Appendices
APPENDICES

APPENDIX 1
COMPOSITION OF STARCH AGAR MEDIUM

<table>
<thead>
<tr>
<th>COMPOSITION</th>
<th>g/L</th>
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<tr>
<td>Peptone</td>
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<td>Yeast extract</td>
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<tr>
<td>Beef extract</td>
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APPENDIX 2
COMPOSITION OF NUTRIENT AGAR MEDIUM

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<td>pH</td>
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APPENDIX 3
COMPOSITION OF ROSE BENGAL CHLORAMPHENICOL AGAR MEDIUM

<table>
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<td>KH₂PO₄</td>
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<td>Agar</td>
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</tr>
<tr>
<td>pH</td>
<td>7.0 ± 0.1</td>
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</table>
APPENDIX 4
DETERMINATION OF α-AMYLASE ACTIVITY
(Bernfeld, 1955)

Principle
The reducing sugars produced by the action of α-amylase react with dinitrosalicylic acid and reduce it to a brown coloured product, nitroaminosalicylic acid.

Reagents
1. Sodium acetate buffer, 0.1 M pH 4.7.
2. 1 per cent starch solution: Prepared a fresh solution by dissolving 1 g starch in 100 ml acetate buffer. Slightly warmed, if necessary.
3. Dinitrosalicylic acid reagent.
   Dissolved by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml of 1 per cent NaOH. Stored at 4°C.
4. 40 per cent Rochelle salt solution (Potassium sodium tartrate).
5. Standard maltose: Dissolved 50 mg maltose in 50 ml distilled water in a standard flask and stored it in a refrigerator.

Procedure
Pipetted out 1 ml of buffered starch solution and 1 ml of enzyme in a test tube. Incubated it at 37°C for 15 minutes. Stopped the reaction by the addition of 2 ml of dinitrosalicylic acid reagent. Heated the solution in a boiling water bath for 5 minutes. While the tubes were warm, added 1 ml of potassium sodium tartrate solution. Then cooled it in running tap water. Made up the volume to 10 ml in all the tubes. Read the absorbance at 560 nm. Terminated the reaction at zero time in the control tubes. Prepared a standard with 50-1000 μg maltose.
APPENDIX 5
BIOCHEMICAL TESTS FOR THE IDENTIFICATION OF BACTERIA
(Bergey's Manual of Determinative Bacteriology,
1984 and Aneja, 1996)

GRAM STAINING

Principle

In 1884, Christian Gram developed this method, to identify Gram positive and Gram negative bacteria. A smear was prepared on the slide, stained with crystal violet and then treated with iodine which acts as a mordant. The Crystal Violet – Iodine complex (CV-I) imparts purple colour to the cells. The intensely stained cells were then washed with ethanol. The ethanol dissolves the lipids and allows the leakage of CV-I complex. Due to the presence of less lipid content in Gram positive bacteria, the lipid is easily dissolved by ethanol. This makes minute pores in the cell wall that are closed by dehydration effect of ethanol. In Gram negative cells, large pores are formed that do not close appropriately, hence dehydration of cell wall protein does not occur completely. This facilitates the release of the unbound crystal violet complex leaving the cell colourless or unstained. If the smear is counter stained with safranin, the Gram negative cells are easily seen due to absorption of safranin and imparting the cells pink colour, while Gram positive cells retain the blue colour of the primary stain.

Reagents

1. Crystal violet
   Solution A : 2 g of crystal violet was dissolved in 20 ml of ethyl alcohol.
   Solution B : Ammonium oxalate - 0.8 g in 80 ml of water.
   Solution A and B were mixed and filtered.

2. Gram’s Iodine
   Potassium Iodide  - 2 g
   Iodine         - 1 g
   Distilled water - 290 ml
   The solution was made up to 300 ml with distilled water.
3. Ethanol 95 per cent
4. Safranin 1 per cent
   Safranin (2.5%) in 95% ethanol - 10 ml
   Distilled water - 90 ml

Procedure
The bacterial smear was heat fixed on a glass slide. Added crystal violet for 30 sec and washed with distilled water for few seconds. The slide was then covered with Grams iodine solution for 30 sec. and washed off with 95 per cent ethyl alcohol. Ethyl alcohol was added drop by drop, until no more colour flew from the smear. The slides were washed with distilled water and drained. Safranin was applied to the smear for 30 sec. (counter stain), washed with distilled water and blotted with absorbent paper. Air dried slide was examined microscopically using oil immersion objective.

