Practical Manual
on
Food and Industrial Microbiology

• Anil Kumar Puniya
• Shilpa Vij

DAIRY MICROBIOLOGY DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
KARNAL - 132 001, HARYANA

NDRI Publication No. 60/ 2010

Published by:
Director,
National Dairy Research Institute
(Deemed University), Karnal- 132001
Haryana, INDIA
Tel: 91-184-2252800, Fax: 91-184-2250042

First edition: March, *2010*

Cover Design: Sumit Singh Dagar, Priti Devi and Malashree. M

Printed at:

Intech Printers & Publishers
353, (Ground Floor), Karan Shopping Complex,
(Mughal Canal), Karnal - 132 001
Contact No. 0184-4043541, 3292951

Preface

"There is nothing like searching, if you want to find knowledge and there is nothing like telling, if you want to spread knowledge".

This is the motto for writing this practical manual on "Food and Industrial Microbiology". This manual has been specially designed for undergraduate and postgraduate students. The main purpose of this lab manual is to guide students through a process of development of microbiological techniques, conducting experiment and interpretation of data. I have tried to engage students in the learning process by applying knowledge. I have also given special emphasis on individual topics so that the students should not be required to re-read the textbooks.

The manual is laid out in sections, e.g., microbiological examinations of different food samples, fermenter in food industry, industrially important microbes and microbial production, each containing a series of experiments. Every practical includes detailed outline in the theory, full list of materials and procedure. At the end of the each section, some review questions are given which will enable the student to assess their potential of what has been done. Glossary, which is given at the end of the manual, covers detailed definition of important terms used in the manual.

The author sincerely thanks Dr. AK Srivastava, Director, NDRI, who entrusted us and granted the permission for writing a practical manual on 'Food and Industrial Microbiology'. I also convey personal gratitude to Dr SL Goswami, (JDR), Dr GR Patil (JDA), Dr. Rameshwar Singh (Head & Registrar), and all other colleagues of DM Division for their support and guidance. Author also thanks ICAR for providing funds for manual preparation under the scheme for strengthening and development of agricultural education.

I also extend my thanks to research scholars Dr Ravinder Nagpal, Mr Sanjay Kumar, Sumit Singh Dagar, Priti Devi and Malashree M, who immensely contributed in retrieving literature and compilation of the manual, without their involvement, it would have been very difficult to complete the task effectively in such a short period of time.

I would also like to thank Monica and Nikhil for their support in preparing the manual.

Shilpa Vij **Anil Kumar Puniya**

TABLE OF CONTENT

SECTION A:

MICROBIOLOGICAL EXAMINATIONS OF DIFFERENT FOOD SAMPLES

Introduction:

Microorganisms are associated with plants and animals in nature. They play important role for survival of plants and animals. But on the other hand growth of certain harmful microorganisms in food can result in spoilage and sometimes cause several diseases on consumption of such food. Food spoilage by microorganisms is due to increase in their numbers, utilizing nutrients, causing enzymatic changes resulting in bad flavours due to breakdown of some food materials or synthesis of new compounds. Due to such microbial activities, food becomes unfit for human consumption. Also food acts as good medium for transmission of many diseases. If the food is contaminated by pathogenic microorganisms, they can grow and increase their population and cause diseases on consumption of such food. Some time microorganisms may not grow in food but they are transported through food. Several food born diseases are the result of microorganism present in food or their growth in them. The presence of microorganisms in food products, with their influence upon quality and methods of preservation, is of great importance to food microbiologists. Control over pathogenic and infectious organisms is essential, but is not enough, since the quality and safety of many foods depend upon the control of spoilage bacteria, yeasts and moulds. So the microbial examination of food and food products is of prime importance for assessing the quality and safety of food products.

1.1 Aim: To examine the microbial flora of vegetables and fruits.

Theory:

Fresh vegetables & fruits are exposed to potential microbial contamination. It has been estimated that 20% of all fruits and vegetables harvested for human consumption are lost through microbial spoilage. The primary agents for microbial spoilage are the bacteria, yeasts and moulds. The latter two are by the far most important etiologic agents of spoilage. Fruits and vegetables carry microbial flora while passing from the farm to the table. The produce is exposed to potential microbial contamination at every step including cultivation, harvesting, transporting, packaging, storage and selling to the final consumers. Fruits and vegetables are susceptible to microbial decomposition because of their nutrient rich characteristics and are capable of supporting the growth of moulds, yeasts and bacteria. The average composition of vegetable and fruits is: Water = 88.3 %, Carbohydrates = 8.6 %, Proteins = 2.0 %, Fat = 0.8%, Ash = 0.3%. Microbial spoilage and contaminating pathogens pose a serious problem in food safety as well as economy. The sanitary control of food quality is concerned with the examination of foods for the presence of pathogens. The examination of foods is performed for the presence of total number of bacteria in the food by SPC, and the presence of coliforms for indicating the presence of pathogens.

General microbiological profile of harvested fruits:

- Bacteria: usually less than 10^6/ g

- Moulds: 10^3-10^4/ g

This exercise deals with the quantitative estimation of viable bacterial cells, yeast and melds in a food sample by serial dilution agar plate technique.

