Materials and Methods
1. Isolation of Microorganisms:

Bacterial, fungal, actinomycetes and yeast cultures were isolated from soil of North-Western Himalayas (J&K, Himachal Pradesh). For isolating bacteria 1:1000,000 soil suspension was used. Nutrient agar (M-1) was used for the purpose of isolation. 0.1 ml suspension was poured in each petridish and the suspension was spread with the help of sterilized spreader for uniform distribution of soil particles throughout the plate. The suspension was allowed to dry for some time and the plates were incubated at 30°C. When the colonies appeared in the plates, they were incubated to freshly prepared test tubes containing nutrient agar slants for the purpose of purification and identification.

Sterol decomposing microbial strains consisting of bacteria, actinomycetes and yeasts, were isolated using enrichment technique.

Sources for isolation were soil from J&K, (Ladakh) and Himachal Pradesh (Manikaran) compost and animal faeces. 25 ml medium (M-2) was dispensed in 250 ml Erlenmeyer flask and inoculated with 0.250 gm soil. The samples were inoculated in medium (M-2) in triplicate sets, one set containing 0.1% cholesterol - source of carbon, second set containing 0.1% sitosterol and the other containing 0.1% stigmasterol as source of carbon. Samples were incubated at 30°C under shaking (220 rpm), two subtransfers of the above cultures were made after 15 days and 7 days respectively, followed by streaking on nutrient agar plates with different dilutions of the cultures for the development of pure and well isolated colonies.
Purification of Micro-organisms :-

Pure culture were isolated by streaking samples of enrichment on plates containing the same media solidified with 2% agar. As soon as the colonies appeared on plates, they were restreaked and finally pure colonies were transferred in agar slants and incubated.

The colonies were streaked to isolate single cell cultures. The cultures were preserved at 4°C on nutrient agar after being tested for purity and ability to utilize the sterol as source of carbon.

Isolation of Fungi :-

i) Dilution plate method :- 1 gm of soil was weighed on sterilized filter paper and was transferred to a 150 ml flask containing 100 ml of sterilized water. Flask was thoroughly shaken to mix the contents and 10 ml of this suspension was pipetted out with sterilized pipette in a 150 ml flask containing 90 ml of sterilized water. Finally 10 ml of the dilution was taken and mixed in 20 ml of the sterilized water thus making a dilution of 1:2000. 1 ml of soil suspension of 1:2000 dilution was plated in sterilized petridishes containing medium (M-5)

ii) Soil Plate Method: (Warcup, 1950) : A small quantity of soil was taken with the help of a sterilized arrow headed needle and was placed in the centre of a sterilized petridish. A drop of sterilized water was added and then soil was spread in the plate with the help of a glass rod 10-12 ml of medium(M-5) was poured in each petridish and plates were gently rotated for uniform distribution of soil particles throughout the plate. The plates were allowed to solidify and then incubated at 26°C ± 2°C.
2. **Microscopy**

An Olympus CH-30 Phase contrast microscope was used for all microscopic observations. The organisms were grown in various media and the bacterial cultures were observed after 24 hours under oil immersion after gram staining.

**Gram Staining Reagents**

**Crystal Violet**

Solution A: Crystal violet : 2.0 gm  
(90% dye content)

Ethyl alcohol (95%) : 20.0 ml

Solution B: Ammonium oxalate : 0.8 gm  
Distilled water : 80.0 ml

Note: Mix Solution A and B

**Gram's Iodine**

Iodine : 1.0 gm  
Potassium iodide : 2.0 gm  
Distilled water : 300.0 ml

Ethyl alcohol (95%):

Ethyl alcohol (100%) : 95.0 ml  
Distilled water : 5.0 ml

**Safranin**

Safranin O : 0.25 ml  
Ethyl alcohol (95%) : 10.0 ml  
Distilled water : 100.0 ml
3. Media Composition:

Nutrient Agar Media: (M-1)

- Peptone : 10 gm
- Beef Extract : 5 gm
- NaCl : 5 gm
- Agar : 20 gm
- Distilled water : 1000 ml
- pH : 7.2

Isolation Media: (M-2)

- $\text{NH}_4\text{NO}_3$ : 1.0 gm
- $\text{K}_2\text{HPO}_4$ : 0.25 gm
- $\text{MgSO}_4\cdot\text{7H}_2\text{O}$ : 0.25 gm
- NaCl : 0.005 gm
- Tween 80 : 1gm
- Cholesterol : 1 gm
- Distilled water : 1000 ml
- pH : 7.2

