Isolation, purification and identification of bacteria from Flacherie infected larvae of *Bombyx mori* L.

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INTRODUCTION

The natural silk yielded by sericigenous insects is one of the most important textile fibers. The natural silk has emerged as the most liked textile fiber in spite of heavy competition by the synthetic fibers. The domesticated silkworms contributed the bulk of global silk. Although bulk of the commercial silk is produced by the mulberry silkworm, *Bombyx mori*, the other silkworms commercially exploited for silk production are eri and muga. India is unique in producing the tasar silk.

Silkworms suffer from various diseases namely pebrine, virosis, bacteriosis and muscardine caused by microsporidia, cytoplasmic polyhedrosis virus, different types of bacteria and fungus respectively and loss due to these diseases is at considerable rate. In India, the extant of cocoon crop loss due to the silkworm diseases is nearly 40% (Sahay *et al.*, 2000). Bacteriosis is caused by Gram negative and Gram positive bacteria. It is transmitted orally. Its impact is more pronounced in larval stage which affects cocoon productivity and quality along with 10-15 % crop loss. Poor leaf quality and poor feeding are predisposing factors of this disease. Rods and Cocci are observed from the body fluid of infected larvae.

Bacteria are microscopic, single celled prokaryotes. They are some of the smallest and most abundant microbes. Despite their small size, the effect of microbes on human, and the world in general, are critical for maintaining life on earth. It is therefore impossible, indeed undesirable, for people to avoid microorganism or their influence. We should, however, understand the activity of microbes as well as their potential for enhancing or diminishing the quality of our lives. Microorganism exists virtually everywhere. They are in our food, in the water we use for drinking and bathing, on our utensil and on our bed sheets, such harmless microorganism comprises the normal flora. As long as bacteria are found practically everywhere, it must be expected that they will occur as mixed culture.

Some bacteria species are very fragile and can be killed by slight changes in the surrounding environment. Some bacteria species are extremely tough, able to withstand sever heat, cold and drying. Some can lie dormant for decades waiting for favourable conditions. Others can extract the nitrogen directly from the air or breakdown some toxic substances. Many microbes release antibiotic substances to suppress particular competitors. In this way some species can suppress other disease causing microorganisms. Decomposer bacteria play an important role in decomposition of organic materials.

Pure culture study of bacteria is an essential aspect for studying bacterial cultures with the objective of learning their characteristics and behaviour or determining their identity or both. Such a study includes microscopic study of pure culture; either stained or unstained, determination of cultural characteristics of an organism, a study of its physiological and biochemical characteristics, determination of pathogenicity and study of pathological effects.

Reports on the identification of bacterial diseases on silkworm are scanty, however investigations on the various types of bacterial infection in mulberry and other sericigenous insects were carried out to develop suitable control measures against the occurrence of bacterial diseases (Izuka, 1983; Baig *et al.,* 1990; Patil, 1990). The pathogenic bacteria responsible for bacterial flacherie in *Bombyx mori* reported to be gram positive *Cocci Staphylococcus* epidermis, *Streptococcus* species and *Staphylococcus aureus* (Izuka, 1983, Kalpana *et al*, 1994), *Serratia marcescens* causing septicemia (Nahar, 1995) and *Streptococcus faecalis* (Patil. 1990), *Streptococcus bombysis* and *Streptococcus pastorianus* (Anitha *et al*, 1994). Reports of mixed infection for *Bombyx mori* are also available (Matsumoto *et al*, 1985, Govindan *et al* 1990).

Even though various spp of pathogen *Bacillus* are available as inocullum, in this study Isolation, purification and identification of bacteria from *Flacherie* infected larvae of *Bombyx mori L.* is envisaged for utilization of research material to carry out the research work.

MATERIALS AND METHODS

Sterilization of culture media and appliances:

Sterility is the hallmark for successful work in the microbiology laboratory. To achieve this, it is mandatory that sterile equipment and sterile techniques are used. Sterilization is the process of rendering a medium or material free of life. There are three types of sterilization process.

1. Heat

(a) – Dry (hot air):

(b) - Autoclave (Moist Wet heat)

2. Filtration

Millipore-cellulose acetate disc, Seitz asbestos pad, Berkefeld-diatomaceous earth, candle filter, sintered glass.

3. Chemical

(a)Ethylene oxide

(b)Beta- propiolactone

I have sterilized the culture media and appliances using autoclave at 121°C/15 lbs pressure for 15 minutes.

Collection of samples:

The mulberry silkworms of 3rd instar showing the symptoms of Flacherie were collected from the silkworm rearings conducted in the Dept.of Sericulture, S.K.University.