MOTILITY TEST
Principle
This technique is meant for microscopic observation of living bacteria. Due to their small size and close refractive index to that of water, they cannot be observed readily under unstained condition.

Procedure
A drop of unknown bacterial strain was placed on the centre of the cover slip with the four corners covered by vaseline. The cavity slide was placed on the cover slip in such a way that the drop does not move or contact the sidewall of the well. The preparation was examined under low and high power objectives of compound microscope.

CATALASE TEST
Principle
During aerobic respiration, microorganisms produce hydrogen peroxide (H₂O₂) which is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen and helps them in their survival. Release of free oxygen gas (O₂) bubbles indicates the presence of catalase.
catalase

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2 \]

Reagents

\[ \text{H}_2\text{O}_2 \ - \ 3 \text{ per cent.} \]

Procedure

The nutrient broth was inoculated with the isolated bacterial culture and incubated at 37°C for 24-48 h. The inoculated tube was held at right angle and 3-4 drops of 3 per cent \( \text{H}_2\text{O}_2 \) was added to the broth. Air bubbles were liberated immediately indicating the presence of catalase.

OXIDASE TEST

Principle

During aerobic respiration, oxidases play a vital role in the operation of electron transport system. Cytochrome oxidase catalyses the oxidation of a reduced cytochrome by molecular oxygen, resulting in the formation of water or hydrogen peroxide.

The test depends on the presence of certain oxidases in bacteria which catalyse the transport of electrons between electron donors in the bacteria and a redox dye tetramethyl - \( p \) - phenylenediamine dihydrochloride. The dye is reduced to a deep purple colour.

Reagents

Tetramethyl - \( p \) - phenylenediamine dihydrochloride.

Procedure

A piece of filter paper was taken in a clean petri dish and 2 or 3 drops of freshly prepared oxidase reagent was added. A loopful of bacterial culture was placed on the filter paper. The appearance of purple colour indicated that the organism was oxidase positive.

INDOLE PRODUCTION TEST

Principle

Tryptophan present in peptone of the culture media is acted upon by the enzyme tryptophanase and converted into indole, pyruvic acid and ammonia.
Indole reacts with Kovac's reagent (para dimethyl aminobenzaldehyde) to produce a cherry red colour product.

\[
\text{Tryptophanase} \quad \text{Tryptophan} \xrightarrow{} \text{Indole + pyruvic acid + } \text{NH}_3
\]

\[
\text{Indole + Kovac's reagent} \xrightarrow{\text{HCl}} \text{Rosindole dye + H}_2\text{O}
\]

(Cherry red compound)

**Reagents**

1. Peptone broth
   - Peptone: 10 g
   - NaCl: 5 g
   - Distilled water: 1000 ml
   - pH: 7

2. Kovac's/ Ehrlich's reagent
   - p-dimethyl aminobenzaldehyde: 5 g
   - Amyl alcohol: 75 ml
   - Concentrated HCl: 25 ml

p-dimethyl aminobenzaldehyde was dissolved in amyl alcohol and concentrated HCl was added.

**Procedure**

Peptone broth was taken in a test tube, sterilized, cooled, inoculated with the isolated bacterial culture and incubated at 37°C for 24 h. After incubation period, 1 ml of Kovac's reagent was added. The tube was shaken gently after an interval of 10-15 minutes and was allowed to stand so that the reagent rises to the top. The tube was examined for the presence of cherry red ring.

**METHYL RED TEST**

**Principle**

Organisms belonging to enterobacteriaceae ferment glucose to pyruvate and produces mixed acids such as acetic acid, lactic acid, succinic acid, formic acid, ethanol, CO\(_2\) and H\(_2\). Because of the abundant acid production, the final pH of the broth drops to less than 4.5 which can be detected by pH indicators.
Reagents

1. MR - VP broth
   - Peptone: 7 g
   - Dextrose: 5 g
   - Potassium phosphate: 5 g
   - Distilled water: 1000 ml
   - pH: 7

2. Methyl red reagent
   - Methyl red: 100 mg
   - Ethanol (95%): 300 ml
   - Distilled water: 200 ml

Methyl red was dissolved in ethanol, distilled water was added and then filtered.

Procedure

MR - VP broth was inoculated with the isolated bacterial culture and incubated at 37°C for 24 h. Five drops of methyl red indicator was added to the tube after incubation period. The change in colour of the broth from yellow to red indicated a positive result, while no change in the colour indicated negative result.