Yeast extract tryptone Medium: (M-3)

- Yeast Extract : 5 gm
- Tryptone : 10 gm
- NaCl : 5 gm
- Tween 80 : 1 gm
- Distilled water : 1000 ml
- pH : 7.2

Sabouraud’s Dextrose Agar Medium: (M-4)

- $\text{KH}_2\text{PO}_4$ : 1.0 gm
- $\text{MgSO}_4\cdot\text{7H}_2\text{O}$ : 0.5 gm
- Peptone : 5.0 gm
- Dextrose : 10.0 gm
Agar : 20.0 gm
Distilled water : 1000 ml

Potato Dextrose Agar Medium : (M-5)

Potatoes : 300 gm
Glucose : 20 gm
Agar : 20 gm
Rose Bengal : 1:30,000
Streptomycin : 30 µg/ml.
Distilled water : 1000 ml

Boiled, finely diced potatoes in 500 ml of water until thoroughly cooked, further filtered through cheese cloth and added water to filtrate of 1.0 lt. The agar was dissolved in the filtrate by heating and glucose was added prior to the sterilization.

Czapek’s Dox Medium : (M-6)
(0.4% yeast extract supplement)

Sucrose : 30 gm
K₂HPO₄ : 1 gm
NaNO₃ : 3 gm
MgSO₄·7H₂O : 0.5 gm
FeSO₄·7H₂O : 0.01 gm
Distilled water : 1000 ml

Yeast Malt Extract Glucose : (M-7)

Yeast extract : 4.0 gm
Malt extract : 10.0 gm
Glucose : 4.0 gm
Distilled water : 1000 ml
The biochemical tests were performed as described in laboratory Manual of Microbiology (Cappuccino et al 1996).

Media used for biochemical test :-

Trypticase nitrate broth medium : (M-8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Reagents :

Solution A : Sulfanilic Acid : 2 - 3 drops
Solution B : Alphanaphthylamine : 2 - 3 drops

Note : Zinc powder to be added for reducing nitrates to nitrite.

Trypticase Soya Agar medium : (M-9)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase</td>
<td>15.0 gm</td>
</tr>
<tr>
<td>Phytane</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Urea Broth Medium : (M - 10)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea broth concentrate</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>(filter-sterilized solution)</td>
<td></td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>90.0 ml</td>
</tr>
</tbody>
</table>

Note : Aseptically add the urea broth concentrate to the
sterilized and cooled distilled water. Under aseptic conditions, disperse 3-ml amounts into sterile tubes.

Starch Agar medium: (M-11)

Peptone : 5.0 gm
Beef extract : 3.0 gm
Starch : 2.0 gm
Agar : 20.0 gm
Distilled water : 1000 ml
pH : 7.0

Triple - Sugar Iron medium: (M-12)

Peptone : 10.0 gm
Tryptone : 10.0 gm
Yeast extract : 3.0 gm
Beef extract : 3.0 gm
Lactose : 10.0 gm
Sucrose : 10.0 gm
Glucose : 1.0 gm
FeSO₄ : 0.2 gm
Sodium Thiosulphate : 0.3 gm
Sodium chloride : 5.0 gm
Phenol Red : 0.024 gm
Agar : 20.0 gm
Distilled water : 1000 ml
pH : 7.4
Methodology adopted for performing biochemical tests:

Nitrate Reduction Test:

\[ \text{NO}_3^- \xrightarrow{\text{Nitrate Reductase}} \text{NO}_2^- \] (Red colour on addition of solution A and B)

i) Using sterile technique, inoculated each experimental organism into its appropriately labelled tube containing nitrate broth medium, by means of a loop inoculation. The last tube will serve as control.

ii) Incubate cultures for 24 to 48 hrs at 37°C.

Observations: On addition of reagents solution A and solution B a cherry red coloration indicates :-

i) Nitrate not reduced by micro-organisms.

ii) Presence of potent nitrate reductase which converts nitrates beyond nitrites to ammonia or even molecular nitrogen. Zinc powder on addition to colourless cultures results in development of red color confirming nitrates not reduced to nitrites. In absence of colour formation, confirms nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas.

Urease Test:

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{C} \\
\text{O} \\
\end{array} \xrightarrow{\text{Urease}} \xrightarrow{+ 2\text{H}_2\text{O}} \begin{array}{c}
\text{NH}_2 \\
\text{CO}_2 + \text{H}_2\text{O} + 2\text{NH}_3 \\
\end{array}
\]

i) Using sterile technique, inoculated each experimental organism into its appropriately labelled tube containing urea broth by
means of a loop inoculation. The last tube will serve as control.

ii) Incubate cultures for 24 to 48 hrs at 37°C.

