring the eppendorfs from oven to the weighing balance, they were always kept in the dessicator to prevent the moisture absorbance. The weight of each conc. of the culture per ml was calculated. The average weight per ml of each concentration was plotted against their O.D.’s. This plot was used to express the LC$_{50}$ values in terms of dry weight per ml in the main bioassays.
Protocol 3.2.10.2. Process of recovery of spore-crystal complex

Whole beer pH 8.4-8.7 (3-5 day old culture)
↓
Adjusted to pH 7.0 with HCl
↓
supernatant (discarded)
Residue
↓
Suspended in 1/10-1/20 volume (based on the original beer)
In 4-6% Lactose
↓
Stirred for 30min
↓
Added slowly while stirring
4 volumes of Acetone
↓
Stirred 30 min
Let stand for 10 min
↓
Filtered with suction
↓
supernatant (discarded)
Residue
↓
Stirred with small volume of Acetone
↓
Filtered with suction
↓
supernatant (discarded)
Residue
↓
Stirred with small volume of Acetone
↓
supernatant (discarded)
Residue
↓
Dried overnight
3.2.12 Range finding bioassays

A number of range finding bioassays were performed prior to the main bioassays to calculate the LC$_{50}$ (lethal concentration which kills 50% of the larvae in 24hrs) values. For these bioassays each isolate was grown on 100ml NYSM in a conical flask and kept on a rotary shaker for 3-5 days, till complete sporulation occurred. The culture was pelleted by centrifugation at 8,500RPM and 4-8°C in RC5C Sorvall centrifuge for 15min. The pellet was washed twice with distilled water and re-suspended in the minimum quantity of distilled water and vortexed. The Optical Density (O.D.) of the suspension was noted using the spectrophotometer (Milton Roy Spectronic 1201). A wide range of concentrations was made noting their O.D.'s. 1ml each of these concentrations was added to the plastic bowls each of which contained 20 mosquito larvae in about 99ml water. Only late III instar larvae of *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* were used in these bioassays. The bioassay bowls were labeled and kept covered with pieces of nylon-net or muslin cloth to prevent any mosquitoes from laying eggs in them. The bowls were left undisturbed for 24hrs after which the live larvae were counted to calculate mortality. The results were based on the count of live larvae instead of the dead ones because of the cannibalistic tendency of larvae at lower concentrations. At times, dead or sluggish larvae are consumed by the active larvae. However, no such behaviour is observed in the untreated controls. Negative control containing 20 larvae in 100ml distilled water without any culture was maintained concurrently each time. If the mortality in the control exceeded 20% the assay was discarded and repeated. The percentage mortality was calculated and then corrected using the Abbot's formula (Mittal *et al*, 1993).
From these bioassays the range where the $L_{C_{50}}$ value of the test culture fell was known so that the main bioassay with minimum 5 replicates could then be performed with a narrow range of concentrations.

**3.2.13 Main Bioassays**

Subsequent to obtaining a narrow range of O.D.'s from the range finding bioassays described earlier where the $L_{C_{50}}$ value lay for an isolate, the main bioassays of that isolates were carried out following WHO procedures (WHO, 1992). For this the culture was grown on 100ml NYSM till sporulation. The beer was centrifuged, the pellet so obtained was washed twice in sterile double distilled water and re-suspended in a small volume of distilled water. The culture was diluted to obtain 3-4 concentrations including a couple of doses lower and higher than the $L_{C_{50}}$ values obtained from the range finding bioassays as described above. For this 20 mosquito larvae of the test mosquito species were added to each plastic bowl (500ml capacity) containing about 247.5 ml distilled water. 2.5ml of the dilution of the culture prepared was then added to these bowls (Fig. 3.2.13). Five replicates were maintained for each concentration. Same number of replicates for the negative controls were also maintained simultaneously, each consisting of 20 larvae in 250ml distilled water without any culture. The bowls were covered with mesh/nylon cloth and kept undisturbed for 24 hrs after which the live larvae were counted. The larvae that did not move on gentle prodding with a blunt glass rod or a tooth-pick, were considered dead. Only the III instar healthy mosquito larvae were used.
in the bioassay and no food was given to them during the test period as the readings were taken within 24hrs (WHO, 1992).