VOGES PROSKAUER TEST

Principle

Glucose undergoes fermentation by a group of bacteria called enterobacteriaceae to produce a non-acidic or neutral end product called acetyl methyl carbinol (CH₃ CO CHO. CH₃) or its reduction product 2, 3 - butylene glycol. Acetyl methyl carbinol undergoes oxidation, on reaction with the guanidine group present in peptone, in the presence of a catalyst α-naphthol and 40 per cent KOH to form a diacetyl compound.

Reagents

1. MR - VP broth
2. Barrit's reagent
**VP Reagent I**

- α-naphthol − 5 g
- Ethanol (absolute) − 95 ml

α-naphthol was dissolved in ethanol with constant stirring.

**VP Reagent II**

- KOH − 40 g
- Creatine − 0.3 g
- Distilled water − 100 ml

Potassium hydroxide was dissolved in 75 ml of distilled water and then creatine was added to the solution and made up to 100 ml with distilled water.

**Procedure**

MR-VP broth was sterilized, inoculated with the isolated bacterial culture and incubated at 37°C for 24 h. Twelve drops of VP reagent I (alpha napthol solution) and 2-3 drops of VP reagent II (40% KOH solution) was added after incubation period. The tube was gently shaken for 30 seconds with the caps off to expose the media to oxygen. The tube was observed for the development of crimson ruby pink colour which indicated positive result while no change in the colour indicated the negative result.

**CITRATE UTILIZATION TEST**

**Principle**

Certain organisms can utilize citrate as sole carbon source for its growth. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and carbon dioxide enzymatically.

The citrate test is performed by inoculating the microorganisms into an organic synthetic medium (Simmons citrate agar) where sodium citrate is the only source of carbon and energy. Bromothymol blue is used as an indicator. When citric acid is metabolized, the carbon dioxide generated combines with sodium and water to form sodium carbonate, an alkaline product, which
changes the colour of the indicator from green to blue and this constitutes a positive test.

\[
\text{CO}_2 + 2\text{Na}^+ + \text{H}_2\text{O} \rightarrow \text{Na}_2\text{CO}_3 \\
(\text{produced during citric acid metabolism}) \quad (\text{alkaline pH})
\]

Bromothymol blue is green when acidic (pH 6.9 and below) and blue when alkaline (pH 7.6 and higher).

**Reagents**

Simmon’s citrate agar medium

- Sodium citrate $- 2 \text{ g}$
- $\text{MgSO}_4$ $- 0.2 \text{ g}$
- $(\text{NH}_4)_2\text{HPO}_4$ $- 0.1 \text{ g}$
- $\text{K}_2\text{HPO}_4$ $- 1 \text{ g}$
- $\text{NaCl}$ $- 5 \text{ g}$
- Bromothymol blue $- 0.08 \text{ g}$
- Agar $- 15 \text{ g}$
- Distilled water $- 1000 \text{ ml}$
- pH $- 6.9$

All the contents were dissolved except phosphates which are to be dissolved separately in 100 ml of water and the volume was made up to 1 litre. The pH was set to 6.9. The medium was poured in the test tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes and the slants were prepared.

**Procedure**

The isolated bacterial culture was streaked onto Simmon’s citrate agar slant. One tube was kept as an uninoculated control for comparison. These tubes were incubated at 37°C for 24-48 h. Following the incubation period, the change in green to deep prussian blue colour in the tubes were noted which indicated positive result. No colour change indicated negative result.
STARCH HYDROLYSIS TEST

Principle

Starch is an insoluble polymer of glucose, which acts as a source of carbon and nitrogen for microorganism, which has an ability to degrade them. Starch degrading microorganism transports the degraded form across the cytoplasmic membrane of the cell. Some bacteria possess the ability to produce amylase that breaks starch into maltose. The amylase is an extra cellular enzyme, which is released from the cells of microorganism.

Reagents

1. Starch agar medium
   - Peptone – 5 g
   - Beef extract – 3 g
   - Starch (soluble) – 2 g
   - Agar – 15 g
   - Distilled water – 1000 ml
   - pH – 7

2. Iodine solution
   - Potassium iodide – 2 g
   - Iodine – 1 g
   - Distilled water – 300 ml

Procedure

Sterile starch agar plate was prepared and the bacterial culture was streaked onto the plate. The plate was incubated at 37°C for 48 h. The surface of the plate was flooded with iodine solution. A clear zone surrounding the organism indicated the positive result. Dark blue colouration of the medium with no clear zone formation indicated the negative result.