Observations: Urea broth medium containing phenol red as pH indicator. As substrate urea is split into its products, ammonia creates an alkaline environment that causes the phenol red to turn to a deep pink, indicating presence of urease enzyme.

Starch hydrolysis

\[
\text{Starch} \xrightarrow{\text{Amylase}} \text{Dextrins} \xrightarrow{} \text{Maltose}
\]

i) Using sterile technique, inoculated experimental organism into its appropriately labelled plate containing starch agar medium, by means of streak method. The last plate serves as control.

ii) Incubate cultures for 24 to 48 hrs at 37°C.

Observations: Starch agar medium containing starch as substrate on being flooded with grams iodine will impart blue-black color indicating absence of amylase. If the starch has been hydrolysed, a clear zone of hydrolysis will surround the growth of the organism indicating presence of amylase.

Oxidase test:

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Cytochrome Oxidase}} \text{H}_2\text{O} \quad \text{(Oxidation of a reduced cytochrome by molecular oxygen O}_2\text{)}
\]

i) Using sterile technique, making a single line inoculation of the experimental organism on the agar surface into its
appropriately labelled plate. The last plate will serve as control.

ii) Incubate the plates in inverted position for 24 to 48 hrs at 37°C.

Observation: Test-reagent p-amino dimethylaniline oxalate added on the colonies grown on plate medium. This light pink reagent serves as an artificial substrate donating electrons and thereby becoming oxidised to a blackish compound in the presence of oxidase and free oxygen. No color change in case of absence of oxidase.

Catalase Test:

\[
2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \uparrow
\]

i) Using sterile technique, inoculated experimental organism into its appropriately labelled tube by means of a streak inoculation. The last tube will serve as control.

ii) Incubate the cultures for 24 to 48 hrs at 37°C.

Observations: Catalase production can be determined by adding the substrate H₂O₂ to appropriately incubated slant cultures. Bubbles of free oxygen gas (O₂) indicate presence of catalase.

Acid formation from Glucose:

i) Using sterile technique, inoculated experimental organism into a medium containing 0.1% glucose as substrate. The last tube will serve as control.

ii) Incubate for 18 to 24 hrs at 37°C.
Observations: Since the substrate is present in minimal concentration, the small amount of acid produced (yellow butt) is oxidised rapidly on the slant surface. Peptone in medium are also used in the production of alkali. In the butt the acid reaction is maintained because of reduced oxygen tension and slower growth of the organisms. Yellow butt indicates acid formation.

4. Methodology for isolating genomic DNA of RRL - 450:
RRL - 450 was grown in yeast extract tryptone medium for 16 hr. Cell pellets were harvested by centrifuging at 10,000×g for 5 minutes. Supernatant was discarded and cells were resuspended in 50 µl lysozyme (50 µg/ml). The micro centrifuge tube (1.5 ml) were further incubated at 37°C for 15 minutes. 450 µl of GES reagent was added and the contents were vortexed vigorously. 250 µl ice cold ammonium acetate (7.5 M) was added and the vials were kept on ice for 15 minutes. 500 µl of chloroform reagent was added and spinned at 10000 × g for 5 minutes in order to separate the phases. Supernatant was transferred to fresh microcentrifuge tube and the above step was repeated three times. One-tenth volume of ice cold ammonium acetate and twice the volume of ethanol was added. DNA was pelleted down at 10,000 × g for 20 minutes, washed with 250 µl 70% ethanol (u/v) and dissolved in 30 - 50 µl TE (pH 8.0).

DNA samples were visualised on 0.7% (w/v) agarose gel
(prepared in TAE buffer) on gel documentation system
(Pharmacia Biotech, USA).

Preparation of Stock Solutions:

50 X TAE:
- Tris - Buffer : 242 gm
- Glacial acetic acid : 57.2 ml
- 0.5 M EDTA (pH 8.0) : 100 ml
- Distilled Water : 1000 ml

0.5 M EDTA:
- EDTA : 18.61 gm
- NaOH : 2 gm
- Distilled Water : 100 ml

Ethidium Bromide:
- Ethidium bromide : 25 mg
- Distilled Water : 5 ml

6X Loading Dye:
- Sucrose : 40%
- Bromophenol Blue : 0.25%

TE:
- Tris-chloride (pH 8.0) : 10 mM
- EDTA (pH 8.0) : 1 mM

GES Reagent:
- Guanidine thiocyanate : 60 gm (5M)
- EDTA : 3.7 gm (0.1 M)
- H₂O : 20 ml
Heat in a 65°C water bath until mixed. Add 1.7 ml of 30% sarcosyl, cool, make up to 100 ml, mix well, filter through a 0.45 μm filter. Store at room temperature in dark.