Materials Required:

Sterile sample bottle or polyethylene bags, scale, sterile dilution blank (99 ml and 9 ml), Petri-dishes, sterile pipette (1 ml), nutrient agar, potato dextrose agar, violet red bile agar, pestle and mortar, incubator, wax marking pencil

Procedure:

- Collect the vegetables and fruit samples at random.
- Weigh 11 g of the vegetable and homogenise the sample in pestle and mortar. Thoroughly mix the sample into 99 ml of sterile diluents to make 10^{-1} dilution.
- Transfer 1 ml of suspension from 10^{-1} dilution to a 9 ml sterile saline blank with a sterile pipette to make 10^{-2} dilution. Similarly prepare serial dilution up to 10^{-8}.

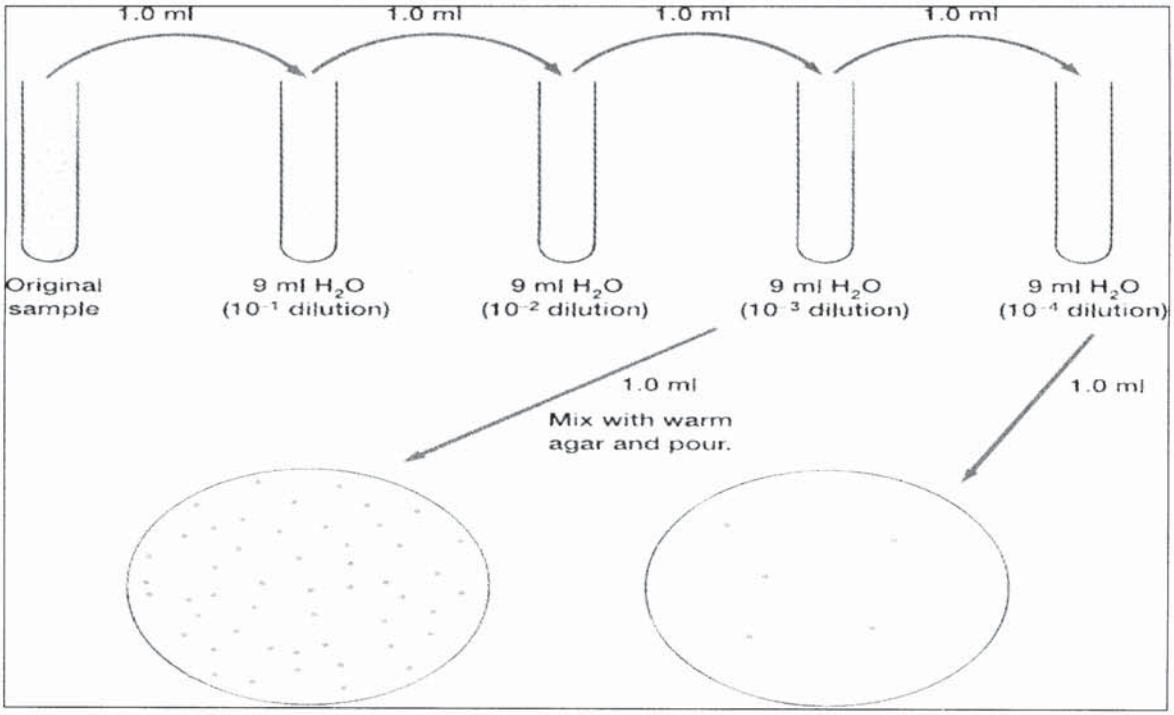


Figure 1 Serial dilution of sample and pour plate technique

Source: http://cms.daegu.ac.kr/sgpark/microbiology/pour.jpg

- Transfer 1 ml aliquot from 10^{-6} and 10^{-8} dilutions to sterile Petri dish for total bacterial count, 10^{-3} and 10^{-5} for coliform count and yeast and melds count.
- Add approximately 15 ml of cooled medium (40°C) to each Petri-plate (Nutrient agar for total bacterial count, VRBA for coliform count, acidified PDA for yeast and melds count) and mix the content by gentle rotation of the Petri dish.
- Invert the plates after solidification and incubate at 37°C for 24-48 h for total bacterial count and coliform count, and at 25°C for 3-5 days for yeast and melds count.