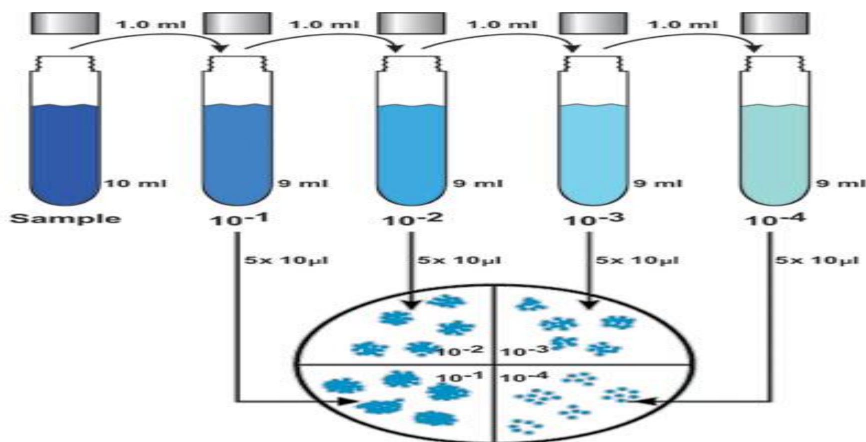
ISOLATION OF MICRO-ORGANISM FROM MULBERRY SILKWORM USING SERIAL DILUTION AGAR PLATING METHOD:

Though various methods are available to isolate and enumerate microorganism (bacteria, fungi, actinomycetes, protozoa, algae, virus, and mycoplasmas,) from soil, food, stuff, milk, and water the Serial dilution agar plating method or viable plate count method is one of the commonly used procedures for the isolation and enumeration of fungi, bacteria and actinomycetes which are the most prevalent micro organism. This method is based upon the principle that when material containing microorganism is cultured. Each viable micro organism will develop into a colony. Hence the number of colonies appearing on the plates represent when the no. of living organism present in the sample.

In serial dilution agar plate method, a known amount (10 ml/100 ml) of material is suspended or agitated in a known volume of sterile water blank (90 ml or so to make the total volume to 100 ml) to make a microbial suspension. The hind gut of silkworm infected with anal lip sealing, a bacterial disease was collected in distilled water and homogenized. Then the isolation of the bacteria was done using serial dilution agar plating method as mention below.

Serial Dilution Technique:

- Homogenized suspension of hind gut of infected silkworm
- 1 ml added to 9.0 Sterile Water = 10-1
- 1 ml added to 9.0 Sterile Water = 10-2
- 1 ml added to 9.0 Sterile Water = 10-3
- 1 ml added to 9.0 Sterile Water = 10-4
- 1 ml added to 9.0 Sterile Water = 10-5



Diagrammatic representation of Serial Dilution Technique

Pour Plate Technique

In this technique the serially diluted samples (I ml) were added in to sterile Petri plates. Then after melted and cooled (42-45°C) agar medium was poured and mixed thoroughly by rotating the plates which were then allowed to solidify. Then the plates were incubated at 37°C for 24 - 48 hrs. The separate colonies were appeared in the agar plate.

Pure culture technique

Dilution Method: A fluid containing a mixture of bacteria was diluted with sterile medium in the hope that ultimately a growth could be obtained that took its origin from a single cell. Development of simple method for obtaining pure culture of bacteria is a vital requirement for growth of bacteria.

Culture Transfer Technique: When bacteria are transferred from one medium to another, the material being transferred is called inoculum and the resultant growth, whether it is in a liquid or in a solid medium is termed a culture or subculture.

Spread Plate Technique: The organism in a dilute microbial mixture can be rapidly separated into individual colonies for isolation of pure cultures by the spread plate technique. The organism are spread over the surface of a solid agar medium in a Petri dish with a sterile, L shaped bent glass rod while the dish is spun on a lazy Susan turn table.

Streak Plate Technique: The streak plate method is a rapid qualitative isolation method. It is essentially a dilution technique that involves spreading a loop full of culture over the surface of an agar plate. Although many types of procedures are performed, the four–way or quadrant streak is described and is to be performed.

Cultivation of Bacteria

Preparation of basic liquid media (broth) for the cultivation of bacteria

Bacteria in contrast to fungi are often cultured in a liquid broth (*i.e.* media lacking agar). The most common constitute of basic media used in routine bacteriological laborites are beef extract (a beef derivatives which is source of organic carbon, N_2, vitamins and inorganic salts) and peptone (a semi digested protein). These may be modified in a variety of ways by supplementing with some specific chemicals or material to provide a medium, suitable for the cultivation or demonstrate of a reaction for specific types or group of bacteria (James and Sherman, 1983).

Culture Media: For cultivation of bacteria from different samples the basic media used is Nutrient Broth media.

REQUIREMENTS

Nutrient broth-

Peptone- 5.0 gm, Beef extract-3.0 gm, Distilled water-1000 ml pH 7.0 .

For preparation of Nutrient Broth media 2% agar is added to the Nutrient Broth. The agar is act as solidify agent.

Procedure:

- Put the weighed amount of peptone 5 gm and the beef extract 3 gm in 500 ml of distilled water.
- Heat with agitation to dissolve the constituent.
- Add more distill water to make 1 litre
- Adjust pH of the medium to 7.0, using a pH meter by adding either acid or alkali, as the case may be
- Pour 10 ml per tube
- Autoclave at 121^0C, 15 lbs pressure for 15 minute / 20 minute.
- Allow the autoclave to cool

Remove the nutrient broth tubes and store at rot (covered with butter paper) for future.