From the readings the % average mortality was calculated and the corrected using Abbot's formula wherever necessary. The corrected values of % mortality were plotted against different doses and the LC$_{50}$ values of the test culture against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* were calculated by probit analysis (Finney, 1971) using MS probit package.

3.2.14 Rearing of test mosquito immature

The mosquito larvae of *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* used in the bioassays were obtained from the insectary of Malaria Research Centre, Field Station Goa, Panjim. This table-top insectary was maintained with the help of trained technicians, insect collectors and lab assistants (Fig.3.2.14). The methodology for mosquito collection from their breeding habitats and their rearing is briefly described here. The three mosquito species were either obtained from the field as larvae or their adult mosquitoes were collected from the human dwellings and then reared in the lab in separate cages for the continuous supply of mosquito immature populations.

**Larval sampling:** Mosquito larvae were collected from the breeding habitats of mosquitoes with the help of laddels/bowls of about 500ml capacity. From the shallow waters larvae were picked up with the help of glass droppers having rubber bulbs. They were transported to the insectary and reared till emergence of adults.
Fig 3.2.13: Laboratory biossays of the mosquito-pathogenic bacilli against mosquito larvae in progress.

Fig 3.2.14: View of the insectary at Malaria Research Centre, Field Station Goa.
**Larval rearing:** Larvae were reared in enamel or plastic trays of 5x 10x 3 inches dimension containing tap water. They were fed on small quantities of the mixture of Yeast Extract and dog biscuit (40:60) and kept in the trays until they reached pupal stages.

**Adult emergence:** The pupae were transferred to the plastic bowls containing tap water and covered with a piece of nylon net having a hole in the center to facilitate the removal of adult mosquitoes with aspirator. This hole was kept plugged with cotton to prevent the emerged adult mosquitoes from escaping. The adults thus emerged were identified and transferred to their respective cages.

**Identification:** The adult mosquitoes were identified based on morphological variations with the help of the keys of Christopher (1933 & 1934); Barraud (1934); Puri (1954, adults and 1960, larvae); Rao, 1984; Das et al, 1990 and Nagpal & Sharma, 1994).

**Collection and transportation of adult mosquitoes from the field:** The adult mosquitoes were also collected early in the morning (5 a.m. to 8 a.m.) from the random and fixed human dwellings with the help of an aspirator/suction tube. In these dwellings, adult mosquitoes were located with the help of a torch-light and sucked into the aspirator tube by holding the mouth of the suction tube close to the mosquito and gently but quickly sucking from the mouth-piece. They were prevented from escaping by immediately covering the suction end with a finger. These mosquitoes were then transferred into the clean test tube by holding the mouth of the test tube very close to suction end and blowing out gently. The test tubes were then plugged with cotton wool labelled and transported to the insectary.
Adult rearing: After their identification, the adult mosquitoes were separated species wise and released into the cages designed especially for them. The adult mosquitoes were fed on the blood of a live chicken daily, which was introduced into the cages in iron frames. Petri-plates containing cotton pad soaked in 5% glucose were also kept inside the cages with few raisins in them, for feeding as a source of sugar.

Egg collection: Plastic bowls containing 300ml tap water were kept inside the adult cages for oviposition by the gravid female mosquitoes. The eggs were transferred to the plastic trays where the larvae emerged. These larvae were either used in bioassays as they grew to III instar stage or were reared further to adults as described above to get the continued supply of adult population for breeding in the cages and for uninterrupted supply of immature.

Maintaining vigor of the mosquitoes in the lab: Vigor of the mosquitoes was maintained by introducing weekly field collected/wild population of adult mosquitoes or through emergence of the larval populations collected from the field and reared in the lab as explained.