5. **Cultivation Conditions**:

The bacterial cultures were maintained on nutrient agar medium (M-1) and transferred every month. Incubation temperature was 30° ± 1°C or as otherwise stated. The cultures were activated by repeated transfer before use. Similarly actinomycete cultures were maintained on potato dextrose agar medium (M-5) at 30° ± 1°C. Several media were tried for optimising the growth conditions of producer strain, an isolate (actinomycete) designated as RRL-450. Time growth studies were done to determine the exponential phase of RRL-450 by growing fresh culture slant in (M-3) yeast - extract tryptone medium on rotary shaker. Samples (1 ml.) were taken after 2 hrs. interval starting from 0 hr to which 9 ml distilled water was added and optical density was measured on Perkin Elmer 377 IR spectrophotometer at wave length 600 nm.

Inoculum Preparation:

Weak growth of *RRL-450* sp. in yeast - extract tryptone medium (M-3) was observed, when inoculum was prepared after scratching potato dextrose agar slant whereas the inoculum prepared by macerating the culture with help of glass beads in presence of Tween 80 when added to yeast extract tryptone medium containing sterol gave moderate growth.
6. **Biotransformation Studies**

Shake flask experiments were conducted in 250 ml Erlenmeyer flask with 25 ml medium, yeast-extract tryptone medium (M-3) containing Tween 80 as surfactant and 0.5 mg/ml cholesterol as substrate dissolved in 1 ml chloroform was autoclaved at 15 lbs for 15 minutes. 8% inoculum grown in M-3 medium was used in all experiments. The flask culture were incubated on rotary shaker having 220 rpm with an eccentric throw of 2.5 cm at 30º ± 1ºC. 48 hr old culture was used as inoculum for inoculating production medium (M-3) and the flasks were further incubated on shaker. The production medium containing 24 hr old culture was treated with enzyme inhibitor (α,α - dipyridyl) dissolved in 1 ml alcohol, was added in the medium. The flasks were kept on shaker for further incubation for 3 - 5 days at 30º ± 1ºC. Culture controls consisted of fermentation blanks in which, organisms were grown under identical conditions without substrate. Substrate control consisted of sterilized medium containing, the same amount of substrate treated with enzyme inhibitor incubated under the same conditions. The samples of reaction mixture were withdrawn periodically for detection and estimation. 250 ml Erlenmeyer flask containing 25 ml production medium (M-3), M-7 for actinomycetes, M-1 for bacteria and M-5, M-6 for fungi was inoculated with 8% of 24 hr old culture and incubated on shaker at 30º ± 1ºC. The medium was treated with enzyme inhibitor (α,α - dipyridyl) dissolved in alcohol. The enzymatic reaction mixture was further incubated on rotary shaker at
220 rpm with an eccentric throw of 2.5 cms. The samples of reaction mixture were withdrawn periodically for detection and estimation.

Biotransformation of standard compounds procured from Sigma Chemicals (U.S.A) and Difco (Bombay) was done using isolate RRL - 450. 250 ml Erlenmeyer flask containing 25 ml of production medium (M-3) was inoculated with 8% of 24 hr old culture and incubated at 30° ± 1°C on a rotary shaker at 220 rpm. After a period of 24 hours, of incubation enzyme inhibitor (α, α'-dipyridyl) dissolved in alcohol was added in each flask. Incubation of the reaction mixture was continued for 5 days. Samples from treated microbial broth were withdrawn at regular intervals and extracted with ethyl acetate thrice and evaporated to dryness on Heidolph’s rotary evaporator under reduced pressure.