GELATIN HYDROLYSIS TEST

Principle

Gelatin is an incomplete protein present as liquid above 25°C and solidified when cooled below 25°C. Hydrolysis (liquefaction) of gelatin is brought about by microbes producing proteolytic exoenzymes known as
gelatinase, which acts on gelatin in the medium. It can be detected by observing liquefaction or testing with a protein precipitating material i.e., flooding the gelatin agar medium with mercuric chloride solution and observing the plates for clearing zone around the line of growth. Liquefaction of gelatin being the commonest proteolytic property is used as an index to determine the proteolytic activity of an organism.

**Reagents**

<table>
<thead>
<tr>
<th>Gelatin agar medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
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<td>Meat extract</td>
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<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Gelatin</td>
</tr>
<tr>
<td>Ferrous chloride</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Distilled water</td>
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<tr>
<td>pH</td>
</tr>
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</table>

All the contents were dissolved in 1000 ml of distilled water and pH was adjusted to 7. The medium was poured into the test tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes.

**Procedure**

Gelatin agar medium was prepared, melted, cooled to 45–50°C, poured into sterile petri dishes and allowed to solidify. Single streak inoculation was made using selected bacterial culture and incubated at 37°C for 4 - 7 days. The surface of the incubated plate was flooded with mercuric chloride solution and allowed to stand for 5 -10 minutes. A clear zone surrounding the organism indicated the positive result. No clear zone formation indicated the negative result.

**UREASE TEST**

**Principle**

Urea is a waste nitrogenous material excreted out by animals. Some bacteria degrade the nitrogen and carbon bond in urea to form carbon dioxide
and ammonia in the presence of a hydrolytic enzyme urease. The carbon
dioxide and ammonia reacts to form ammonium carbonate, an alkaline end
product, which increase the pH of the medium. This can be detected by
incorporating a pH indicator in the medium, which changes the colour during
alkaline conditions.

\[
\text{H}_2\text{N} - \xrightarrow{\text{Urease}} - \text{C} = \text{O} + \text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2
\]

Reagents

Christensen’s urea agar medium

- Peptone - 2 g
- Sodium chloride - 0.2 g
- \(\text{KHPO}_4\) - 0.1 g
- Glucose - 1 g
- Phenol red (0.2% solution) - 6 ml
- Agar - 15 g
- Distilled water - 1000 ml
- pH - 7

All the ingredients were dissolved by heating, adjusted the pH to 7 and
autoclaved at 121°C for 15 minutes and cooled to 50°C.

Urea (20% aqueous solution) -100 ml

Urea was added to the sterile molten base, cooled, mixed well and taken
in a sterile test tube and allowed to solidify in a slanting position to form slants.

Procedure

Sterile Christensen’s urea agar tube prepared was inoculated with the
isolated bacterial culture and incubated at 37°C for 24-48 h. The slant was
examined for deep pink colour which indicated the presence of urease and
yellow colour indicated the negative result.
NITRATE REDUCTION TEST

Principle

Certain bacteria use nitrates in place of oxygen as an external terminal electron acceptor. Nitrate can be easily reduced to nitrite by nitrate reductase. In case of aerobic bacteria, oxygen is first used to prevent nitrate reduction and then utilize nitrate. The nitrite may further give rise to nitrogen, ammonia and nitrogen oxide.

\[
\text{Nitrate reductase} \quad \text{NO}_3^- + 2e^- + 2H^+ \rightarrow \text{NO}_2^- + H_2O
\]

Reagents

1. Nitrate broth
   - KNO₃ (nitrite free) - 0.2 g
   - Peptone - 5 g
   - Distilled water - 100 ml

2. Test reagent
   - Solution A: Dissolved 8 g of sulphanilic acid in 1 litre acetic acid
   - Solution B: Dissolved 5 g of alpha naphtholamine in 1 litre acetic acid
   Equal volumes of solution A and solution B were mixed (immediately before use).

Procedure

To 5 ml of sterile nitrate broth the isolated bacterial culture was inoculated and incubated at 37°C for 96 h. After incubation, 0.1 ml of test reagent was added to the culture tube. Development of red colour in the tube indicated the presence of nitrates and absence of red colour indicated a negative result.

CARBOHYDRATE FERMENTATION TEST

Principle

Microbes use carbohydrates as energy source depending on their enzyme components. Major products of carbohydrate catabolism are lactic, formic or acetic acid with the production of H₂ or CO₂ as gas. Fermentative
degradation is carried out in a fermentation broth containing pH indicator and Durham's tube for gas collection.

Reagents

Carbohydrate fermentation medium

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<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>*Carbohydrate</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.018 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
</tbody>
</table>

(* a specific carbohydrate namely glucose, maltose, lactose, sucrose, mannitol and xylose was added separately).