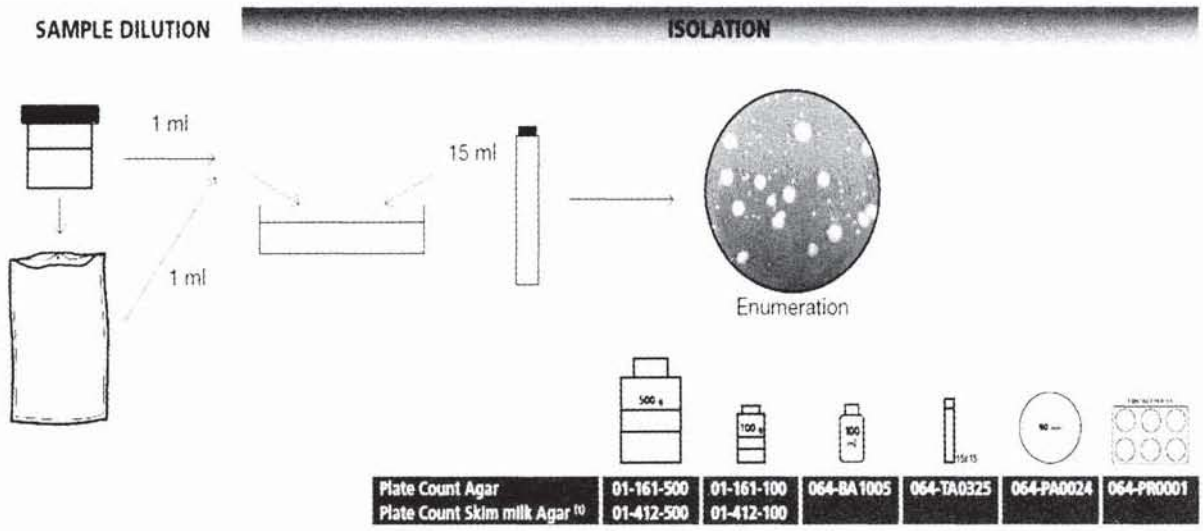


Figure 1.2 Enumeration steps for bacterial count in food sample

Source: www.scharlau.com

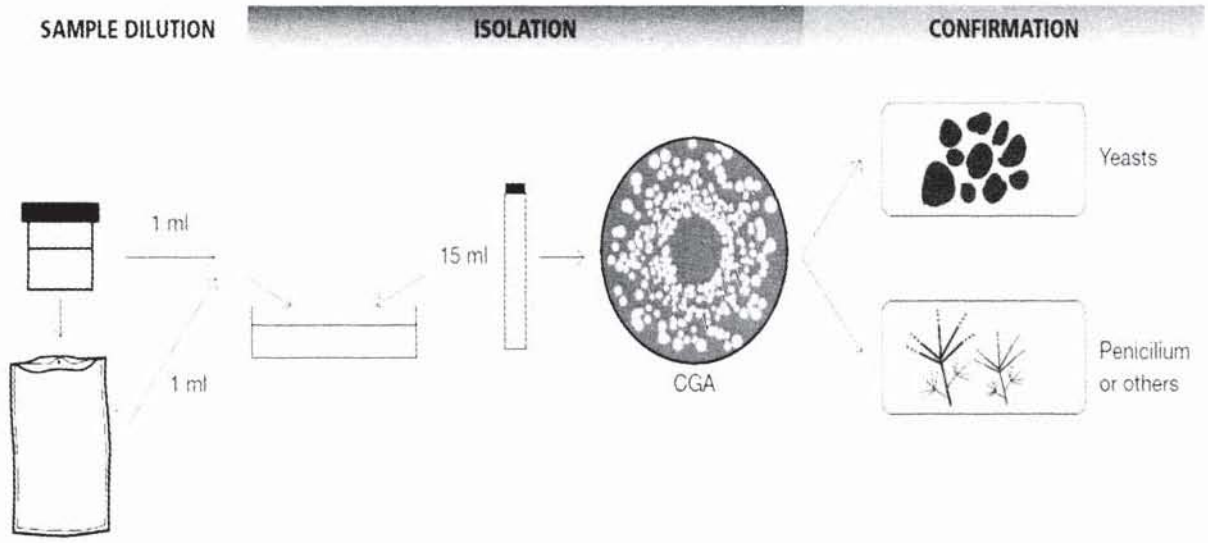


Figure 1.3 Enumeration steps for fungal count in food sample

Source: www.scharlau.com

Observation table:

Type of count	Dilution	Agar used	Incubation – temp./ time	Count per plate (cfu/ g or ml)	Total count (cfu/g or ml)	Average count (cfu/g or ml)

Results and interpretations:

1.2 Aim: To study the microbial flora of meats and eggs.

Theory:

Meats and eggs are the most perishable of all important foods because of the presence of ample amount of nutrients required for the growth of bacteria, yeasts and melds. Moreover these nutrients are available in more available form. It has also been pointed out that during slaughter, dressing and cutting, microorganisms come chiefly from the exterior of animals and its intestinal tract but that more added from knives, cloth air, workers.

A great variety of kinds of organisms are added and so it can assumed that under ordinary conditions most kind of potential spoilage organisms are present and it will be able to grow if favourable conditions present themselves. Most common organism responsible for meat spoilages are bacteria, melds and yeasts respectively.

Bacteria most often involved in spoilage are *Acinetobacter, Hafnia, Campylobacter, Enterococcus, Salmonella, Serratia, Staphylococcus, Vibrio, Pseudomonas, Vagococcus, Carnobacterium, clostridium, Aeromonas, Alcaligenes etc.* with respect to fungal agent involved *Thamnidium, Mucor, Penicillium, Rhizopus, Candida, Rhodotorula, Chrysosporium* and *Cladosporium.*

The hen's egg is an excellent example of a product that normally is well protected by its intrinsic parameters. Externally a fresh egg has three structures outer waxy shell membrane, the shell, and the inner shell membrane. Freshly laid eggs are generally sterile. However in a relatively short period after laying numerous microorganisms may be found on the outside and under the proper condition may enter eggs, grow and cause spoilage.

Among bacteria found are the members of following genera: *Pseudomonas, Acinnetobacter, Proteus, Aeromonas, Alcaligenes, Escherichia, Micrococcus, Salmonella, Serratia, Enterobacter, Flavobacterium and Staphylococcus.* Among the melds generally found are the members of the genera *Mucor, Penicillium, Hormodendrum, Cladosporium and Torula.* The entry of microbes in eggs is favoured by high humidity. Under such conditions growth on the surface is also favoured followed by the penetration in shell and inner membrane.