Conversion of substrate into AD or ADD was estimated as follows:

Conversion (%) = [(weight of AD(D)/weight of substrate) × (MW substrate/MW AD(D))] × 100

7. **Estimation of Biomass:**

The biomass from the culture broth was separated by centrifugation at 10,000 xg. It was washed with water, recentrifuged and transferred with a little water to a pre-weighed dish. Dry weight of the biomass was determined after drying in a vacuum oven at 60°C.
FLOW CHART SHOWING DIFFERENT STEPS IN BIOTRANSFORMATION

1. Culture from slant
2. Transfer in flasks containing liquid medium with substrate
3. Incubation on rotary shakers at 220 rpm for 24/48 hrs
4. Transferred in flasks containing production medium
5. Incubation for 24 hrs.
6. Enzyme inhibitor (2, 2-dipridyl) was added in 24 hr. grown culture
7. Downstream processing
8. Sampling at hrs.
9. Extractions were done with equal volume of ethyl acetate
10. 24, 48, 72, 96, 120
11. Analysis
12. TLC, Mass spectrometry, NMR, IR, HPLC
8. Qualitative Estimations of Biotransformation Products by TLC:

5 ml of the fermentation broth was extracted with equal amount of ethyl acetate and centrifuged. Supernatant extract (10 μl) was applied on a silica gel G plate by a micro pipette. The plate, developed with benzene : ethyl acetate (4:1) was heated at 115°C for 10-15 minutes after being sprayed with 1% ceric ammonium sulfate in concentrated sulphuric acid.

Quantitative Analysis:

HPLC (High Performance Liquid Chromatography):

HPLC analysis was performed on HPLC system consisted of an Agilent 1100 instrument equipped with a binary pump, an autosampler, an automatic electronic degasser, an automatic thermostatic column oven, a diode array detector, and a computer with HP chemstation software for analysis. The LC separations were achieved using Silica Si₆₀ (5μm, 125-4μm) Merck column. The mobile phase consisted of isopropyl alcohol : hexane (2:3) delivered at a flow rate of 0.5 ml/min. The samples were analysed at 30°C to achieve efficient separation. The UV chromatograms were recorded at 210 nm and 240 nm. Fig. 2 shows the LC (UV) DAD chromatogram at 210 (B) and 240 (C) nm of three reference compounds viz. cholesterol, AD and ADD which were eluted at retention times of 3.8, 7.5 and 10.4 min. respectively. During LC-MS analysis UV chromatograms were acquired at 210 and 240 nm as cholesterol did not exhibit any absorbance at 240 nm like AD and ADD.
Assay Procedure:
For quantification of cholesterol, ADD and AD in various fermentation broths, dried residues obtained after extraction of fermentation broths were dissolved in 1:1 solvent mixture of isopropyl alcohol and hexane followed by filtration through 0.2 μm filters before injection into the HPLC system.
Calibration curves (fig) were established by injecting six concentrations each of these compounds in the concentration range of 100 - 500 pg on column. Calibration curves were obtained for all the three reference compounds where R²= curve co-efficients of 0.99660, 0.99979, 0.99863 were obtained respectively for cholesterol, AD and ADD respectively (n=6).
The method was validated by analysing fermentation samples (n=6 each) fortified with cholesterol, AD and ADD at two concentration levels viz 0.05 μg/g and 0.5μg/g. The mean recoveries ranged from 90 to 94 (CV=4.56 to 9.78%) percent for the samples fortified at concentration level, 0.05 μg/g and 92 to 98% for samples fortified at concentration level 0.5 μg/g (CV = 3.36 to 6.57%)

9. Isolation of Biomodified Products [androsta-1, 4-diene-3, 17-dione (ADD), androst. 4 - ene -3, 17-dione (AD) and spectral analysis]:
Fermentation broth initially containing 0.5mg/ml of cholesterol was extracted thrice with equal volume of ethyl acetate. The extracts were combined, dried over anhydrous sodium
sulphate and distilled under reduced pressure after filtration to yield crude product. The dry extract was chromatographed on alumina (50×2.5cm column, 100 g alumina) and eluted with benzene containing increasing quantities of ethyl acetate (0.1 to 1.0%). 60 fractions, each approximately 20 ml, were collected and scanned by TLC for their components.

Spectral analysis:
The pure compounds isolated by column chromatography were identified by melting points, mixed melting points, co-TLC with authentic samples and from their UV, IR and NMR spectra.

Ultra-violet spectroscopy: UV spectra (in ethanol) were measured with a Pye-Unichem - SP8 - 100 spectrophotometer.

Infra-red spectroscopy: IR spectra (as KBr Pellets) were recorded with Perkin Elmer 377 IR spectrophotometer.

NMR (Nuclear magnetic reasonance) spectroscopy: NMR spectra (taken in CDCl₃ with TMS as internal standard) was recorded with a Varian T60A instrument.
Calibration Curves
Fig.

IR of androsta 1,4-diene-3, 17-dione (ADD) and androst 4-ene, 3, 17-dione (AD)
Fig.