Procedure

Four types of sugar fermentation broth containing Durham's tube were inoculated with the isolated bacterial strain and one uninoculated tube of each fermentation broth was kept as a comparative control. The tubes were incubated at 37°C for 24 – 48 h and after incubation period, the tubes showing acid and gas formations were recorded. Acid production is indicated by change in colour of the medium from red to yellow and the accumulation of gas in Durham's tube indicated the positive result.

APPENDIX 6
LACTO PHENOL COTTON BLUE STAINING
(Cappuccino and Sherman, 1999)

Principle

Lactophenol cotton blue is a stain commonly used for making semi permanent microscopic preparation of fungi. It stains the fungal cytoplasm and provides a light blue background against which the walls of the hyphae can readily be seen. It contains four constituents, phenol which serves as a fungicide, lactic acid which acts as a cleaning agent, cotton blue which stains
the cytoplasm of the fungus and glycerol which gives a semi permanent preparation.

Requirements
Reagents

20 ml of lactic acid and 40 ml of glycerol was dissolved in distilled water. 20 g of phenol crystals were added to the above contents and heated to dissolve thoroughly. Two ml of 1per cent aqueous cotton blue was added and mixed well.

Procedure

A drop of lacto phenol cotton blue was placed on a clean glass slide. Using a flamed needle, a small amount of fungal culture (5 - 7 days old) with spores was added into the stain. The mold was mixed gently with the stain using two teasing needle and a cover slip was placed on the preparation and examined under microscope.

APPENDIX 7

COMPOSITION OF POTATO DEXTROSE BROTH

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>COMPOSITION</th>
<th>g/L</th>
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<tr>
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<tr>
<td>2</td>
<td>Dextrose</td>
<td>20</td>
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APPENDIX 8

ESTIMATION OF REDUCING SUGAR BY DINITROSALICYLIC ACID METHOD
(Miller, 1959)

Principle

The reducing sugars produced by the action of α-amylase react with dinitrosalicylic acid and reduce it to a brown coloured product, nitroaminosalicylic acid.

Reagents

1. Dinitrosalicylic acid reagent :Dissolved by stirring 1g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml of 1 per cent NaOH. Stored at 4°C.
2. 40 per cent Rochelle salt solution (Potassium sodium tartarate)
3. Standard maltose: Dissolved 50 mg maltose in 50 ml distilled water in a standard flask and stored it in a refrigerator.

Procedure

Pipetted out 0.5 to 3 ml of the extract in test tubes and equalized the volume to 3 ml with water in all the tubes. Added 3 ml of dinitrosalicylic acid reagent to all the test tubes. Heated the solution in a boiling water bath for 5 minutes. While the tubes were warm, added 1 ml of 40 per cent potassium sodium tartarate solution. Then cooled it in running tap water. Made up the volume to 10 ml in all the tubes. Read the absorbance at 510 nm. Prepared a standard with 50-500 µg maltose.

APPENDIX 9
PROFORMA FOR VISUAL EVALUATION OF THE DESIZED FABRICS

<table>
<thead>
<tr>
<th>Fabrics</th>
<th>General appearance</th>
<th>Texture</th>
<th>Stiffness</th>
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</table>

G – Good  F – Fair  P – Poor  R – Rough  S – Soft  C – Course
L – Low  M – Medium  H – High

CM - Conventional method  CE - Commercial enzymatic method
BA - Bacterial amylase treatment  FA - Fungal amylase treatment
## APPENDIX 10
### PROFORMA FOR VISUAL EVALUATION OF THE DYED FABRICS

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<thead>
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CM  - Conventional method
CE  - Commercial enzymatic method
BA  - Bacterial amylase treatment
FA  - Fungal amylase treatment
APPENDIX 11
ORIGINAL AND DESIZED FABRICS

GHADA

Original  CM  CE  BA  FA

KHADI COTTON

Original  CM  CE  BA  FA

LONG CLOTH

Original  CM  CE  BA  FA

VOILE

Original  CM  CE  BA  FA

POLY COTTON

Original  CM  CE  BA  FA
CM-Conventional Method  CE-Commercial enzymatic method  BA-Bacterial amylase treatment  FA-Fungal amylase treatment
APPENDIX 12
DYED FABRICS

GHADA
CM
CE
BA
FA

KHADI COTTON
CM
CE
BA
FA

LONG CLOTH
CM
CE
BA
FA

VOILE
CM
CE
BA
FA

POLY COTTON
CM
CE
BA
FA
CM-Convention Method  CE-Commercial enzymatic method  BA-Bacterial amylase treatment  FA-Fungal amylase treatment