3.1 Chemicals and reagents

- **Bacterial Strains**: The LAB strain used in these studies was *Enterococcus faecium* MTCC 5153 (Halami, 2004) and *Lactobacillus acidophilus* isolated from curd.

- **Media**
  - **MRS Media** (Hi Media)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Gms / Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease peptone</td>
<td>10.00</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.00</td>
</tr>
<tr>
<td>Beef extract</td>
<td>10.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.00</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium citrate</td>
<td>2.00</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.00</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.10</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>0.05</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>2.00</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>6.0 ± 0.2</td>
</tr>
</tbody>
</table>

- **Rose Bengal Chloramphenicol Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Gms / Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycological peptone</td>
<td>5.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10.00</td>
</tr>
<tr>
<td>Monopotassium phosphate</td>
<td>1.000</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate</td>
<td>0.500</td>
</tr>
</tbody>
</table>
Rose bengal 0.050
Chloramphenicol 0.100
Agar 15.500
Final pH (at 25°C) 7.2±0.2

- **Chemicals**

Chemicals for media preparation were beef extract, peptone, tryptone, and yeast extract, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, glucose, tween 80, sodium chloride, magnesium sulfate, and manganese sulfate.

Chemicals for analysis include phosphate buffer (pH 6.5), sodium lauroyl sarcosinate (sarcosyl NL-30), CTAB (cetyl trimethyl ammonium bromide), glutaraldehyde, ONPG (Sigma, SRL), bovine serum albumin (BSA), Bradford’s reagent, glucose, galactose, sucrose, fructose, lactose, Benedict’s Reagent and Biuret Reagent, Molisch’s Reagent Iodine solution, Fehling’s reagent A, Fehling’s reagent B, Barfoed’s reagent, Seliwanoff’s reagent, Bial’s reagent, Phenylhydrazine hydrochloride, Sodium acetate, Glacial acetic acid, Glucose, fructose.

### 3.2 Instrumentation

Instruments required for the determination of growth of different organisms under study were located in the Department of Microbiology, Govt. Holkar Science College, Indore (M.P.). The instrument used were UV-Vis spectrophotometer, pH of designed media was adjusted by using digital pH meter. Sterilization of the MRS media was done in autoclave at 121°C for 15-20 minutes, while membrane filtration technique was used for the designed media which were found to be heat sensitive. Culture preservation was done by sub-culturing method and the slants were prepared for short-term use. For more than three months time, the cultures were preserved in the agar stabs which were stored in normal refrigerated conditions.

### 3.3 Collection and selection of different Probiotic organisms

The probiotic bacteria most commonly studied include the members of the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Escherichia coli* and *Enterococcus* strains. In our studies we have selected six probiotic strains namely *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Saccharomyces cerevisiae* and *Escherichia coli*. 
E.coli. These strains are then screened to select most appropriate strain for our studies. For screening we have fixed sixteen characters which are:

- Probiotic property
- Natural presence in fermented food products which are consumable
- Normal constituent of human digestive system
- Ease in isolation and identification
- Growth at wide range of temperature and pH
- Provision to exclude competing bacteria
- Growth on agricultural waste
- Ability to survive in human gastrointestinal tract
- Ability to produce antimicrobial substances
- Genome sequenced
- Sensitivity to common antibiotics
- Aerotolerance
- Organotropic nutrition
- Non pathogenic
- Ability to produce lactic and acetic acid to control intestinal pH
- Important research going on the strain.

3.4 Collection and selection of different waste materials

The waste materials were collected from the locality according to their easy and mass availability. Some points have been fixed for the selection of waste materials. These were as follows:

- **Easy availability.**

The waste should be such that it is easily available in the locality. This point will reduce the transport charges of it which will make the product less expensive.

- **Large volume**
To be used for any industrial production the considered waste material should be present in large amounts.

- **High organic content.**

On the basis of general information available about the waste materials, its organic content is determined and the point is taken into consideration.

- **High nutrient content.**

Based on pre-studies and general information the nutrient content of the waste materials is determined and the point is taken into consideration.

- **Tendency to form suspension.**

Since to be used as media for the growth of microorganisms a liquid suspension is required therefore if the waste material is in solid form it should have the ability to form suspension with the water. It should have components soluble in water.

- **Having problematic decomposition.**

Studies will be more effective and useful if the waste material considered is such that its natural decomposition is problematic, then by giving a suggestive measure for its bulk usage, its problem of decomposition is minimized.

- **Problem in disposal.**

If the waste materials is such that there is a serious problem with respect to its disposal then a suggestion regarding its alternative beneficial use will be effective.

- **Industrial waste material.**

One of the main criteria for the waste materials selection is their easy and bulk availability and both of these conditions can be meet when the waste material is of industrial origin.

This point will further make a possible suggestion regarding the disposal and use of these bulk waste materials.
The main criteria to select waste materials is to monitor the growth of selected organisms on them.

About 20 different waste materials have been analyzed on the basis of above grounds, they are fruit and vegetable peels, agricultural waste, industrial waste (dairy industry, sugar industry, ghee industry), hotel-kitchen waste, house hold kitchen waste etc.

**Preparation of media from fruit and vegetable peels**

- Different fruit peels were collected from different sources in plastic bags.
- 10 gms of each were weighed accurately in different containers.
- The peels were crushed in 50 ml distilled water with the help of pestles and mortar.
- After making a fine paste 50 ml more distilled water were added and the final volume of the solution were adjusted to 100 ml.
- The above solutions were filter sterilized.
- 10 ml of each were dispensed in different sterilized test tubes and labeled accordingly.

**Analysis of growth of the two selected organisms on prepared media.**
Many studies require the quantitative determination of bacterial populations. The two most widely used methods for determining bacterial numbers are the standard, or viable, plate count method and spectrophotometric (turbidimetric) analysis. Although the two methods are somewhat similar in their results but quite distinct from each other. For example, the standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead and alive.

**STANDARD PLATE COUNT**

The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer diluent until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons (too few may not be representative of the sample), and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs). The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., $10^{-4}$ to $10^{10}$) is normally plated because the exact number of bacteria is usually unknown. Greater accuracy is achieved by plating duplicates or triplicates of each dilution.

1. The bottom of six petri plates were 1-6 and four tubes of saline as $10^{-2}$, $10^{-4}$, $10^{-6}$, and $10^{-8}$.
2. Using aseptic technique, the initial dilution was made by transferring 1 ml of bacterial sample to a 99ml sterile saline blank. This is a 1/100 or $10^{-2}$ dilution.
3. The $10^{-2}$ dilution is then shaken by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and break up any clumps.
4. Immediately after the $10^{-2}$ dilution has been shaken, aseptically 1ml is transferred to a second 99ml saline blank. Since this is a $10^{-2}$ dilution, this second blank represents a $10^{-4}$ dilution of the original sample.
5. The $10^{-4}$ dilution was shaken vigorously and 1ml was transferred to the third 99ml blank. This third dilution represents a $10^{-6}$ dilution of the original sample. The process was repeated once more to produce a $10^{-8}$ dilution.
6. The $10^4$ dilution was shaken again and 1.0 ml was aseptically transferred to one petri plate and 0.1 ml to another petri plate. The same was done for the $10^{-6}$ and the $10^{-8}$ dilutions.

7. One molten agar tube from the 48 to 50°C water bath were taken and aseptically poured into 10 petri plates. The agar and sample were immediately mixed by gently moving the plate in a figure-eight motion or a circular motion while it rests on the tabletop. This process was repeated for the remaining five plates.

8. After the pour plates have cooled and the agar has hardened, they were inverted and incubated at 25°C for 48 hours or 37°C for 24 hours.

9. At the end of the incubation period, all of the petri plates containing between 30 and 300 colonies were selected. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC). The colonies on each plate were counted. A Quebec colony counter was used.

10. The number of bacteria (CFU) per milliliter or gram of sample were calculated by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

11. The results were recorded.

TURBIDIMETRY DETERMINATION OF BACTERIAL NUMBERS

Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, the amount of transmitted light decreases as the cell population increases.

The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading, called absorbance or optical density, indirectly reflects the number of bacteria.

This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of $10^7$ cells or greater.
1. The original tube of bacterial culture and four tubes of the sterile MRS broth were kept in a test-tube rack. Each tube of MRS were containing 5 ml of sterile broth. These tubes (tubes 2 to 5) of broth were used to make four serial dilutions of the culture.