Tests for Bacteria identification:

The isolated bacteria were identified on the basis of

(i) Cultural characters
(ii) Morphological characters
(iii) Biochemical characters
(iv) Molecular character

The cultural characters include shape, size and colour of colony as appeared in plates. The morphological characters include shape, size and gram reaction of vegetative cells of isolated bacteria. The biochemical characters include pattern of reaction with different enzymes (Conn *et al.,* 1957)

Microscopic study of living bacterial preparation

The isolated bacteria were examined under phase contrast microscope. It is useful to observe cell activities like motility and binary fission and the natural size and shape of the cells as heat fixation and exposure to chemicals during staining cause some degree of distortion.

STAINING METHOD:

To study their properties and to differentiate microorganism into specific groups for diagnostic purpose, biological stains and staining procedure in conjunction with light microscopy have become major tools in microbiology.

Chemically, a stain (dye) may be defined as an organic compound containing a benzene ring plus a chromophore and auxochromo group.

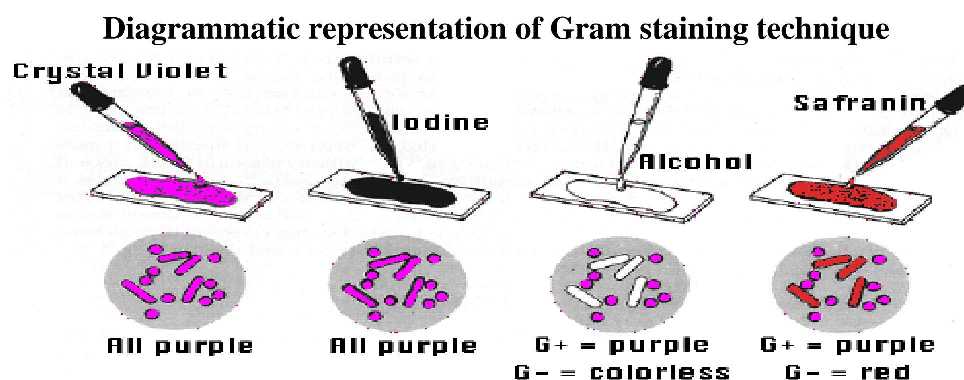
Gram Staining:

The gram stain is the most useful stain in the microbiology. It allows one to learn both the reaction of the stain and morphology of the bacteria.

Different staining requires the use of at least three chemicals reagents that are applied sequentially to a heat fixed smear. The first reagent is called the primary stain. Its function is to impart its colour to all cells. In order to establish a colour contrast, the second reagent is used is the decolourizing agent. Based on the chemical composition of cellular components, the decolourizing agent may or may not remove the primary stain from the entire cell or only from certain cell structures. The final reagent, the counters stain has a contrasting colour to that of the primary stain.

The procedure consists of staining with crystal violet (a purple dye), mordanting with iodine, decolorizing with acetone alcohol and counter staining with safranine (a red dye). Bacteria are divided in to two groups by this procedure. The stain reaction correlates with the type of cell wall makeup those bacteria that are not decolorized retain the purple colour of crystal violet, and are gram positive. Those decolorized by acetone alcohol are stained red by safranin counter stain and are gram negative.

Diagrammatic representation of Gram staining technique



Requirements:

1. Slide 2. Pipette 3. Bacterial growth culture

Reagents:

1. Crystal violet 2. Burker's iodine 3. Safranin, 4. Alcohol (95%)

Procedure:

1. A bacterial smear from a young culture was prepared. The bacterial sample was taken from one colony with a sterile loop in a small drop of saline on a clean glass slide and spread out to leave a thin film.
2. Smear was air dried.
3. The smear was flooded with CRYSTAL VOILET for one minute.
4. Washed off the stain with a gentle flow of water drain.
5. The smear was flooded with GRAM'S IODINE for one minute.
6. Washed off the stain with a gentle flow of water drain.
7. Decolourized with Acetone-Alcohol until the solvent flows colourlessly from the slide.
8. Washed off quickly with a gentle flow of water drain.
9. Flooded the smear with SAFRANIN for one minute.
10. Washed off the stain with a gentle flow of water drain.
11. Blotted the smear with blotting paper and air dried before viewing.

Reagent Preparation:

(A) CRYSTAL VIOLET : Crystal violet -2 gm, Ammonium oxalate - 1 gm.
Distill water -100ml.

(B) BURKER'S IODINE: Iodine -1 g, Potassium iodine -2.0 g
Distill water -100ml

(C) AQUEOUS SAFRANIN

2.5% solution of safranin in 95 % Ethanol 10ml
Distill water 100ml

BIOCHEMICAL TEST:

In the present study we used Catalase production, Amylase productivity, Nitrate reduction, Proteolytic activity and Utilization of Citrate test. Although there are various biochemical tests for identification of bacteria which are as follows.