The sanitary control of meat and eggs quality is primarily concerned with testing meat products for the presence of specific microorganisms. Meat products are the primary vehicle responsible for the transmission of microbial diseases of the gastrointestinal system like salmonellosis and botulism, for this reason, meat products are routinely examined for the presence of bacteria.

Materials Required:

Raw meat samples, 22.5 ml saline, 9 ml saline, Plate Count Agar (PCA), Malt Extract Agar (MEA), Oxford Perfringens agar, *Bacillus cereus* agar, VRBGA (plus 4ml overlay molten), Sterile Petri dishes, Pipettes 0.1 and 1ml automatic plus tips, Glass rod spreader plus alcohol.

Procedure:

Sampling:

- Take number of subsamples from different parts of the meat and eggs including areas known to be subject to contamination or particularly favourable for microbial growth.
- Use sterile swabs and templates for taking surface swab samples.
- Moisten the first swab with sterile peptone water and rub firmly across the exposed area several times in all directions.
- Use the second swab dry and rub over the same area.
- Introduce both swabs into a bottle containing 3 or 4 glass beads and an appropriate known volume of diluents (e.g., 0.1 % peptone, 0.9% NaCl).
- Shake vigorously.
- Otherwise directly cut small pieces of meat or egg yolk and add it to the sterile diluents

Plating:

Microbial flora of raw meats

- Analyse two different raw meats.
- Check whether your agar plates need pre-drying.
- Weigh 2.5 g of the raw meat provided into a Stomacher bag and add 22.5ml of saline. This is the 10^{-1} dilution. Homogenise using the Stomacher for 30 seconds.
- Allow the large particles to settle (fatty material will float to the surface) and then dilute your sample in 9ml sterile saline up to 10^{-8} dilutions.

Spread plate inoculation:

- Dispense 0.1ml of each dilution (10^{-8} to 10^{-1} to save on pipette usage) onto the surface of dried Plate Count Agar (PCA), Malt Extract Agar (MEA), *and Bacillus cereus* agar.

Pour plate inoculation:

- Pipette 1ml of each dilution into 8 Petri dishes. Add approx. 20ml molten Tryptose Sulphite Cycloserine Agar (also known as Perfringens agar).
- Carefully swirl to mix the samples.
- Pipette 1ml of each dilution into 8 Petri dishes. Add approx. 20ml molten VRBGA.
- Carefully swirl to mix the samples.

NOTE: This will need a 4ml VRBGA overlay (stock volume 50ml).

- Incubate the plates aerobically at 37°C; except for the TSCA agar which will be incubated anaerobically and the MEA which will be incubated at 25°C.

Observations:

- Arrange the plates in order of lowest to highest dilution.

- Count the number of colonies on the plates that have colonies 30 to 300. Designate plates with fewer than 30 colonies as too few to count and plates with more than 300 colonies as too numerous to count.
- Calculate the average number of bacteria per gram of sample as follows:
- Number of bacteria/ gram = $\frac{\text{Number of colonies}}{\text{Dilution factor x Weight of sample}}$

Observation Table:

Record in the table the number of colonies per plate.

Dilution	**Number of colonies/ plate**
10^{-3}	
10^{-4}	
10^{-5}	
10^{-6}	

Results and interpretations:

1.3 Aim: To study the microflora of wheat and flour.

Theory:

The microbial flora of wheat, rye, corn and related products may be expected to be that of soil, storage environments, and those picked up during the processing of these commodities. While these products are high in proteins and carbohydrates, their low a_w is such as to restrict the growth of all microorganisms if stored properly. The microbial flora of flour is relatively low, since some of the bleaching agents reduce the load.

When conditions of aw favour growth of bacteria of genus bacillus and melds of several genera are usually the ones that develop. Many aerobic spore formers are capable of producing amylase, which enables them to utilize flour and related products as sources of energy, provided that sufficient moisture is present to allow growth to occur with less moisture, mould growth occurs and may be seen as typical mycelial growth and spore formation. Members of the genus rhizopus are common and may be recognized by their black spores. Most common microflora of dough consist of mainly lactic acid bacteria more than half belonging to the genus Lactobacillus, Leuconostoc and Streptococcus. Moulds are generally found in low numbers.

General microbiological profile of flour and baked goods (Breads) is:

A) Flour:

- Moulds = 10^2-10^4/ g
- Yeast = 10-10^2/ g
- Total Bacteria = 10^2-10^6/ g
- Coliforms = 0-10/ g

B) Baked Goods:

- Moulds = 10-10^3/ g
- Yeast = 10-10^3/ g
- Total Bacteria = 10-10^3/ g
- Coliforms = 10-10^2/ g

Material Required:

Sterile sample bottle or polyethylene bags, sterile dilution blank (99 ml and 9 ml), Petri-dishes, sterile pipette (1 and 10 ml), nutrient agar, potato dextrose agar, violet red bile agar, pestle and mortar, incubator, wax marking pencil

Procedure:

- Weigh 11 g of the sample and put in sterile pestle and mortar. Thoroughly mix the sample into 99 ml of sterile diluents to make 10^{-1} dilution.