2. 5ml of culture was transferred to the first tube of MRS, thoroughly mixing the tube afterwards. Then 5ml from that tube were transferred to the next tube, and so on until the last of the 4 tubes has 5ml added to it. These tubes were ½, 1/4, 1/8, and 1/16 dilutions.

3. These directions were followed for using spectrophotometer.
   a. The wavelength was preset somewhere at 600 nm.
   b. The spectrophotometer were standardized.
   c. Readings were taken by using micro-cuvettes.
   d. The BLANK used to standardize the machine was sterile MRS broth: it is called the BLANK because it has a sample concentration equal to zero. 1ml of the sterile MRS were pipetted into one of the micro-cuvettes and it was placed into the cuvette holder, the cover was closed and read.
   e. 1ml of the original bacterial specimen were pipetted into a second micro-cuvette, and read. After that the micro-cuvette content was discarded into container. Repeat this with the ½, ¼, 1/8, and 1/16 dilutions.
USE OF THE SPECTROPHOTOMETER

Light entering a cloudy solution will be absorbed. A clear solution will allow almost all of the light through. The amount of absorbance can be determined by using a spectrophotometer, which measures what fraction of the light passes through a given solution and indicates on the absorbance display the amount of light absorbed compared to that absorbed by a clear solution. Inside, a light shines through a filter (which can be adjusted by controlling the wavelength of light), then through the sample and onto a light-sensitive phototube. This produces an electrical
current. The absorbance meter measures how much light has been blocked by the sample and thereby prevented from striking the phototube. A clear tube of water or other clear solution is the BLANK and has zero absorbance. The amount of substance in the solution is directly proportional to the absorbance reading. A graph of absorbance vs. concentration will produce a straight line.

3.5 Analysis of the composition of the selected waste materials:

The waste materials supporting the growth was analyzed for their components (nutritive substances), by qualitative and quantitative analysis, for Carbohydrates by Cole’s Method,(Cole,1933) and for Proteins by Biuret Method,(Reigler,1914).

3.5.1 Qualitative analysis of Carbohydrates:

1) Molisch’s Test:

In a test tube, 2 ml of the test solution and 2 drops of α-naphthol solution were added. The tube were carefully inclined and conc. H₂SO₄ was poured dropwise using a dropper, along the sides of the tube. Violet colour at the junction of the two liquids were observed.

2) Fehling’s Test:

In a test tube 2 ml of the test solution and add equal volumes of Fehling A & Fehling B were added and placed it in a boiling water bath for few minutes. When the content of test tube started boiling, they were mixed together and observed for any change in color or precipitate formation. The production of yellow ‘or brownish-red precipitate of cuprous oxide indicated the presence of reducing sugars in the given sample.

3) Benedict’s Test:

To 2 ml of Benedict’s reagent, 5 drops of the test solution were added and mixed well. The test tube were placed in a boiling water bath for 5 minutes after that the solution were cooled and observed for any change in color or precipitate formation. The colour change from blue to green, yellow, orange or red (depending upon the amount of reducing sugar present in the test sample) were observed.
4) Barfoed’s Test:

To 2 mL of the test solution about 2 mL of Barfoed’s reagent were added. Mixed well and boiled for one minute in the water bath. The solution were allowed to stand for a few minutes. Formation of a red precipitate of cuprous oxide in the bottom and along the sides of the test tube immediately, indicated the presence of monosaccharides.

Since Barfoed’s reagent is weakly acidic, it is reduced only by monosaccharides.

5) Seliwanoff’s Test:

To 2 mL of Seliwanoff’s reagent, two drops of test solution were added and the mixture was heated to just boiling. A cherry red condensation product was observed indicating the presence of ketoses in the test sample.

6) Bial’s Test:

To 5 mL of Bial’s reagent 2-3 mL of test solution were added and warmed gently in a hot water bath for 2 minutes. The formation of a bluish green product is indicative of pentoses. Hexoses generally react to form muddy brown products.

7) Iodine Test:

2 drops of iodine solution were added to about 2 ml of the test solution. A blue black colour was observed which is indicative of presence of polysaccharides.

Table 3.1 Qualitative tests used for carbohydrate analysis
<table>
<thead>
<tr>
<th>No</th>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
<th>Reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Molisch's-test</td>
<td>A deep violet coloration is produced at the junction of two layers.</td>
<td>Presence of carbohydrates.</td>
<td>This is due to the formation of an unstable condensation product of beta-naphthol with furfural (produced by the dehydration of the carbohydrate)</td>
</tr>
<tr>
<td>2</td>
<td>Iodine-test</td>
<td>Blue colour is produced at the junction of two layers.</td>
<td>Presence of polysaccharide.</td>
<td>Iodine forms coloured adsorption complexes with polysaccharides</td>
</tr>
<tr>
<td>3</td>
<td>Fehling's test</td>
<td>A red precipitate is formed.</td>
<td>Presence of reducing sugar.</td>
<td>This is due to the formation of cuprous oxide by the reducing action of the sugar.</td>
</tr>
<tr>
<td>4</td>
<td>Benedict's test</td>
<td>Formation of a green, red, or yellow precipitate</td>
<td>Presence of reducing sugars.</td>
<td>If the saccharide is a reducing sugar it will reduce Copper [Cu] (11) ions to Cu(1) oxide, a red precipitate</td>
</tr>
<tr>
<td>5</td>
<td>Barfoed's test</td>
<td>A deep blue colour is formed with a red ppt, settling down at the bottom or sides of the test tube.</td>
<td>Presence of reducing sugars [appearance of a red ppt as a thin film at the bottom of the test tube within 3-5 min. is indicative of reducing mono-</td>
<td>If the saccharide is a reducing sugar it will reduce Cu (11) ions to Cu(1) oxide</td>
</tr>
</tbody>
</table>
### Seliwanoff test

To 3ml of Seliwanoff’s reagent, add 1ml of the test solution, boil in water bath for 2 minutes.

- **A cherry red colored precipitate within 5 minutes is obtained.**
  - **Presence of ketoses**
  - *Sucrose gives a positive ketohexose test*
- **A faint red colour produced.**
  - **Presence of aldoses**

When reacted with Seliwanoff reagent, ketoses react within 2 minutes forming a cherry red condensation product, while aldopentoses react slowly forming the coloured condensation product.

### Bials-test

Add 3ml of Bial’s reagent to 0.2ml of the test solution, heat the solution in a boiling water bath for 2 minutes.

- **A blue-green product.**
  - **Presence of pentoses**
- **A muddy brown to gray product.**
  - **Presence of hexoses.**

The furfurals formed produces condensation products with specific colour.

### 3.5.2 Quantitative estimation of sugar

The use of ferricyanide for the quantitative estimation of sugar was introduced by Hagedorn and Jensen [1923]. Their method has been extended to other sugars by Hanes [1929] and Hulme and Narain [1931]. These methods involve a preliminary heating for 15 minutes and a subsequent back titration. Lane and Eynon [1923] introduced methylene blue as an internal indicator for the detection of the end-point when titrating Fehling's solution with reducing sugars. The indicator is not reduced until the whole of the ferricyanide has been reduced and the end-point, being a change from a blue or violet solution to one that is colourless, is easily determined.
Solutions.

(1) Potassium ferricyanide, 1 %. This should be stored in a dark bottle and kept in a dark cupboard when not in use. I have not determined its stability, but have been unable to detect any change over a period of 6 weeks.

(2) Sodium hydroxide, 2-5 N. This is best prepared by decanting or filtering the clear fluid from some 45 % NaOH that has stood for a few days and diluting down to about 11 %. 10 cc. are titrated with N HCl against methyl red. The bulk is then diluted so that 10 cc. of the NaOH require 25 cc. of the acid.

(3) Methylene blue, 1 % in water. This is conveniently stored in a bottle fitted with a rubber cork, carrying a dropping pipette with a rubber teat.

Apparatus.
100 cc. flasks with rather narrow necks.
Graduated 2 and 5 cc. pipettes.
Burette

Titration.

20 cc. of the ferricyanide and 5 cc. of the NaOH were taken in a 100 cc. flask. The solution were heated to boiling on a wire gauze over a Bunsen flame. The sugar solution were added slowly until the yellow colour has appreciably decreased. Then a small drop of methylene blue were added. Addition of the sugar were continued until the fluid was decolorized.

Calculation of results.

If x be the volume of sugar solution added (in cc.), then the amount of the sugar required to reduce the ferricyanide is:

- glucose, 20.12 + 0.035 x mg;
- maltose anhydride, 26.8 + 0.06x mg.;
- lactose anhydride, 23.6+ 0.1x mg.