Catalase Production: The slants (nutrient agar) inoculated with an organism incubated for 24 hrs. To 24hrs old slant culture and 1ml of 10% (vol/vol) aqueous H_2O_2 solution was added. Bubbles of oxygen released from the surface of the growth in the culture tube indicating the preserve of catalase in the organism.

Media composition

Peptone: 5gm, Beef extract:3gm, Nacl:5gm
Distilled water 1000ml, pH 7

Amylolytic activity: The bacteria were streaked on the starch agar medium in petridishes. After 3-5 days incubation at 30^0C the plates were flooded with Lugols iodine solution. A colourless zone around the streak indicated the production of the Co-enzyme amylase by the bacteria.

Media composition:

Starch agar medium: 10g starch added gradually to 800ml boiling distilled water with constant stirring. The volume reduced 500ml by boiling.

In another 500 ml distilled water add -K_2HPO_4 1.0 g, $MgSO_4 .7H_2O$ -1.0 g,NaCl -1.0 g, $CaCO_3$ -3.0 g, $(NH_4)_2SO_4$ - 2.0 g, Agar-20 g, pH 7.2.

Lugols iodine solution

Iodine -1 gm, Potassium iodine- 2 gm, Distilled water-300 ml.

Proteolytic activity:

The bacteria were streaked on plates containing gelatine agar medium and incubated at 30^0 C. After good grow bacteria the plates were flooded with a protein-precipitating reagent. The production of a clear zone around the streaks was taken as an indication for the presence of proteolytic enzymes.

MEDIA COMPOSITION

1. Protein precipitating reagent: $HgCl_2$:15g, Distilled water -100ml.
Conc Hcl 20 ml.

2. Gelatin Agar medium: Beef extract 3.0 g, Peptone 5.0 g, Gelatin 4.0 g, Agar 20 g
Distilled water 1000 ml, pH 7.0

Voges proskauer test for the production of Acetyl Methyl Carbinol:

The bacteria were grown in 10 ml of the medium (AS GIVEN BELOW) in the tubes. For 4 to 7 days at 30°C to test the presence of methyl carbinol of 2:3 butylene - glycol, 1.0 ml of the culture broth was mixed with .6ml of 5% napthol and 0.2 ml of 4.0% KOH, shaken vigorously and allowed to stand presence of acetyl methyl carbinol results appearance of pink color. After 15 to 20 minute methyl red test is performed with the same medium having 0.02% methyl red. A positive methyl red reaction is indicated by a distinct red reaction (acidic). Yellow color is regarded as negative reaction (pH alkaline).

Media composition: Peptone 5 g,K_2HP0_4-5 g, Glucose 5 g
Distill water 1000 ml. pH 7.2 .

Indole production:

The DM broth was supplemented with 0.1% casein hydrolysis and was distributed in 10 ml quantities in tubes sterilized by autoclaving at 10lbs /inch^2 pressure for 10 min, cooled and inoculated with loopful of bacteria.The culture was incubated for 3to 7 days at 30^0 C.

The presence of indole in the culture filtrate was tested by Ehrlich Bohmi reagent. The reagent was introduced carefully to form a layer on the broth. A red colored ring at the function of the 2 liquids indicated the presence of indole.

Media composition:

1. Ehrlich Bohme Reagents.

A - Para - di methyl amino benzyaldehyde 1.0 g, Ethanol (95%), Conc HCl

B.- saturated solution of Potassium Peroxy sulfate

1 ml solution of A and 1 ml solution of B is mixed and added to 10 ml of culture of filtrate.

Utilization of citrate

The bacteria were grown in 10 ml of sterile Koser's synthetic medium taken in tubes. Growth evidenced by turbidity indicated the citilazation of citrate as a sole source of carbon.

MEDIA COMPOSITION:

1. Koser's medium

NaCl 5 gm, $MgSO_4$ 0.2 gm, (NH_4)H_2PO_4 1.0 gm,Na-Citrate2.0 gm, K_2HPO_4 1.0 gm.
Distill water:1000 ml. pH6.8 to 7.0

Acids and gas production by the bacteria:

To test the utilization of different sugars the DM broth was supplemented with 0.1% of each sugar and was distributed in 10 ml quantities in tubes. Durham's tubes were poured with the same media and inserted into the tube in inverted position so that gas produced by the bacteria would be seen at at the top of the Durham's tubes. The change of colour from pink to yellow is the indication of the utilization of sugars. The sterilized by autoclaving at 10lbs /inch^2 pressure for 10 min, cooled and inoculated with loopful of bacteria. The culture was incubated for 3 to 7 days at 30^0 C.

Cellulose decomposition: White filter paper strips 50 mm x 80 mm are prepared. One strip inserted as carbon source in tubes containing 10 ml devis mingivli (without glucose). Tubes are plugged and sterilized inoculated and incubated 48 to 96 hours. Breakage of filter paper strip at surface level on slight shaking indicates cellulose decomposition.

Nitrate production:

The reduction of nitrate to nitrite is a function of the enzyme nitrate reductases.

Media composition:

K_2HPO_4 7.0 g, KH_2PO_4, 3.0 g, Na Citrate 0 .5 g,$(NH_4)_2SO_4$, 1.0 g, $MgSO_4.7H_2O$ 0.2 g
Distilled water 1 lit, pH7.0.