- Transfer 1 ml of suspension from 10^{-1} dilution to a 9 ml sterile saline blank with a sterile pipette to make 10^{-2} dilution. Similarly, prepare serial dilution up to 10^{-8}.
- Transfer 1 ml aliquot from 10^{-6} and 10^{-8} dilutions to sterile Petri dish for total bacterial count, 10^{-3} and 10^{-5} for coliform count and yeast and melds count.
- Add approximately 15 ml of cooled medium (40°C) to each Petri-plate (Nutrient agar for total bacterial count, VRBA for coliform count, acidified PDA for yeast and melds count) and mix the content by gentle rotation of the Petri dish.
- Invert the plates after solidification and incubate at 37°C for 24-48 h for total bacterial count and coliform count, and at 25°C for 3-5 days for yeast and melds count.

Observation Table:

Type of count	**Dilution**	**Agar used**	**Incubation – temp./ time**	**Count per plate (cfu/ g or ml)**	**Total count (cfu/g or ml)**	**Average count (cfu/g or ml)**
1. Bread						
2. Flour						

Results and Interpretations:

1.4 Aim: Detection of endotoxins in food samples by Limulus Amoebocyte Lysate test.

Theory:

Microbial toxins are organic poisons produced by microorganisms. When such poisons are ingested, absorbed, or otherwise introduced into the body of different living organisms, they cause damage to tissues and/ or interfere with normal physiological functions. Microbial toxins are complex in terms of structure and chemical composition, and they possess antigenic properties. Numerous types of microbial toxins have been described; these are bacterial toxins, fungal toxins (mycotoxins), algal toxins (phycotoxins), etc. Various types of toxins are produced by bacteria and potent toxins producers are *Corynebacterium diphtheriae, Clostridium tetani, Clostridium botulinum, E. coli, Staphylococcus aureus* etc.

Exotoxins are soluble substances produced within cells but secreted to the cell's exterior environment during periods of active growth. Endotoxins are bound to the bacterial cell wall as a structural component – or, in certain types of microbes, contained within the cell's cytoplasm.

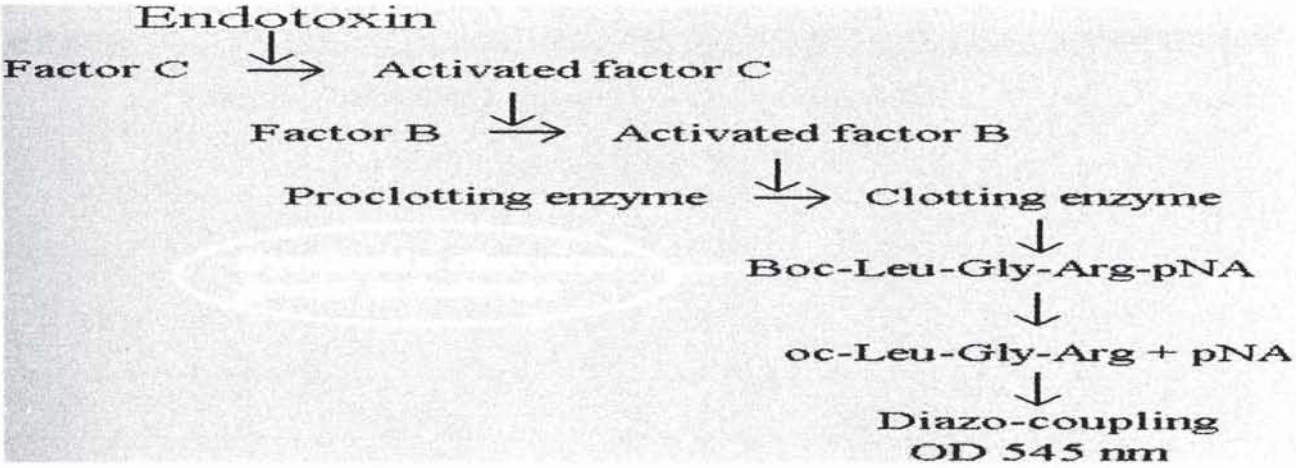


Figure 1.4 Flow chart showing LAL test mechanism

Source: http://www.endotoxin.gmxhome.de/lal.jpg

Exotoxins are proteinaceous substances, specially characterized by their lack of chemical association with other macromolecules. These are sensitive to denaturation by heat and other chemical substances. Exotoxins can be neutralized by their homologous antibodies.

Endotoxin is complex lipo-polysaccharide-protein component of the bacterial cells wall. These are relatively heat-stable and cannot be neutralized by homologous antibodies. Limulus polyphemus present in the blue blood of the horseshoe crab is a nucleated cell, called amoebocyte. The cytoplasm of amoebocyte is densely packed with granules. Limulus lysate, extract of amoebocyte granules, contains all the necessary clotting factors. Limulus lysate clots in the presence of bacterial lipopolysaccharides (endotoxins). Hence this test can be used to rapidly detect the endotoxins present in food samples. This test is very specific and sensitive and can detect up to 10^{-15} g endotoxins per millilitre of sample.

Material required:

Food samples, Clean and dry test tubes, Limulus lysate reagent, Water bath incubator (37°C), wax marking pencils, sterile 0.1 and 1 ml tips, auto-pipettes

Procedure:

- Prepare the serial decimal dilution of samples using normal saline.
- Take equal volume of diluted sample and limulus lysate reagent in clean and dry test tubes and mix the content gently.
- Incubate the test tube in a water bath incubator at 37°C for 4 h.
- After incubation is over, invert the test tube and see the flow behaviour of the fluid in the test tube.
- If the mixture remains unchanged and runs down the test tube wall, indicate the negative LAL test and thus endotoxins are absent in the sample dilution.
- If a firm and opaque gel is formed and sticks to the bottom of the tube, indicates the positive LAL test and thus endotoxins are present in the sample dilution.