Thus if 4 cc. of a glucose solution are required, then

4 cc. contain 20.12 + 0.14 mg. and

100 cc. contain 0.506 g.

Or generally if x cc. are required, then g. per 100 cc. is 2.012/x + 0.035.
3.5.3 Qualitative analysis of Proteins:-

**Biuret Test:**
The Biuret Test positively identifies the presence of proteins (not less than two peptides). The reaction in this test involves the complex formation of the proteins with Cu²⁺ ions in a strongly alkaline solution.

**Procedure:**
- To 2 mL protein solution, 5-6 drops of dilute CuSO₄ (Fehling’s solution A diluted 1/10 with water) were added.
- 3 mL 40% NaOH solution were added.
- Color change was observed.

If the sample to be tested is insoluble in water, then the procedure given below were applied:
- 3 ml acetone and 1.5 ml water measured into a test tube.
- 1 drop of dilute NaOH and a little piece of protein to be tested were added.
- The contents were boiled continuously over a small flame for 2 min and cooled.
- 0.5 ml 40% NaOH and 2 drops of a 1/10 diluted Fehling’s solution A were added.
- Color change was observed.

**Nitroprusside Test:**
The nitroprusside test is specific for cysteine, the only amino acid containing sulfhydryl group (-SH). This group reacts with nitroprusside in the presence of excess ammonia.

**Procedure:**
- 2 ml test solution were taken into the test tube.
- 0.5 ml nitroprusside solution were added and shaked thoroughly.
- 0.5 mL ammonium hydroxide were added.
- Color change was observed.
Sakaguchi Test:
The Sakaguchi reagent is used to test for a certain amino acid and proteins. The amino acid that is detected in this test is arginine. Since arginine has a guanidine group in its side chain, it gives a red color with a-naphthol in the presence of an oxidizing agent like bromine solution.

Procedure:
- 1 mL NaOH and 3 mL arginine solution was mixed and 2 drops of a-naphthol was added.
- The solution were mixed thoroughly and 4-5 drops of bromine solution were added.
- Color change was observed.

Ehrlich Test:
Aromatic amines and many organic compounds (indole and urea) give a colored complex with this test.

Procedure:
- 0.5 ml of the protein solution were taken in a test tube.
- 2 mL Ehrlich reagent were added.
- Color change was observed
- The test were repeated with urea solution.

Hopkin’s Cole Test:
The indole group of tryptophan reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains glyoxylic acid CHOCOOH as an impurity) in the presence of concentrated H2SO4 to give a purple color.

Procedure:
- To a few mL of glacial acetic acid containing glyoxylic acid, 1-2 drops of the protein solution were added.
- 1-2 mL H2SO4 were poured down the side of the sloping test tube to form a layer underneath the acetic acid.
- The development of a purple color at the interface proved a positive reaction.
Millon’s Test:
Millon’s test is specific to phenol containing structures (tyrosine is the only common phenolic amino acid). Millon’s reagent is concentrated HNO₃, in which mercury is dissolved. As a result of the reaction a red precipitate or a red solution is considered as positive test. A yellow precipitate of HgO is NOT a positive reaction but usually indicates that the solution is too alkaline.

Procedure:
- 2 mL protein solution were taken in a test tube and 1-2 drops of Millon’s reagent were added.
- The tube were kept in a boiling water bath for 10 min.
  o A brick red color is a positive reaction.
  o Note that this is a test for phenols, and the ninhydrin test should also be positive if it is to be concluded that the substance is a phenolic amino acid.

Xanthoproteic Test:
Some amino acids contain aromatic groups that are derivatives of benzene. These aromatic groups can undergo reactions that are characteristics of benzene and benzene derivatives. One such reaction is the nitration of a benzene ring with nitric acid. The amino acids that have activated benzene ring can readily undergo nitration. This nitration reaction, in the presence of activated benzene ring, forms yellow product.

Procedure:
- To 2 mL amino acid solution in a boiling test tube, equal volume of concentrated HNO₃ were added.
- The solution were heated over a flame for 2 min and observed for the color.
- Solution were cooled under the tap and run in sufficient 40% NaOH to make the solution strongly alkaline.
- The color of the nitro derivatitive of aromatic nucleus were observed.

3.5.4 Quantitative estimation of proteins
3.5.4.1 Quantitative estimation of proteins Biuret method
Protein molecules are long chains of amino acids joined by peptide bonds. The biuret reagent reacts with any compound containing two or more peptide bonds to give a violet-coloured complex. Any compound containing two carbonyl groups (C=O) linked through either a nitrogen or a carbon atom will give a positive reaction. Therefore this test is not fully specific for proteins. However, the intensity of the reaction is an indication of the number of peptide bonds present in a protein.

The biuret reagent, which is blue in colour, contains a strong solution of sodium or potassium hydroxide (NaOH or KOH) and a smaller amount of dilute copper sulfate solution. The name of the test actually comes from another compound called biuret (H₂NCONHCONH₂), which also gives a positive reaction. The biuret compound itself is not part of the reagent, but just gives the test its name. The reagent changes colour in the presence of proteins or peptides because the amino group (H₂N-) of the protein or peptide chemically combines with the copper (II) ions in the biuret reagent. A purple/violet copper (II) complex indicates a positive result. This reagent changes to pink when combined with short-chain polypeptides. A negative result occurs with free amino acids because there are no peptide bonds present.

The color reaction of protein molecules with cupric ions, known as the Biuret color reaction, has been known since 1878. Since the Riegler publications of 1914, several attempts have been made to stabilize the cupric ions in the alkaline reagent. Kingsley modified the procedure in 1939 and 1942 to include the use of sodium potassium tartrate as a complexing agent. This procedure was later modified by Weichselbaum and Gornall. The present method is based on these modifications.

**Principle**

Protein + Cu²⁺ → Colored Complex

Protein in serum forms a violet colored complex when reacted with cupric ions in an alkaline solution. The intensity of the violet color is proportional to the amount of protein present when compared to a solution with known protein concentration.

**Reagent Content**

Sodium Hydroxide 600mM, Copper Sulfate 12mM, Sodium Potassium Tartrate 32mM, Potassium Iodide 30mM, Non-reactive ingredients.
Procedure (Manual)
1. Test tubes were labeled as "Blank", "Standard", "Sample, etc.
2. 1.0ml of working reagent were pipette out into each tube.
3. 0.02ml (20ul) of standard and sample were added to appropriate tubes and mixed by inversion.
4. Tubes were allowed to stand at room temperature (18-25°C) for 5 minutes.
5. Spectrophotometer were set at 540nm and zero were adjusted with the reagent blank.
6. Absorbance readings of each tube were read and recorded.

Calculation
(Abs. = Absorbance)

\[
\text{Abs. of Unknown} \times \frac{\text{Conc. of standard}}{\text{Abs. of Standard}} = \text{Total Protein (g/dl)}
\]

3.5.4.2 Quantitative estimation of proteins by Lowry’s Method

Reagents Required
1. BSA stock solution (1mg/ml).
2. Analytical reagents:
   (a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)
   (b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepare analytical reagents by mixing 2 ml of (b) with 100 ml of (a)
3. Folin - Ciocalteau reagent solution (1N) Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water)

Principle
The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin-Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml and is probably the most widely
used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

**Procedure**

1. Different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ml.

2. From these different dilutions, 0.2 ml protein solution were pipette out to different test tubes and 2 ml of alkaline copper sulphate reagent (analytical reagent) were added. The solutions were mixed well.

3. This solution was incubated at room temperature for 10 mins.

4. Then 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) were added to each tube and incubated for 30 min. Colorimeter were adjusted to zero with blank and the optical density (measure the absorbance) were taken at 660 nm.

5. Plot of the absorbance against protein concentration were drawn to get a standard calibration curve.

6. The absorbance of unknown sample were checked and the concentration of the unknown sample were determined using the standard curve plotted.

**Table 3.2 Quantitative estimation of proteins by Lowry’s Method**

<table>
<thead>
<tr>
<th>BSA (ml)</th>
<th>Water (ml)</th>
<th>Sample conc. (mg/ml)</th>
<th>Sample vol (ml)</th>
<th>Alk. CuSO₄ (ml)</th>
<th>Lowry reagent (ml)</th>
<th>O.D. 660 nm</th>
</tr>
</thead>
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<tr>
<td>0.25</td>
<td>4.75</td>
<td>0.05</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>4.5</td>
<td>0.1</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.2</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.4</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.6</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.8</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>1.0</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>
3.6 Studies on the characteristics of the probiotics after growth on waste materials:

The probiotics have some peculiar properties (such as β-galactosidase production, Bacteriocin production etc.) which are important for their functions and which can be exploited for beneficial purposes. The studies also involved the determination of these properties on waste materials. This step involved the “ONPG Assay” Craven et al. (1965), and “Bacteriocin assay” methods (Tagg & McGiven, 1971).