Griess Losvay Reagent

Solution A : Sulfonic acid 0.5 g, Glacial acetic acid 30 ml, Distilled water 100 ml.

Solution B: α – napthalamine 0.1 g dissolved in 100 ml of boiling distilled water, cold and 30 ml of glacial acetic acid added. Equal volume of A and B mixed before use. Grow the bacteria on 10 ml DM broth medium containing 0.2% potassium nitrate for 24 hours at 30^0C. Then add 1 ml of Griess Losvay reagent - A cherry red coloration indicate the presence of NO_2 (Nitrite.)

MEASUREMENT OF BACTERIAL GROWTH:

Counting of bacteria using haemocytometer:

The Haemocytometer was originally devised for counting blood cells and the microbial spores (bacteria fungus, etc). It is a special microscope slide with a counting chamber 0.1mm deep. So the volume of the liquid over
one square mm is 0.1 cubic mm. The counting chamber has a total of nine squares, each of 1 mm x 1 mm engraved over it. The central square of the counting chamber *i.e*. 1 square mm area is divided into25 small square by triple lines *i.e*. each of these small squares has an area of 1/25 square mm which is bounded by triple lines. Each of these small squares *i.e*. 1.25 sq. mm area is further divided into 16 smaller squares each of which has an area = 1/25x 1/16= 1/400square mm.

Method: The number of bacterial cells in small squares was 135+145 +151 +139+ 122 respectively. Now in 5 small squares (R_1, R_2, R_3, R_4 R_5,) Or, In 1 smaller square, no of Bacteria - (R_1, R_2, R_3, R_4 R_5,)/80 = N, Each smaller square has 1/4000 cu mm. of cells, No. of bacteria in 1 cu mm. = N x 4000, But the bacterial suspension is diluted a number of times (T) for convenience which should be multiplied.
Thus, number of bacterial cells in 1cu mm. of suspension = T x N x 4000

Isolation of Bacterial DNA:

Reagents used in DNA isolation:

Media; Trypton yeast extract media, Trypton - 1.0 gm, Yeast extract - 500 mg
NaCl - 500 mg, pH - 7.0, Distilled water - 100 ml

EDTA (0.5 M): EDTA – 181.6 g (disodium EDTA), Water – 800 ml,
Stir on magnetic stirrer, adjust pH to 8.0 with NaOH EDTA not dissolved until pH reach at 8.0

Tris Cl (I M): Tris base -121.4 g, Water - 800 ml, pH 8.0 by adding concentrated HCl

TF (Tris – EDTA) Buffer: 1 M Tris Cl 1 ml, 0.5 M EDTA 0.2 ml, Water 98.8 ml.

Procedure:

1. Cultures grow in 10 ml tube at 37 ºC for over night.
2. Then incubate at 4 ºC for 20min
3. After that 10ml of each strain is transferred to 1.5 ml centrifuge tube *i.e*. in 6 tubes.
4. Centrifuge at 10,000 rpm for 2 minutes at 4 ºC
5. Mix 3 tubes of each strain together and finally get 1 tube for each strain.
6. Centrifuge at 10,000 rpm for 2 minutes at 4 ºC
7. Take palate, at 400 µl Tris EDTA (0.5M) buffer. EDTA buffer is used to chelate the divalanet bond.
8. Add 40 µl of 10% SDS, 5 µl of proteinase K (20mg/ml stock solution) and 5 µl of RN ase (10mg/ml) and add 50 µl of 20 mg /ml stock lysozyme and incubate on ice for 2 mins.
9. Incubate it at 56 °C for 45 mins to break the cells. After vortex for 2 mins then incubate at 75 °C for 15mins.
10. cool down at room temperature
11. At 400 µl of Tris saturated pheniol and centrifuge at 12,000rpms for 10 mins.

12. Take supernatant is contain DNA.
13. Add 400 µl of PCI and centrifuge it for 12,000rpm for 10 mins.
14. Take supernatant and add 400 µl CI
15. Centrifuge at 12, 000 rpm for 10 mins.
16. Take the supernatant and add 1/10 volume of 3M sodium acetate and 2 volume of absolute ethanol.
17. Incubate it at -20 °C for 2hrs or -70 °C for 1hr.
18. Centrifuge it for 12000 rpm for 20-30 mins at 4 °C.
19. Discard supernatant
20. Wash the palate with 750 µl of 70% ethanol for 20 mins
21. Centrifuge it at room temperature at 9000rpms for 5 mins.
22. Discard the supernatant and dry the palate for 15-20 mins
23. Dissolve in 20-50 µl of tris EDTA (0.5M) PH 8.5.