Observation:

Sample(s)	Dilutions	Opaque Gel formed	Results

Results and Interpretations:

1.5 Aim: To check the presence of antibiotics in milk samples through DSM DELVO test.

Theory:

Antibiotic contamination in milk can seriously affect consumers' health by causing allergic reactions to residues or by the development of resistant strains of microorganisms. Therefore, subsequently antibiotic contamination in milk can also cause significant economic losses for producers and manufacturers of milk and milk products.

For determination of antibiotic residues in milk, use commercially available Antibiotic Detection Kit: Delvo-X-Press bL-II, COPAN TEST, CHARM FARM TEST or CHARM AIM-96, BITA STAR KIT

The growth of the *Bacillus stearothermophilus* spores at 64°C initiates an acidification process which causes the turning of a pH indicator from purple to yellow. The presence of antibacterial substances will cause delay or inhibition of the spores, depending on the concentration of the residues. In the presence of residues the spores will not multiply and the pH indicator will remain purple.

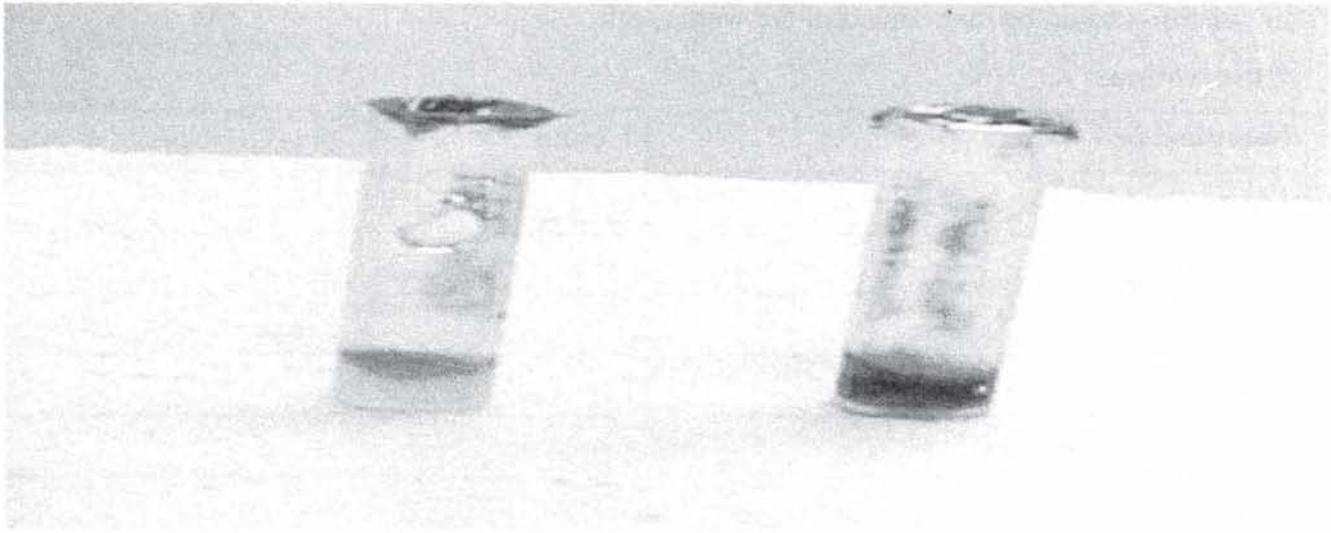


Figure 1.5 Yellow colours: no antibiotic; Purple colour: antibiotic present

Source: www.cvm.umn.edu/.../delvoptestpics/home.html

Material Required:

DSM DELVO test kit, raw milk samples, water bath, agar well strips

Procedure:

- Add 1 nutrient tablet to each of the agar wells in the strip.
- Inoculate 100 ml of milk into the agar well plus nutrient tablet.
- Seal the wells for incubation

- Incubate the strip of wells in a water bath at 64°C ± 0.5°C for 2 h 30 min* (at the time the negative control has been changed to yellow)
- Examine the strip for color change from purple to yellow. A yellow reading indicates that no inhibitory substances are present; a purple reading indicates that antibiotic residues are present and a yellow/purple reading indicates a doubtful result.

*For best sensitivity a control time reading is advised using a negative control sample.

The sample incubation period is crucial to the accuracy of the Delvo® SP test method. This particularly applies to the detection of sulphonamides as the sensitivity to sulphonamides is greatly reduced by an increased incubation time. An incubation time of 2 hr 30 min (at the time the negative control has been changed to yellow) is recommended with a 15 minute extension time of the test in the case of a suspect sample. For reading times of 2 h 45 min or 3 hours the sensitivity of the test will diminish. The use of antibiotic free skim milk powder as a control is also advised. · It is essential that the correct temperature is maintained in the water bath (64°C ±0.5°C). The use of a proper water-bath lid, a sloping lid is advised·

The temperature control in the water-bath should be digital temperature readout · Good circulation should be maintained in the water-bath

Observations:

Samples	**Colour**

Results and Interpretations:

1.6 Aim: To study the methylene blue reduction test for a given milk samples.

Theory:

A milk sample that contains a large population of actively metabolizing microorganisms will contain a markedly decreased concentration of dissolved oxygen because of the vigorous growth of the organisms. In other words, the oxidation-reduction potential of the sample is greatly lowered. The dye methylene blue a redox indicator; loses its colour in an anaerobic environment and is then said to be reduced.