3.6.1 ONPG Assay for Beta—galactosidase.

Beta-galactosidase (Lactase) catalyzes the following hydrolysis:

\[
\beta-D-galactoside + H_2O \rightarrow galactose + alcohol
\]

β-Galactosidases are widespread in microorganisms, animals and plants. These enzymes from the Escherichia coli strain K12 has been particularly studied at Anfinsen’s laboratory in connection with genetic experiments on gene regulation of protein synthesis. (Craven, Steers and Anfinsen 1965). The enzyme has been reviewed in detail by Wallenfels and Weil (1972). Lactase may be used as a reagent for determining lactose in blood and other biological fluids.

Another important application is in food processing. Its special interest is its use in the treatment of milk to meet the needs of the large percentage of the world population affected with lactose intolerance.

Industrial applications necessitate the enzyme’s immobilization on which considerable investigation has been reported (Byrne and Johnson 1975; Narinesingh et al. 1975; Paine and Carbonell 1975; Faulsitch et al. 1974; Bunting and Laidler 1972, Lilly 1971, Khare and Gupta 1988, and Park and Hoffman 1990).
β-Galactosidase

\[
\text{β-D-Galactoside} + \text{H}_2\text{O} \rightarrow \text{β-D-Galactose} + \text{R-OH}
\]

β-D-Galactoside            β-D-Galactose            Alcohol

Figure 3.7 Conversion of beta-galactoside to beta-galactose

**Method** by Craven *et al.* (1965). One unit causes the hydrolysis of one micromole of o-nitrophenyl-β-D-galactopyranoside per minute at 25°C and pH 7.5 under the specified conditions.

**Reagents**

- 0.3 M Sodium phosphate buffer, pH 7.5 with 0.003 M magnesium chloride
- Substrate diluent: 0.01 M Tris-acetate pH 7.5 with 0.01 M magnesium chloride
- Enzyme diluent: 0.01 M Tris-HCl, pH 7.5 with 0.01 M magnesium chloride, 0.01 M mercaptoethanol and 0.01 M sodium chloride
- 0.10 M Sodium/potassium phosphate buffer, pH 7.0
- 1.0 M Mercaptoethanol
- 0.014 M o-Nitrophenyl-β-D-galactopyranoside (ONPG) in substrate diluent. Prepare fresh daily.

**Enzyme**

A one mg/ml stock solution in 0.10 M sodium/potassium phosphate buffer pH 7.0 was prepared. Immediately prior to use, diluted further to 0.02 - 0.04 ΔA/min. in enzyme diluent. The protein
concentration of the chromatographically purified enzyme (Code: BGC) was determined as follows:

\[
\text{mg protein/ml} = A_{280} \times 0.478
\]

Note: This enzyme is not stable when diluted. All dilutions should be made as quickly as possible and used immediately.

Procedure

The spectrophotometer were adjusted to 405 nm.

The following chemicals were pipetted into cuvette:

Table 3.3 ONPG Assay for Beta-galactosidase.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 M sodium phosphate buffer</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>1.0 M mercaptoethanol</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>0.014 M ONPG</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Reagent grade water</td>
<td>1.1 ml</td>
</tr>
</tbody>
</table>

Incubated at 25°C for 3 - 5 minutes to achieve temperature equilibration. 0.1 ml freshly diluted enzyme were added and the increase in $A_{405}$ for 4 - 5 minutes were recorded. $\Delta A_{405}/\text{min}$ were calculated.

Calculation

\[
\text{Units/mg protein} = \frac{\Delta A_{405}/\text{min}}{31 \times \text{mg enzyme/ml reaction mixture}}
\]
3.6.2 Bacteriocin assay methods (Tagg & McGiven, 1971)

The production of bacteriocins by lactic acid bacteria has been known for many years. According to the original definition, the term bacteriocin refers to proteins of the colicin type, characterized by lethal biosynthesis, intraspecific activity, and adsorption to specific receptors.

**Preparation of culture supernatants:** The bacteriocin-producing strains were grown in MRS broth (pH 5.5) at 37 °C for 18-20 h. The lactobacilli culture was centrifuged at 10,000 rpm for 5 min, and then the supernatant was adjusted to pH 6.5-7.0 with 1 N NaOH.

3.6.2.1 Bacteriocin assay by agar-spot test

Bacteriocin activity was detected by the *agar-spot test.*

The test was performed as follows:
- 200 μl of each probiotic culture at the early exponential growth phase (OD600 of 0.2-0.3) were taken in MRS broth
- It was mixed with 4 ml of MRS soft agar (0.6% agar, prewarmed to 48 °C)
- Poured on an MRS agar plate.
- Then, 3 μl of each culture supernatant were dropped onto the solidified soft agar.
- The plates were incubated for 48 h.

Bacteriocin inhibition was indicated by a clear zone in the soft agar layer.

3.6.2.2 Bacteriocin assay by Agar-well-diffusion assay

The antagonistic effects of culture supernatants of bacteriocin producing probiotic strains on various gram positive, gram negative organisms were tested by the agar-well-diffusion assay as described by Toba et al.

The test was performed as follows:
- The melted agar was taken and inoculated with culture.
- Poured on a sterilized petridish and allowed to solidify
- 0.5 mm-diameter wells were made with the help of cork-borer.
- Each well was filled with 100 μl of culture supernatant of bacteriocin-producing probiotic strains after neutralization with NaOH
- The plates were incubated at 37 °C for 48 hrs.
- The inhibition zones around the wells were measured and recorded.

3.6.3 Tracing of growth curve
A growth curve is an empirical model of the evolution of a quantity of cells over time. Growth curves are widely used in microbiology for quantities such as population size or biomass. Values for the measured property can be plotted on a graph as a function of time. When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a typical bacterial growth curve.

The growth curve were traced for both the organisms *E. faecium* and *L. acidophilus* on all the three selected media. The media were taken in 10% concentration and in 100 ml volume. The flasks were incubated in shaking conditions for uniform growth. Following steps were performed:

1. 500 ml flask were taken and 300 ml of the selected media was added in 10% concentration, into it.
2. After sterilization, the flask was inoculated with 2% of freshly grown culture of *Lactobacillus acidophilus* in MRS media.
3. It was mixed well and 3 ml of media from it were taken in a previously sterilized test tube.
4. Turbidimetric analysis of the taken sample were performed at 600 nm and the reading was recorded as 0 hr reading.
5. The flask was incubated in shaking condition at 37°C.
6. After 30 minutes of incubation again the sample were taken aseptically and performed turbidimetric analysis of the taken sample at 600 nm and the absorbance were recorded.
7. Repeated the sixth step after every 30 minutes of incubation.
8. The graph of growth was plotted taking O.D.600 at X-axis and time in hrs on Y-axis.

The same experiment was repeated with *Enterococcus faecium*.

### 3.6.4 Testing for antibiotic sensitivity

With the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility test as a routine. Antibiotic sensitivity is a term used to describe the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly inoculated with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the antimicrobial impregnated absorbent paper...
The disc diffusion method of AST is the most practical method and was performed in this study.

Antibiotic sensitivity were tested for both the organisms, *E. faecium* and *L. acidophilus*. The test were performed as follows:-

1. A test tube containing 10ml of selected media were prepared.

2. After sterilization, inoculated with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

3. The tube was then incubated at 37°C for 24 hours.

4. MRS agar plates were prepared and allowed to solidify.

5. 0.1 ml of freshly grown culture of *Lactobacillus acidophilus* from step three were taken and spreaded on the above plates.

6. Different antibiotic discs were taken and aseptically placed on the above inoculated MRS agar plates. (The discs should be diagonally opposite to each other having proper distance between them for inhibitory zone formation)

![Diagram](158.png)
7. Each plate was incubated at 37°C for 24 hours.