Gel electrophoresis of bacterial DNA:

1. Dissolve 0.7 gram agarose in 100 ml of 1x TAE buffer (0.7% gel)
2. Heat the solution until clear solution comes
3. Keep the solution for cooling at room temperature
4. Add 6 µl of Ethedium bromide in agarose solution when temperature come up to 60 °c
5. Cast the gel in gel tray with the comb in place.
6. When the gel is solidified remove the comb and place the gel tray in gel tank
7. Fill the gel tank with 1 x TAE buffer until the gel submerged in the buffer
8. Mix the DNA sample with 6 x gel loading dye (Bromophenol blue 25 mg, Xylene Cyanol 25 gm, Glycerol 3ml and distilled water 10 ml) and load in to the well
9. Run the gel submerged in 1 x TAE buffer for 2 hrs at 80 V.
10. After that remove the gel from gel tank and view under ultra violet light in gel documentation.

Random Amplification of Polymorphic DNA (RAPD) by Polymerase Chain Reaction (PCR):

The amplification reactions were performed in 0.2 ml PCR tubes using a programmable thermocycler (Techne, TC - 512). The reaction was carried out in volume of 50.0 µl containing 39.0 µl sterile water, 5.0 µl 10x Taq buffer A, 2.0 µl 10 nm dNTP mix, 2.0 µl RAPD primer (Bangalore genei), 1.0 µl DNA template (50ng/ µl) and 1.0 µl Taq DNA polymerase (Bangalore genei) (3U/ µl).

The thermocycler was programmed for 5 minutes at 94°C, for 8 cycles of 45 sec. at 94°C, 1 min at 35°C, 1.5 min at 72°C, for 35 cycles of 45 sec. at 94°C, 1 min at 38°C, 1 min at 72°C. and for 10.0 min at 72°C and finally hold at 4°C. Once the PCR is completed, loaded 8 µl PCR product and analysed it on a 2.0% Agarose gel run at 50-100 V for 30 minutes. The gel was observed under UV transilluminator.

RESULTS

Isolation of bacteria from diseased silkworm:

A total of four different bacteria were isolated from silkworm suffered with flachery disease on the basis of shape and colour of colony appeared in agar plates (fig. 1). The bacteria isolated were coded as MSF 1, MSF 2, MSF 3 and MSF 4 (Table 1).

Table: 1. Code of isolated bacteria from diseased silkworm.

Sample collected	Isolates	Code
From HDP	2	**MSF 1, MSF 2**
From ATP	**2**	**MSF 3, MSF 4**

Identification of isolates: The isolates were identified on the basis of their cultural, morphological biochemical and molecular characters. MSF 1 and MSF 2 showed irregular white and stick colony, MSF 3 showed round white and sticky colony where as MSF 4 showed round creamy white colony. MSF 1, MSF 2 and MSF 4 were rod in shape where as MSF 3 was round in shape. MSF 1 and MSF 3 was gram positive where as MSF 2 and MSF 4 was gram negative (Table 2, and Fig 2 and 3)

The bacterial isolates were subjected to Catalase, Nitrate reduction, Amlyase production, proteolytic activity and citrate utilization tests for their biochemical identification. In biochemical reaction all the isolates had given positive result in catalase test except MSF 1. MSF 1 and MSF 3 were able to reduced nitrate and used starch as their carbon source, whereas as MSF 2 and MSF 4 have shown negative results in nitrate reduction test. MSF 1, MSF 2 and MSF 4 have shown positive reaction of Amylase production while MSF 3 was negative in Amylase production. Proteolytic activities have seen only in MSF 1 bacterial isolate. Citrate utilization test was positive in MSF 1, MSF 2 and MSF 3 (Table 3 and fig. 4).

On the basis of above morphological, cultural and biochemical characters, the bacterial isolates MSF 1, MSF 2, MSF 3 and MSF 4 were tentatively identified as Bacillus sp., Xanthomonas sp., Staphylococcus sp. and Serratia respectively according to Murry (1974) manual of determinative bacteriology.

MSF 1 (Bacillus spp.) : Round, white sticky colony, singly long rod, aerobic, gram positive, nitrate, amylate , citrate and proteolytic activity positive, catalase negative.

MSF 2 (Xanthomonas sp.) Round, irregular, white sticky colony, rod in chain, aerobic, gram negative, Amylate, catalse and citrate positive, nitrate and proteolytic negative,

MSF 3 (Staphylo Coccus spp.) : Round, white sticky colony, coccus in group, anaerobic, gram positive, Catalase, nitrate and citrate positive, amylase and proteolytic negative.

MSF 4 (Serratia spp.) : Small round, creamy white, rod in pair, anaerobic, gram negative, catalase and amylase positive, nitrate, proteolytic and citrate negative.