H¹NMR of androsta 1-4-diene-3, 17-dione (ADD) and androst 4-ene, 3, 17-dione (AD)
Figure 8: LC-APCI-MS of Cholesterol

Figure 9: LC-APCI-MS of AD

Figure 10: LC-APCI-MS of ADD

Total Ion Current Trace (A) of a fermentation broth sample and LC-UV-DAD chromatogram of the same at 210 (B) and 240 nm (C) where in the presence of Cholesterol, AD and ADD have been observed.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Chemicals/Reagents</th>
<th>Product Code</th>
<th>Obtained From (Company name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beef Extract</td>
<td>21705</td>
<td>Qualigens</td>
</tr>
<tr>
<td>2</td>
<td>Malt Extract</td>
<td>RM 004</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>3</td>
<td>Magnesium Sulphate (MgSO₄.7H₂O)</td>
<td>RM 684</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>4</td>
<td>Yeast Extract</td>
<td>RM 027</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>5</td>
<td>Glucose</td>
<td>RM 016</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>6</td>
<td>NaCl</td>
<td>Art 5819</td>
<td>Loba Chemic</td>
</tr>
<tr>
<td>7</td>
<td>Sucrose</td>
<td>RM 134</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>8</td>
<td>K₂HPO₄</td>
<td>RM 168</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>9</td>
<td>NaNO₃</td>
<td>RM 1722</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>10</td>
<td>Iodine</td>
<td>39568</td>
<td>Sarabhai M.Chemicals</td>
</tr>
<tr>
<td>11</td>
<td>Potassium iodine</td>
<td>RM 1086</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>12</td>
<td>Safranin</td>
<td>RM 1315</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>13</td>
<td>Peptone</td>
<td>RM 667</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>14</td>
<td>NH₄NO₃</td>
<td>RM 5657</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>15</td>
<td>Cholesterol</td>
<td>034811</td>
<td>Sisco Laboratories Ltd.</td>
</tr>
<tr>
<td>16</td>
<td>β-sitosterol</td>
<td>C.A.S. 83-46-5</td>
<td>Acros Organics Ltd.</td>
</tr>
<tr>
<td>17</td>
<td>Stigmasterol</td>
<td>S 440-9</td>
<td>Aldrich Chemical Co. Ltd.</td>
</tr>
<tr>
<td>18</td>
<td>α,α – Dipyridyl</td>
<td>117501000</td>
<td>Acros Organics Ltd.</td>
</tr>
<tr>
<td>19</td>
<td>1,4 - Androsta-diene-3,17-dione</td>
<td>A-7505</td>
<td>Sigma Aldrich Ltd.</td>
</tr>
<tr>
<td>20</td>
<td>Androst-4-ene-3,17-dione</td>
<td>A9630</td>
<td>Sigma Aldrich Ltd.</td>
</tr>
<tr>
<td>21</td>
<td>Tris - acetate</td>
<td>RM 6292</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>22</td>
<td>Tris - Buffer</td>
<td>Art 6390</td>
<td>Loba Chemic</td>
</tr>
<tr>
<td>23</td>
<td>Glacial Acetic acid</td>
<td>39704</td>
<td>Sarabhai M.Chemicals</td>
</tr>
<tr>
<td>24</td>
<td>Ethylene diamine tetra-acetic acid, disodium salt E.D.T.A, disodium salt.</td>
<td>RM 1370</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>25</td>
<td>Ethidium Bromide</td>
<td>C.A.S. 1239-45-8</td>
<td>Acros Organics Ltd.</td>
</tr>
<tr>
<td>26</td>
<td>Bromophenol Blue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Guanidine thiocyanat</td>
<td>G 6639</td>
<td>Sigma Aldrich Ltd.</td>
</tr>
<tr>
<td>28</td>
<td>Sodium dodecyle sulphate</td>
<td>L 3771</td>
<td>Sigma Aldrich Ltd.</td>
</tr>
<tr>
<td>29</td>
<td>Lysozyme</td>
<td>L - 6876</td>
<td>Sigma Aldrich Ltd.</td>
</tr>
<tr>
<td>30</td>
<td>8-hydroxy quinoline</td>
<td>RM 4307</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>31</td>
<td>Glass Beads undrilled</td>
<td>RM 2228</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>32</td>
<td>Crystal violet</td>
<td>RM 114</td>
<td>Hi-media Laboratories Ltd.</td>
</tr>
<tr>
<td>33</td>
<td>Tryptone</td>
<td>Art 6405</td>
<td>Loba Chemic</td>
</tr>
</tbody>
</table>