8. The inhibitory zones formed were measured.

![Diagram showing petri dish with antibiotic disc and inhibitory zone](image)

**Figure 3.9 Correct measurement of zones for Antibiotic sensitivity testing**

Calculations:

Zone size = (Diameter of disc – Total inhibitory zone measured) in mm

**3.6.5 Testing for competitive exclusion.**

The term competitive exclusion (CE) is used to describe the process by which beneficial bacteria exclude bad bacteria or pathogens. CE implies the prevention of entry and establishment of a bacterial population into the gut. To succeed, the good bacteria must be better suited to establish or maintain itself in that gut environment. The test were performed as follows:

1. A test tube containing 10ml of selected media were prepared.

2. After sterilization, inoculated with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

3. The tube was then incubated at 37°C for 24 hours.

4. Nutrient agar plates were prepared and allowed to solidify.
5. After solidification each plate were inoculated with 0.1 ml of freshly grown cultures of different organisms to be tested (One plate with one culture)
6. The plate was labeled according to the organism inoculated into it.
7. The culture of *Lactobacillus acidophilus* from step three were taken and inoculated as a point inoculation on the above nutrient agar plate.
8. Each plate was incubated at 37°C for 24 hours.
9. The plate was observed for growth of both, the test organisms and the point inoculated probiotic culture. The zone of inhibition if formed indicated the competitive exclusion by probiotic culture

### 3.7 Determination of the main factors affecting the growth of probiotics on waste materials

The main factors affecting the growth of probiotics on waste materials was determined and the conditions for better growth was standarised. Influence of growth conditions on growth of *E. faecium* and *L. acidophilus* were studied. The same experiments were repeated for all the three selected media.

#### 3.7.1 Effect of pH

1. Five test tubes containing 10ml of selected media were taken.
2. The pH was adjusted to 2, 4, 6.8 & 10 and labeled them accordingly.
3. After sterilization, each tube was inoculated with freshly grown culture of *Lactobacillus acidophilus* in MRS media.
4. Each tube was incubated at 37°C for 24 hours.
5. Turbidimetric analysis and standared plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*.

#### 3.7.2 Effect of Temperature

1. Five test tubes containing 10ml of selected media were taken.
2. After sterilization, each tube was inoculated with freshly grown culture of *Lactobacillus acidophilus* in MRS media.
3. All five inoculated tubes were kept at different temperature (8°C, 15°C, 25°C, 37°C & 45°C) for incubation of 24 hours.

4. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*.

**3.7.3 Effect of salt concentration**

1. Four test tubes containing 10ml of selected media were taken.

2. Different concentration (0.8%, 1.0%, 1.5% & 2.0%) of salt were adjusted in each tube by adding respective amounts of salt.

3. After sterilization, each tube was inoculated with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

4. Each tube was incubated at 37°C for 24 hours.

5. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*.

**3.7.4 Effect of Methylene blue concentration**

1. Four test tubes containing 10ml of selected media were taken.

2. Different concentration (0.1%, 0.2%, 0.3% & 0.4%) of Methylene blue were adjusted in each tube by adding respective amounts of Methylene blue.

3. After sterilization, each tube was inoculated with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

4. Each tube was incubated at 37°C for 24 hours.

5. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*. 
3.7.5. Effect of Inoculum size

1. Five test tubes containing 10ml of selected media were taken.

2. The media volume were kept to 100 ml in each flask.

3. Each flask were labeled according to the size of inoculums to be added(for e.g. 2%, 5%, 10% 20% & 50%)

4. After sterilization, each tube was inoculated with freshly grown culture of \textit{Lactobacillus acidophilus} in MRS media

5. Each tube was incubated at 37\(^\circ\)C for 24 hours.

6. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with \textit{Enterococcus faecium}.

3.7.6. Effect of Incubation time

1. Five test tubes containing 10ml of selected media were taken.

2. Each tube were labeled according to the time to be provided for incubation(for e.g. 12hrs, 18hrs, 24hrs, 48hrs & 72hrs)

3. After sterilization, each tube was inoculated with freshly grown culture of \textit{Lactobacillus acidophilus} in MRS media

4. Each tube was incubated at 37\(^\circ\)C for 24 hours.

5. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with \textit{Enterococcus faecium}.

3.7.7. Effect of flask volume

1. 10% media were prepared in large volume in a single flask.

2. Three different small capacity flask were taken and the media in different volumes were added in each flask.

3. The flask were labeled accordingly.
4. After sterilization, each flask were inoculated with 2% inoculum.

5. Each flask were incubated at 37°C for 24 hours.

6. Turbidimetric analysis and standared plate count were performed for the analysis of growth.

The same experiment was repeated with Enterococcus faecium.

3.8 Optimisation of the waste materials to be used as the growth media for probiotics:

The waste materials supporting the growth of probiotics was optimized by using the response surface methodology. (G. E. P. Box and K. B. Wilson)

Different experiments were performed individiually with different waste materials studied taking Lactobacillus acidophilus. Each experiment were then repeated on Enterococcus faecium. Series of experiment includes following:-

3.8.1 Optimisation of the waste materials with respect to its concentration.

Concentration of the waste material was an important factor to be considered for large scale production. During the studies the minimum concentration of waste material supporting the growth was determined. These studies can lead to the decrease in the transportation charges and can overall decrease the cost of the product. The experiment were performed in following steps:-

For solid waste materials:-

1. Different amounts of the solid waste material were weighed in different containers (e.g. 2g, 5g, 10g, 20g & 50g)
2. Crushed in about 50 ml distilled water and thick paste were made.
3. 50ml more distilled water were added to make suspension.
4. If thick particles were found the contents were filtered using muslin cloth.
5. After getting a clear liquid it was transferred to a fresh container.
6. The solution was filter sterilized, and inoculated each with freshly grown culture of Lactobacillus acidophilus in MRS media.
7. Each flask were incubated at 37°C for 24 hours.
8. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with Enterococcus faecium.

For liquid waste materials:-

1. Different flasks were taken and added with different volumes of the liquid materials to be tested (For e.g. 2ml, 5ml, 10ml, 20ml & 50ml)

2. The total volume of the container were adjusted to 100ml by distilled water.

3. The solution were filter sterilized, and inoculated each with freshly grown culture of Lactobacillus acidophilus in MRS media.

4. Each flask were incubated at 37°C for 24 hours.

5. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with Enterococcus faecium.

Optimisation of the waste materials with respect to different nutrient sources.

In the above step the minimum concentration of the waste materials supporting the growth was determined. Now the aim was to find out whether this growth can be increased by adding certain nutrients which are not the normal components of the taken waste material or if present they are in insufficient amount to support the adequate growth of the organism. Thist process of addition of the required nutrients to the waste materials solution is called as enrichment of the waste material. For this purpose the waste material different nutrient components were selected. The basis of selection were following:

- Normal requirement of the probiotic culture.

- Presence of the component in chemically defined media generally used for the laboratory cultivation of organism.

- Low cost of the component.

- Easy availability.

On the basis of above points following components were selected:
The experiment were performed in following steps:-

For solid waste materials:-

1. Standard amount of the solid waste material was weighed in different containers.
2. Crushed them in about 50 ml distilled water and thick paste were made.
3. 50ml more distilled water were added to make suspension.
4. If thick particles were found the contents were filtered using muslin cloth.
5. After getting a clear liquid it was transferred to a fresh container.
6. 1 gm of above selected components were added in each of the different flasks.
7. The solution was filter sterilized, and inoculated each with freshly grown culture of *Lactobacillus acidophilus* in MRS media.
8. Each flask were incubated at 37°C for 24 hours.
9. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*.

For liquid waste materials:-

1. Different flasks were taken and added with the selected volume of the liquid materials to be tested.
2. The total volume of the container were adjusted to 100ml by distilled water.
3. 1 gm of above selected components were added in each of the different flasks.
4. The solution was filter sterilized, and inoculated each with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

5. Each flask were incubated at 37°C for 24 hours.

6. Turbidimetric analysis and standard plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*.

### 3.8.2 Optimisation of the waste materials with respect to different concentration of selected nutrient source.

The nutrient component favoring the growth were determined in the above step. Now the experiment were done to find out the best concentration of the selected nutrient component. Since in the above step different nutrients were used but their concentration were kept same. It may be possible that changing the concentration may increase the growth. So the experiment were done on this aspect.

The experiment were performed in following steps:-

For solid waste materials:-

1. The standard amount of the solid waste material were weighed in different containers.

2. Crushed them in about 50 ml distilled water and thick paste were made.

3. 50 ml more distilled water were added and to make suspension.