Table: 2. Culture and morphological character of bacterial isolates

Culture and morphological characters					
Bacterial isolates	**Colony colour**	**Colony shape**	**Vegetative cell shape**	**Gram reaction**	**Aerobic/anaerobic**
MSF 1	White, sticky	irregular	Long rod in single	+ve	**Aerobic**
MSF 2	White, sticky	irregular	Rod in chain	-ve	**Aerobic**
MSF 3	White, sticky	Round	Coccus in group	+ve	**Anaerobic**
MSF 4	**Creamy white**	**Small round**	**Rods in pair**	**-ve**	**Anaerobic**

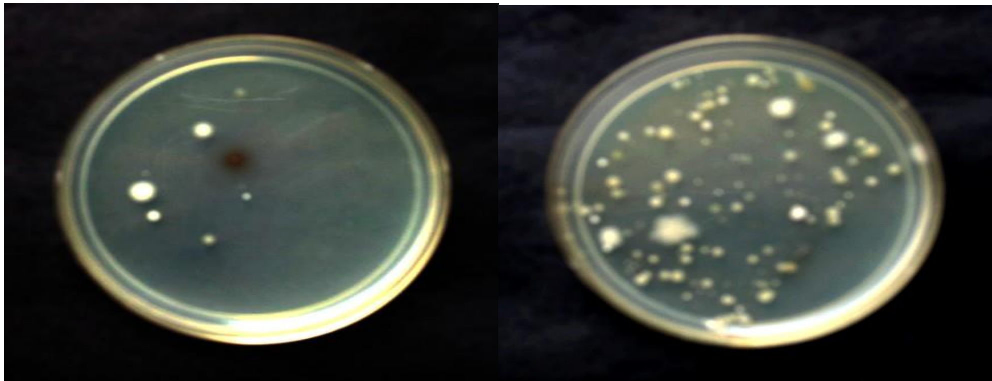


Fig.1. Isolated bacterial colony

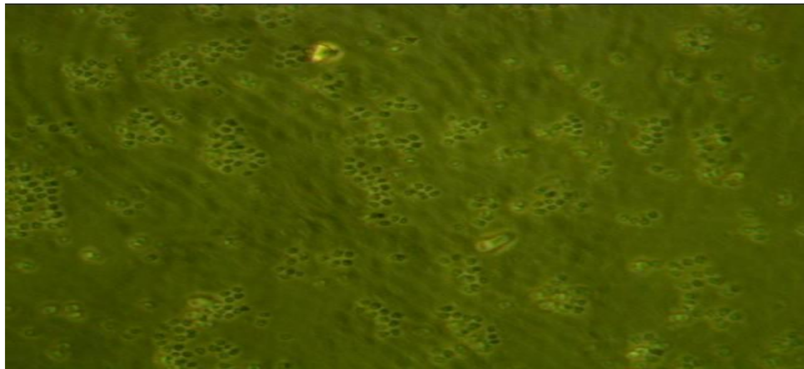


Fig. 2 . Bacteria: Coccus in groups

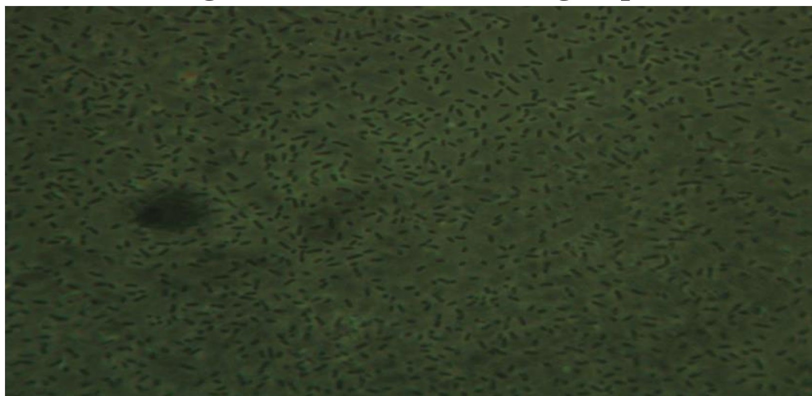


Fig. 3. Bacteria: Long single rods

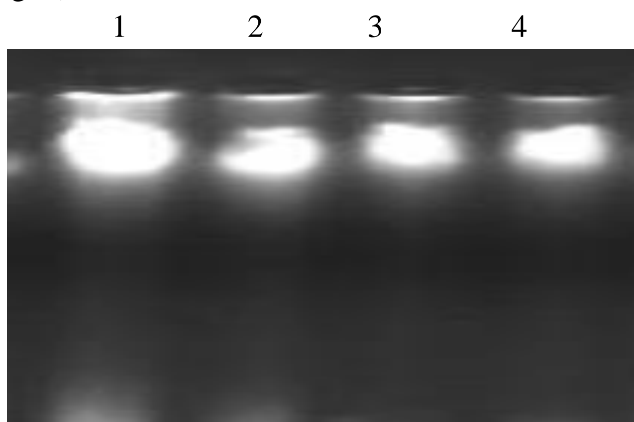
Table 3. Biochemical character of bacterial isolates

Bacterial Isolates	Biochemical character				
	Catalase Test	Nitrate reduction	Amylase production	Protolytic activity	Citrate Utilization
MSF 1	–	+	+	+	+
MSF 2	+	–	+	–	+
MSF 3	+	+	–	–	+
MSF 4	+	–	+	–	–

Fig. 4. MSF 1 shows positive reaction in Amylase production

Gel electrophoresis of genomic DNA

The analysis of genomic DNA of all bacterial samples were done by using 0.7% agarose gel containing ethidium bromide at 70 v for 1 hrs. Under UV light presence of DNA were visualized by band in all samples (Fig. 5)