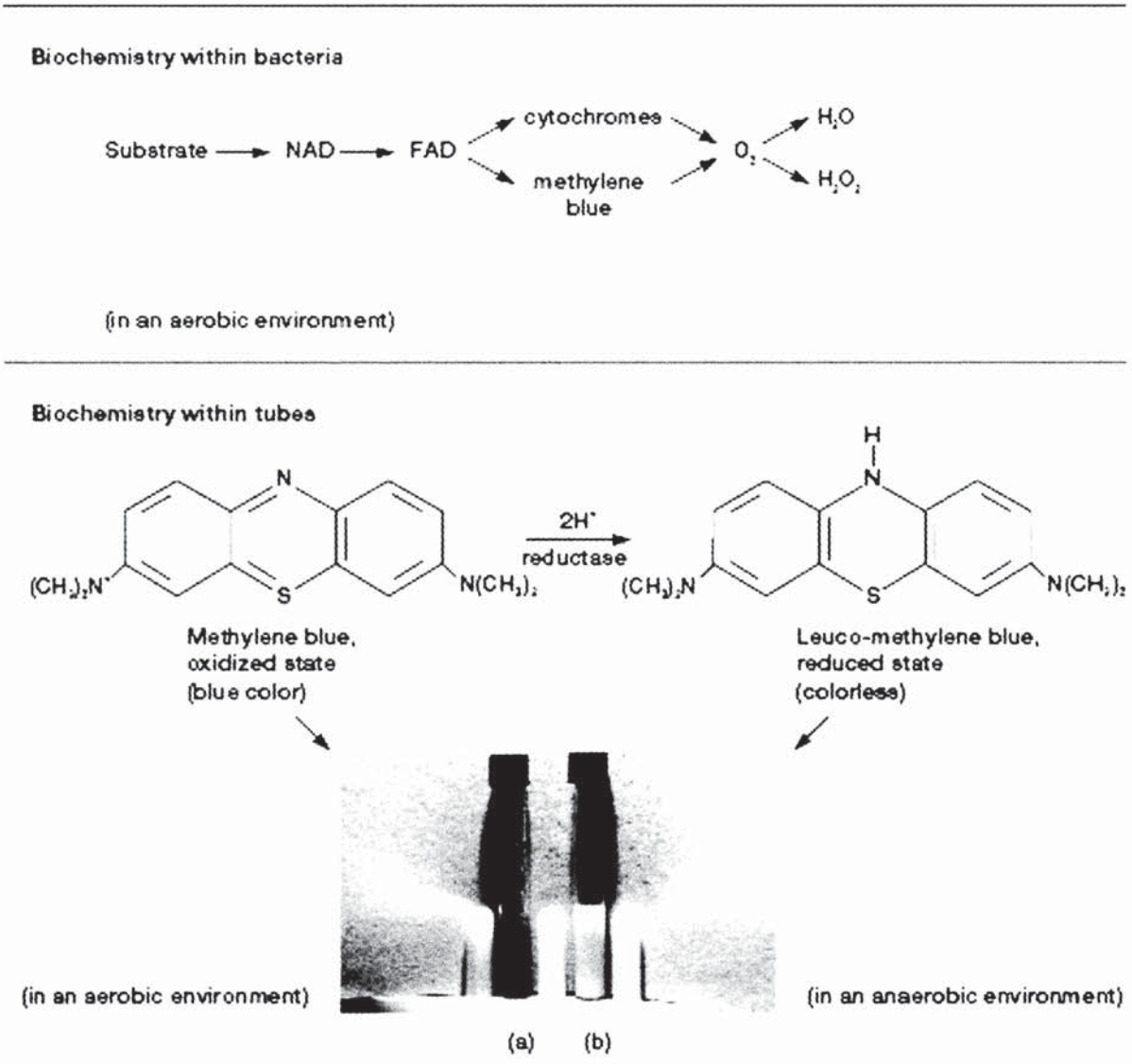


Figure1.6. Methylene blue reduction test for bacteria (a) No reduction (b) complete reduction

Source: Prescott et al. Microbiology, 2nd Edition, 2002

The methylene blue reductase test is designed to screen the quality of raw milk, which may contain large population of enteric organisms and *Streptococcus lactis*, which are potent

reducers of the dye. The speed at which reduction occurs following addition of methylene blue to a sample of milk indicates the milks quality. This determination is made as follows:

1. Reduction within 30 minutes is indicative of very poor quality.
2. Reduction occurring between 30 minutes and 2 hour is indicative or poor quality.
3. Reduction occurring between 2 and 6 hour is indicative or fair quality
4. Reduction occurring between 6 and 8 hour is indicative or good quality

Materials Required:

One raw milk sample and one pasteurized milk sample stored at room temperature for 48 hour, Methylene blue solution (1:250,000), Sterile screw-cap tubes, test tube pack, sterile 10 ml and 1 ml pipettes, mechanical pipette device, 37°C water-bath, Bunsen burner; and glassware marking pencil.