4. If thick particles were found the contents were filtered using muslin cloth.

5. After getting a clear liquid it was transfered to a fresh container.

6. Different grams of above selected components were added in each of the different flasks (For e.g. 2g, 5g, 10g, 20g & 50g)

7. The solution was filter sterilized, and inoculated each with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

8. Each flask were incubated at 37°C for 24 hours.

9. Turbidimetric analysis and standard plate count were performed for the analysis of growth.
The same experiment was repeated with *Enterococcus faecium*.

For liquid waste materials:-

1. Different flasks were taken and added with the selected volume of the liquid materials to be tested.

2. The total volume of the container were adjusted to 100ml by distilled water.

3. Different grams of above selected components were added in each of the different flasks (For e.g. 2g, 5g, 10g, 20g & 50g)

4. The solution was filter sterilized, and inoculated each with freshly grown culture of *Lactobacillus acidophilus* in MRS media.

8. Each flask were incubated at 37°C for 24 hours.

9. Turbidimetric analysis and standared plate count were performed for the analysis of growth.

The same experiment was repeated with *Enterococcus faecium*.

3.9 Comparision of the growth of probiotics on waste materials and the standard media available in markets:

After optimization of the selected waste materials it was necessary to compare its efficacy with the chemically defined media available. These studies can provide an estimation for increase or decrease in growth if the two selected organisms when their growth or cultivation was performend on optimized waste material.

The comparision was done by studying the growth on the selected media by *spectrophotometric analysis* and the *standard plate count method*. (American Public and health Association(APHA,1993)).

The experiment were performed in following steps:-

1. Each of the optimized media were prepared as in the above steps in different flasks.

2. The flasks were labelled according to the waste material media added into it.

3. A different flask were prepared in the same volume with MRS Broth.
4. After sterilization, 2% inoculum was added in each flask.

5. Each flask was then incubated at 37°C for 24 hours.

6. Turbidimetric analysis and standard plate count for the analysis of growth was performed.

The same experiment was repeated with Enterococcus faecium.

3.10 Checking for the contamination of the media designed by waste materials:

Shelf life of the media is a very important factor from its storage point of view. Since the optimized media contains all the growth factors required for the growth of the probiotic cultures these can be effectively utilized by other contaminating organisms also. A frequent contamination of the optimized media can lead to decrease in its shelf life. Thus checking for the contamination of the media is required.

Contamination check will be done by the incubation of the un-inoculated media under proper conditions for proper time. During contamination check, the organisms grown in the media were determined.

The experiment were performed in following steps:-

1. Each of the optimized media were prepared as in the above steps in different flasks.
2. The flasks were labeled according to the waste materials media added into it.
3. After sterilization, the flasks were kept at room temperature and left undisturbed.
4. Each flask were monitored each day, for any change in its consistency and turbidity.
5. When turbidity was found, aseptically the sample was withdrawn from the flask and plated on the nutrient agar media.
6. Gram staining were also performed to study the contaminants.

Procedures for Gram staining:-

1. Preparation of Reagents:

   o **Gram Crystal Violet Solution:** 20 g of crystal violet were dissolved in 100 ml of ethanol to make a crystal violet stock solution. Similarly, 1 g of ammonium oxalate was dissolved in 100 ml of water to make an oxalate stock solution. Working solution was obtained by mixing 1 ml of
the crystal violet stock solution with 10 ml of water and 40 ml of the oxalate stock solution. The working solution was stored in a drop bottle.

**Gram Iodine Solution:** 1 g of iodine, 2 g of potassium iodide, and 3 g of sodium bicarbonate was dissolved in 300 ml of water.

**Gram Decolorizer Solution:** Equal volumes of 95% ethanol and acetone were mixed.

**Gram Safranin Solution:** 2.5 g of safranin was dissolved in 100 ml of 95% ethanol to make a stock solution. Working solution was obtained by diluting one part of the stock solution with five parts of water.

**2. Preparation of a slide smear:**
- A drop of the suspended culture to be examined was transferred on a slide with an inoculation loop. When the culture was to be taken from a Petri dish or a slant culture tube, first a drop or a few loopful of water was added on the slide and aseptically transferred a *minute* amount of a colony from the Petri dish.
- The culture was spreaded with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter.
- The culture was air-dried and fixed over a gentle flame, while moving the slide in a circular fashion to avoid localized overheating. The applied heat helps the cell adhesion on the glass slide to make possible the subsequent rinsing of the smear with water without a significant loss of the culture. Heat can also be applied to facilitate drying the smear. However, ring patterns can form if heating is not uniform, e.g., taking the slide in and out of the flame.

**3. Gram Staining:**
- About 5 drops of crystal violet stain was added over the fixed culture. Kept for 60 seconds.
- The stain is poured off and *gently* rinsed the excess stain with a stream of water by a plastic wash water bottle.
- About 5 drops of the iodine solution was added on the smear, enough to cover the fixed culture. Allowed to stand for 30 seconds.
- Poured off the iodine solution and rinsed the slide with running water.
- A few drops of decolorizer was added so the solution trickles down the slide. Rinsed it off with water after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide.
- Then the slide was counterstained with 5 drops of the safranin solution for 20 seconds.
The red safranin solution was washed off with water. Blotted with blotting paper to remove the excess water. Alternatively, the slide may shaken to remove most of the water and air-dried.

4. The prepared slide was examined under a microscope.

Figure 3.10 The procedure of gram staining.

3.11 Determination of reduction in environmental pollution by the use of waste materials as Growth Media.

Study of change in Physical, chemical and biological properties of soil dumped with waste materials was done before and after their use, which will show the reduction in environmental pollution.

Soil is the main resource upon which the 'natural environment' and agricultural production depends. Soil pollution is defined as the build-up in soils of persistent toxic compounds, chemicals, salts, radioactive materials, or disease causing agents, which have adverse effects on plant growth and animal health. Discharge of industrial waste into the soil leads to serious soil
pollution. In general, waste includes garbage, domestic refuse and discarded solid materials such as those from commercial, industrial and agricultural operation. Since a significant amount of urban solid waste tends to be paper and food waste, the majority is recyclable or biodegradable in landfills. Similarly, most agricultural waste is recycled and utilized by microbes. Thus study was performed on the beneficial use of these waste materials and to observe their effect on soil when dumped freely.

Study of parameters was done according to the methods given by American Public and Health Association (APHA, 1986):-

3.11.1 Sampling of soils

Sampling and analysis plans were devised accurately. Before sampling, vegetation and other non-soil material (including rocks and concrete) was removed. Collection of samples was accomplished with minimal disturbance, in trays.

![Sampling of soil](image)

Figure 3.11 Sampling of soil

3.11.2 Studies on soil: - Different parameters of soil have been analysed. In the initial experiments the normal garden soil was taken and then it was enriched with the selected waste materials. Following properties were studied:

3.11.2.1 Physical properties of soil

- Texture

The size distribution of primary mineral particles, called soil texture, has a strong influence on the properties of a soil. Particles larger than 2 mm in diameter are considered inert. Particles smaller than 2 mm in diameter are divided into three broad categories based on size. Particles of 2 to 0.05 mm diameter are called sand; those of 0.05 to 0.002 mm diameter are silt; and the <0.002 mm particles are clay. The texture of soils is usually expressed in terms of the percentages of sand, silt, and clay. Soil texture, a stable and an easily determined soil
characteristic, can be estimated by feeling and manipulating a moist sample, or it can be determined accurately by laboratory analysis. Soil horizons are sometimes separated on the basis of differences in texture. Soil texture refers to the proportion of the soil “separates” that make up the mineral component of soil. These separates are called sand, silt, and clay. These soil separates have the following size ranges:

- Sand = <2 to 0.05 mm
- Silt = 0.05 to 0.002 mm
- Clay = <0.002 mm

Sand and silt are the “inactive” part of the soil matrix, because they do not contribute to a soil’s ability to retain soil water or nutrients. Because of its small size and sheet-like structure, clay has a large amount of surface area per unit mass, and its surface charge attracts ions and water. Because of this, clay is the “active” portion of the soil matrix. Thus for this study using the sieves, clay portion of soil were taken and weighed approximately 10 kg. This soil were taken in a tray and then analysed for further parameters.