Lane 1: Genomic DNA of MSF 1m Lane 2: Genomic DNA of MSF 2
Lane 3: Genomic DNA of MSF 3 Lane 4: Genomic DNA of MSF 4

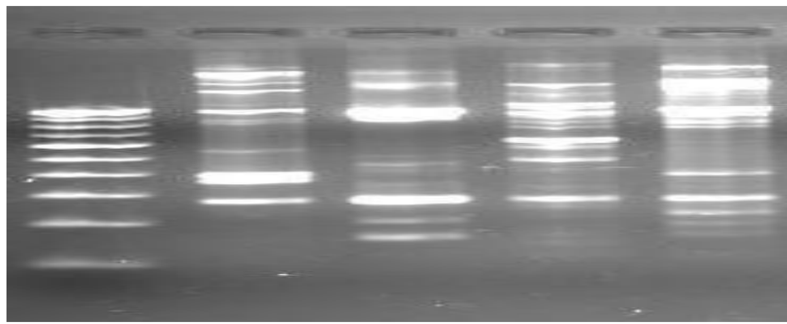
Fig. 5. Genomic DNA of bacteria

Gel electrophoresis of PCR product:

The genomic DNA of all bacterial samples were amplified by using two RAPD primers (RBa-C, 1 and 2). After running agarose gel of 2%, the gel was observed under UV light. The different samples were shown different band pattern in both the primer.

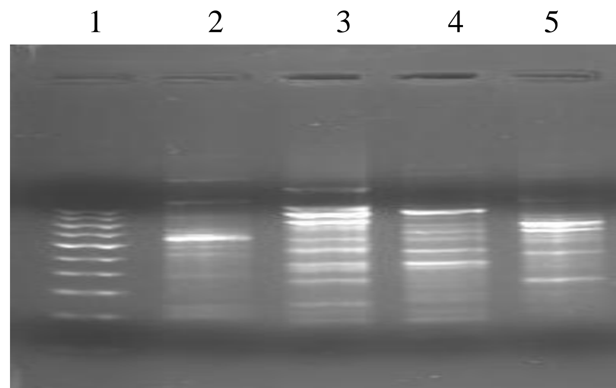
The band patterns were analyzed by using UV doc software for band range by putting a standard of 100 bp ladder. Primer 1 gave polymorphic band of range 113 bp -3261 bp in all the samples. The minimum base pair 113 was observed in SAL 2 and maximum base pair 3261 in MSF 1 (Fig. 6 and table 4). The primer (RBa-C 2)have shown the amplification of all four samples of bacterial DNA where polymorphic bands observed between the range of 139 bp -2267 bp. The minimum base pair 139 was observed in MSF 2 & 3 and maximum base pair 2267 in MSF 4 (Fig. 7 and table 5)

1 2 3 4 5



Lane 1: 100 bp ladder, Lane 2 to 4: MSF 1, MSF 2, MSF 3 and MSF 4 with primer 1

Fig.6. Polymorphic bands of bacterial DNA with primer RBa-c 1



Lane 1: 100 bp ladder Lane 2 to 4: MSF 1, MSF 2, MSF 3 and MSF 4 with primer 2

Fig.7. Polymorphic bands of bacterial DNA with primer RBa-c 2

Table 4 . Analysis of polymorphic bands of different bacterial DNA amplified by RAPD primer RBa-C 1.

Sl. No	Name of primer	Lane No.	Name of sample	No. of fragment	Band range (bP)
1	**RAPD Primer (RBa-C) 1**	1	100 bp Ladder	10	100-1000
2		2	MSF 1	10	157-3261
3		3	MSF 2	8	113-2001
4		4	MSF 3	10	239-2003
5		5	MSF 4	8	214-1263

Table. 5. Analysis of polymorphic bands of different bacterial DNA amplified by RAPD primer RBa-C 2.

Sl. No	Name of primer	Lane No.	Name of sample	No. of fragment	Band range (bP)

1	**RAPD Primer (RBa-C) 2**	1	100 bp Ladder	10	**100-1000**
2		2	MSF 1	5	**248-2042**
3		3	MSF 2	8	**139-1825**
4		4	MSF 3	6	**139-880**
5		**5**	**MSF 4**	**8**	**281-2267**

INFERENCE

Four different types of bacteria were isolated from the mulberry silkworm showing the symptoms of flachery of bacteria disease. The bacteria isolated were characterized by morphological, cultural and biochemical methods. On the basis of above characters the bacteria codes as MSF 1, MSF 2, MSF 3 and MSF 4 were identified as Bacillus sp., Xanthomonas sp., Staphylococcus sp. and Serratia sp. respectively. These bacterial isolates were also characterized molecularly by their DNA finger prints. The amplified DNA finger prints of the four bacterial isolates were different from each other, which indicate that the bacterial sp. identified as Bacillus sp., Xanthomonas sp., Staphylococcus sp. and Serratia sp. on the basis of morphological, cultural and biochemical characters are different at molecular level.

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