- **Structure**

Sand particles do not cohere (stick together) as do the finer clay particles. The nature of the arrangement of primary particles into naturally formed secondary particles, called **aggregates**, is soil structure. A sandy soil may be structureless because each sand grain behaves independently of all others. A compacted clay soil may be structureless because the particles are clumped together in huge massive chunks. In between these extremes, there is the **granular** structure of surface soils and the **blocky** structure of subsoils. In some cases subsoils may have **platy** or columnar types of structure. Structure may be further described in terms of the size and stability of aggregates. Structural class is based on aggregate size, while structural grade is based on aggregate strength. Soil horizons can be differentiated on the basis of structural type, class, or grade.

The reason for aggregates formation and their holding together is that Clay particles cohere to each other and adhere to larger particles under the conditions that prevail in most soils. Wetting and drying, freezing and thawing, root and animal activity, and mechanical agitation are involved
in the rearranging of particles in soils—including destruction of some aggregates and the bringing together of particles into new aggregate groupings. Organic materials, especially microbial cells and waste products, act to cement aggregates and thus to increase their strength. On the other hand, aggregates may be destroyed by poor tillage practices, compaction, and depletion of soil organic matter. The structure of a soil, therefore, is not stable in the sense that the texture of a soil is stable. Good structure, particularly in fine textured soils, increases total porosity because large pores occur between aggregates, allowing penetration of roots and movement of water and air. Thus we have taken the clay particles for our study to make it more and more useful from ecological point of view.

• Consistence

Consistence is a description of a soil's physical condition at various moisture contents as evidenced by the behavior of the soil to mechanical stress or manipulation. Soil consistence refers to the ease with which an individual particle can be crushed by the fingers. Descriptive adjectives such as hard, loose, friable, firm, plastic, and sticky are used for consistence. The consistence of a soil is determined to a large extent by the texture of the soil, but is related also to other properties such as content of organic matter and type of clay minerals.

Soil consistence, and its description, depends on soil moisture content. Terms commonly used to describe consistence are:

- Moist soil
- Wet soil
- Dry Soil

Consistence was determined by measuring the moisture content of the soil. It was done as follows:

1) A cleaned and dried petridish was taken and weighed. Its weight was recorded as \( W_1 \).

2) From the properly sampled soil the petridish was filled completely and weighed. Its weight was recorded as \( W_2 \).

3) Then the above petridish was placed in the Hot air oven at 160° C for 4-5 hours and weighed. Its weight was recorded as \( W_3 \).
4) Now the moisture content was determined according to the following formula:

\[ W = \frac{w_2 - w_3}{w_3 - w_1} \times 100 \]

\( W \) = moisture content percent
\( w_1 \) = weight of container with lid (in gms)
\( w_2 \) = weight of container with lid with wet soil (in gms)
\( w_3 \) = weight of container with lid with dry soil (in gms)

- Color

The color of objects, including soils, can be determined by minor components. Generally, moist soils are darker than dry ones and the organic component also makes soils darker. Thus, surface soils tend to be darker than subsoils. Gray hues indicate poor aeration. Soil color charts have been developed for the quantitative evaluation of colors.

In well aerated soils, oxidized or ferric (Fe+3) iron compounds are responsible for the brown, yellow, and red colors in the soil.

When iron is reduced to the ferrous (Fe+2) form, it becomes mobile, and can be removed from certain areas of the soil. When the iron is removed, a gray color remains, or the reduced iron color persists in shades of green or blue.

Soils that are dominantly gray with brown or yellow mottles immediately below the surface horizon are usually hydric.

We have taken the moist garden soil that’s why it was dark in colour and after the incorporation of the waste materials, due to increase in its organic content, the colour still grows darker.
3.11.2.2 Chemical properties of soil

- **Major Elements**

Eight chemical elements comprise the majority of the mineral matter in soils. Of these eight elements, oxygen, a negatively-charged ion (anion) in crystal structures, is the most prevalent on both a weight and volume basis. The next most common elements, all positively-charged ions (cations), in decreasing order are silicon, aluminum, iron, magnesium, calcium, sodium, and potassium. Ions of these elements combine in various ratios to form different minerals. More than eighty other elements also occur in soils and the earth's crust, but in much smaller quantities.

The organic fraction of a soil, although usually representing much less than 10% of the soil mass by weight, has a great influence on soil chemical properties. Soil organic matter is composed chiefly of carbon, hydrogen, oxygen, nitrogen and smaller quantities of sulfur and other elements. The organic fraction serves as a reservoir for the plant essential nutrients, nitrogen, phosphorus, and sulfur, increases soil water holding and cation exchange capacities, and enhances soil aggregation and structure.

The most chemically active fraction of soils consists of colloidal clays and organic matter. Colloidal particles are so small (< 0.0002 mm) that they remain suspended in water and exhibit a very large surface area per unit weight. These materials also generally exhibit net negative charge and high adsorptive capacity.

- **Cation Exchange**

Silicate clays and organic matter typically possess net negative charge because of cation substitutions in the crystalline structures of clay and the loss of hydrogen cations from functional groups of organic matter. Positively-charged cations are attracted to these negatively-charged particles, just as opposite poles of magnets attract one another. Cation exchange is the ability of soil clays and organic matter to adsorb and exchange cations with those in soil solution (water in soil pore space). A dynamic equilibrium exists between adsorbed cations and those in soil solution. Cation adsorption is reversible if other cations in soil solution are sufficiently...
concentrated to displace those attracted to the negative charge on clay and organic matter surfaces. The quantity of cation exchange is measured per unit of soil weight and is termed **cation exchange capacity**. Organic colloids exhibit much greater cation exchange capacity than silicate clays. Various clays also exhibit different exchange capacities. Thus, cation exchange capacity of soils is dependent upon both organic matter content and content and type of silicate clays.

Cation exchange capacity is an important phenomenon for two reasons:

1. exchangeable cations such as calcium, magnesium, and potassium are readily available for plant uptake and
2. cations adsorbed to exchange sites are more resistant to **leaching**, or downward movement in soils with water.

Movement of cations below the rooting depth of plants is associated with weathering of soils. Greater cation exchange capacities help decrease these losses. Pesticides or organics with positively charged functional groups are also attracted to cation exchange sites and may be removed from the soil solution, making them less subject to loss and potential pollution.

Calcium (Ca\(^{++}\)) is normally the predominant exchangeable cation in soils, even in acid, weathered soils. In highly weathered soils, such as oxisols, aluminum (Al\(^{+++}\)) may become the dominant exchangeable cation.

The energy of retention of cations on negatively charged exchange sites varies with the particular cation. The order of retention is: aluminum > calcium > magnesium > potassium > sodium > hydrogen. Cations with increasing positive charge and decreasing hydrated size are most tightly held. Calcium ions, for example, can rather easily replace sodium ions from exchange sites. The cations of calcium, magnesium, potassium, and sodium produce an alkaline reaction in water and are termed bases or **basic cations**. Aluminum and hydrogen ions produce acidity in water and are called **acidic cations**. The percentage of the cation exchange capacity occupied by basic cations is called **percent base saturation**. The greater the percent base saturation, the higher the soil pH.
Soil pH

Soil pH is probably the most commonly measured soil chemical property and is also one of the more informative. Like the temperature of the human body, soil pH implies certain characteristics that are associated with a soil. Since pH (the negative log of the hydrogen ion activity in solution) is an inverse, or negative, function, soil pH decreases as hydrogen ion, or acidity, increases in soil solution. Soil pH increases as acidity decreases.

A soil pH of 7 is considered neutral. Soil pH values greater than 7 signify alkaline conditions, whereas those with values less than 7 indicate acidic conditions. Soil pH typically ranges from 4 to 8.5, but can be as low as 2 in materials associated with pyrite oxidation and acid mine drainage. In comparison, the pH of a typical cola soft drink is about 3.

Soil pH has a profound influence on plant growth. Soil pH affects the quantity, activity, and types of microorganisms in soils which in turn influence decomposition of crop residues, manures, sludges and other organics. It also affects other nutrient transformations and the solubility, or plant availability, of many plant essential nutrients. Phosphorus, for example, is most available in slightly acid to slightly alkaline soils, while all essential micronutrients, except molybdenum, become more available with decreasing pH.

Aluminum, manganese, and even iron can become sufficiently soluble at pH < 5.5 to become toxic to plants. Bacteria which are important mediators of numerous nutrient transformation mechanisms in soils generally tend to be most active in slightly acid to alkaline conditions.

For our studies the pH was determined as follows:

1) 10 gms soil sample were taken and added to 100 ml distilled water.

2) The contents were stirred vigorously and the solution was allowed to stand for 10 minutes.

3) After settling of the coarse particles the pH of the supernatant was measured by using the pH meter.

4) The process were repeated three times with different part of soil of same sample.

5) The readings were recorded as pH1, pH2 and pH3 of same sample.
6) The mean pH were then determined as follows:

\[
\text{mean pH} = \frac{pH_1 + pH_2 + pH_3}{3}
\]

- **Organic matter**

The organic soil matter includes all the dead plant material and all creatures live and dead.

Soils have varying organic compounds in varying degrees of decomposition. Organic matter holds soils open, allowing the infiltration air and water and may hold as much twice its weight in water. Many soils, including desert and rocky-gravel soils, have no or little organic matter. Soils that are all organic matter, such as peat (histosols), are infertile (Henry D., 1984). In its earliest stage of decomposition the original organic material is often called raw organic matter.

The final stage of decomposition is called humus. Humus refers to organic matter that has been decomposed by bacteria, fungi and protozoa to the final point where it is resistant to further breakdown. Humus usually constitutes only 5 percent of the soil or less by volume but it is an essential source of nutrients and adds important textural qualities to soil critical to plant growth.

Humus also hold bits of un-decomposed organic matter which feed arthropods and worms that further improve the soil. Humus has high cation exchange capacity that on a dry weight basis is many times greater than clay colloids and acts as a buffer against changes in pH.

- **Plant nutrients**

Plants require a number of essential nutrients for growth and development. Both the soil and the atmosphere can provide these nutrients. Some of these minerals are needed in large amounts (major elements or macronutrients) and others are needed in smaller amounts (trace elements or micronutrients).
Table 3.4 Macronutrients and Micronutrients of Plants.

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>Micronutrients</th>
</tr>
</thead>
<tbody>
<tr>
<td>phosphorus</td>
<td>copper</td>
</tr>
<tr>
<td>nitrogen</td>
<td>molybdenum</td>
</tr>
<tr>
<td>potassium</td>
<td>chlorine</td>
</tr>
<tr>
<td>sulfur</td>
<td>zinc</td>
</tr>
<tr>
<td>calcium</td>
<td>boron</td>
</tr>
<tr>
<td>magnesium</td>
<td>iron</td>
</tr>
<tr>
<td>hydrogen</td>
<td>cobalt</td>
</tr>
<tr>
<td>carbon</td>
<td>manganese</td>
</tr>
</tbody>
</table>

Alternatively the elements are classified into 3 groups.

- Major Elements: N, P, K
- Secondary Elements: Ca, Mg, S
- Trace or Minor Elements: Fe, Mn, Cu, Zn, B, Mo

3.11.2.3 Biological properties of soil

The living component of an acre of soil may contain 900 pounds of earthworms, 2400 pounds of fungi, 1500 pounds of bacteria, 133 pounds of protozoa, 890 pounds of arthropods and algae. (Pimentel, D. et al. 1995)

Most living things in soils, including plants, insects, bacteria and fungi, are dependent on organic matter for nutrients and energy. The presence of these organisms affects the other properties of
soil. In this study we have studied the normal biota of soil and the change in it when incorporated with waste materials. The studies were done as follows:

1) Two different media plates were prepared one was of Nutrient agar and the other was Rose Bengal Chloramphenicol Agar for bacterial and fungal growth respectively.

2) The soil sample was serially diluted in sterile distilled water.

3) 0.1 ml of the properly diluted sample was inoculated on each of the above media plates separately, using spread plate technique.

4) After inoculation the Nutrient agar plate was incubated at 37°C for 24-48 hrs while the Rose Bengal Chloramphenicol Agar plate was incubated at 25°C for 3-5 days.

5) After incubation the plates were observed for the presence of bacterial and fungal colonies.

6) The colonies were counted by using colony counter and the enumeration were done by using following formula:

\[
\text{cfu/ml} = \frac{\text{(no. of colonies x dilution factor)}}{\text{volume inoculated}}
\]

7) In the next step the identification of bacterial and fungal colonies were done as follows:

**Identification of bacterial colonies**

Identification of bacterial colonies were done by studying colony morphology, cell morphology (by Gram stain reaction), and several morphological, nutritional, and biochemical properties.

**A. Colony morphology**

Bacterial colonies grow from a single cell and are composed of millions of cells. Each colony has a characteristic size, form or shape, edge, texture, degree of opacity, and color. These characteristics describe the morphology of a single colony and may be useful in the preliminary identification of a bacterial species. Colonies with a markedly different appearance (when grown on the same medium) can be assumed to contain different bacterial species. However, since many species have a similar colony morphology, the reverse (that colonies that look alike are the
same species) is not always true. Figure 3.12 shows some of the terms used to describe colony morphology.

**Form**

- Circular
- Irregular
- Filamentous
- Rhizoid

**Elevation**

- Raised
- Convex
- Flat
- Umbonate
- Crateriform

**Margin**

- Entire
- Undulate
- Filiform
- Curled
- Lobate

Figure 3.12 Colony morphology.

**B. Gram stain, cell morphology, and cell size**

Differences in cell wall structure cause the different staining reactions for Gram-positive and Gram-negative bacteria, on the basis of gram staining the bacteria can be divided into gram positive and gram negative. We can also determine the cell morphology by studying the gram stained slide. The three most commonly recognized cell morphologies are cocci, bacilli, and spirilla. The cocci have a spherical shape. The bacilli are shaped like rods or cylinders. There is often great variation in the length of bacilli. Many are long and slender, while others are so short that they may resemble cocci. The spirilla resemble a corkscrew, although the number of spirals varies considerably from species to species. In addition to cell morphology, the specific arrangement of cells is an important identifying characteristic of many bacteria. Bacterial cells
are found singly, in pairs, in clusters, or in chains when viewed under the microscope. In addition to morphologies of the cell and of the colony, other morphological characteristics can be useful in initial identifications. Additional identifying morphological characteristics of bacteria include the presence/absence of structures external to the cell wall, such as flagella, a capsule or slime layer, and fimbriae and pili. Certain Gram-positive and a single Gram-negative bacterium are capable of forming endospores when essential nutrients are depleted. The ability of bacterial cells to form endospores is another characteristic used in identification.

### C. Biochemical properties

Biochemical tests that investigate the enzymatic activities of cells can be used in the identification of bacteria. These are the specific series of tests, defined by a specific flow chart, performed to aid in the identification of the genus and species of the bacterium. Some general tests were performed to determine,

1. the ability of the bacterial species to use glucose as a carbon source, and
2. the specific oxygen requirements for growth of each species.

Bacteria are capable of using different carbon sources to obtain the energy needed to sustain life. One main carbon source is glucose, but not all bacteria can metabolize glucose to harness energy. There are several pathways that bacteria can use to metabolize glucose. The end products
and side products of each pathway provide an indication of the specific pathway the bacterium uses to metabolize glucose. For these test bacteria were injected into a medium containing glucose and the pH indicator phenol red, the color changes that occur, and the presence of CO₂, provide evidence of the pathways used by the bacteria. Bacteria are capable of growing in a variety of environmental conditions. The oxygen requirements for bacterial growth are a factor useful in identification. Bacteria are grouped into several categories based on their oxygen needs. Three of these categories are:

- obligate aerobes that require oxygen for survival and growth
- obligate anaerobes that do not tolerate oxygen and growth ceases in the presence of oxygen
- facultative anaerobes that can survive in aerobic and anaerobic conditions but prefer to grow in the presence of oxygen.

The colonies formed on the respective plate were analysed on the basis of above tests and then preliminary identification of them were done.

**Identification of fungal colonies**

The isolated fungus is identified primarily by its colony morphology and microscopic structures. Colonial morphology traits include color, size, texture, and topography of the colony. Yeast colonies resemble bacterial colonies (moist, rounded, opaque and raised), whereas mold colonies are described variously as wrinkled, heaped, folded, etc. The pigments of both the underside and top of the colony were noted. The examination of microscopic structures of fungi usually provides definitive identification for molds. Microscopic morphological features that were the type, size, shape and arrangement of spores and the size and color of hyphae. It was also noted down whether the hyphae have cross walls (septations).

Microscopic observation of molds is done by using several techniques, always prepared in a biological safety hood. Transparent preparations are a rapid method for observing the arrangement of spores. The colony was kept on the slide and then a drop of dye mounting fluid such as lactophenol cotton blue (LPCB) was placed and mixed. The preparation was then examined under low and high magnification on the microscope for spore type and arrangement. In a wet mount preparation, portions of colonies were teased apart with a dissecting needle, transferred to a drop of LPCB and observed under a coverslip on the microscope.