Procedure:

- Label the test tubes a raw milk and pasteurized milk.
- Using a different 10-ml pipette each time transfer 10 ml of each type of milk into its appropriately labeled test tube.
- Add 1 ml of methylene blue dye to each test tube.
- Insert stoppers, invert the test tubes gently about four times, and place in water bath. Record the time of incubation; that is, record the amount of time elapsed for the color to turn white.
- Allow the tubes to stabilize for 5 minutes remove them from the water bath invert them gently once and replace them in the water bath.

Precaution:

- Bacterial culture must be fresh
- Reagents should be fresh

Observations:

- Observe the milk samples for methylene blue reduction every 30 minutes for 3 to 6 hours; a reduction is demonstrated by a change in the color of the sample to white.
- Record in the chart the time required for reduction in both milks sample
- Based on your observations, determine and record in the chart the quality of each sample as very poor, poor, fair or good

Type of milk sample	Reduction time	Quality of milk

Results and Interpretations:

1.7 Aim: Presumptive, confirmatory and completed test for water quality.

- Determine the presence of coliform bacteria in a water sample
- The possible number of coliform bacteria present in a water sample
- Describe steps (i.e. presumptive, confirmed, and completed) for determining coliforms in a water sample.

Theory:

The number of total coliforms (*Enterobacter, Klebsiella, Citrobacter, and Escherichia*) in a water sample can be determined by a statistical estimation called the most probable number. This test involves a multiple series of Durham fermentation tubes and is divided into three parts: the presumptive, confirmed, and completed tests. In the presumptive test, dilutions from the water sample are added to lactose or lauryl tryptose broth fermentation tubes. After 24 to 48 hours of incubation at 35°C, one looks for bacteria capable of fermenting lactose with gas production, presumably coliforms. (The lauryl tryptose broth is selective for Gram-negative bacteria due to the presence of lauryl sulfate.) In the confirmed test, one transfers material from the highest dilution of those lactose broth tubes that showed growth and gas production into brilliant green lactose bile broth, which is selective and differential for coliforms. The tube is incubated for 48 ± 3 hours at 35°C. Gas formation in the Durham tube is a confirmed test for total coliforms. In the completed test, a sample from the positive green lactose bile broth is streaked onto Levine's EMB or LES Endo agar and incubated for 18 to 24 hours at 35°C. On EMB agar, coliforms produce small colonies with dark centers. On LES Endo agar, coliforms produce reddish colonies. Samples are then inoculated into brilliant green lactose bile broth and onto a nutrient agar slant. These tubes are incubated for 24 hours at 35°C. If gas is produced in the lactose broth, and the isolated bacterium is a gram-negative (based on a Gram stain) nonsporing rod, the completed test is positive. An estimate of the number of coliforms (most probable number) can also be done in the presumptive test.

In this procedure, 15 lactose broth tubes are inoculated with the water sample. Five tubes receive 10 ml of water, 5 tubes receive 1 ml of water, and 5 tubes receive 0.1 ml of water. A count of the number of tubes showing gas production is then made, and the figure is compared to a table developed by the American Public Health Association. The number is the most probable number of coliforms per 100 ml of the water sample. (It should be noted that the MPN index usually comes from the presumptive test if raw sewage is being tested and comes from confirmed or completed tests for other types of samples.)

Materials Required:

10-ml single-strength lactose broth in Durham fermentation tubes (lauryl tryptose broth)-10, 10-ml double-strength lactose broth in Durham fermentation tubes-5, 125-ml water sample,

Gram-staining reagents, Petri plate containing Levine's EMB agar, Tryptic agar slant, tubes containing brilliant green lactose bile broth with Durham's tubes, 1 sterile 10-ml pipettes, 2 sterile 1-ml pipettes, wax pencil, test-tube rack, 35°C incubator, inoculating loop and needle, Bunsen burner,

Procedure:

I. Presumptive Test

- Mix the bottle of water to be tested 25 times. Inoculate five of the double-strength lactose (or lauryl tryptose) broth tubes with 10 ml of the water sample; five single-strength tubes with 1 ml of the water sample; and five single-strength tubes with 0.1 ml of the water sample.
- Carefully mix the contents of each tube without spilling any of the broth by rolling the tubes between the palms of your hands.
- Using the wax pencil, label all tubes with your name, date, and the amount of water added.
- Incubate the three sets of tubes for 24 to 48 hours at 35°C.
- Observe after 24 ±2 and 48 ±3 hours. The presence of gas in any tube after 24 hours is a positive presumptive test.
- The formation of gas during the next 24 hours is a doubtful test. The absence of gas after 48 hours is a negative test.
- Determine the number of coliforms per 100 ml of water sample.
- For instance, if gas was present in all five of the 10-ml tubes, only in one of the 1-ml series, and none in the 0.1-ml series, your test results would read 5–1–0.
- Standard table (Page 111) indicates that the MPN for this reading would be 30 coliforms per 100 ml of water sample.

II. Confirmed Test

- Using an inoculating loop, from the tube that has the highest dilution of water sample and shows gas production transfer one loopful of culture to the brilliant-green lactose bile broth tube. Incubate for 48±3 hours at 35°C.
- The formation of gas at any time within 48 hours constitutes a positive confirmed test.

III. Completed Test

- From the positive brilliant green lactose bile broth tube, streak a LES Endo or Levine's EMB plate.
- Incubate the plate inverted for 24 hours at 35°C.

- If coliforms are present, select a well-isolated colony and inoculate a single-strength, brilliant green lactose bile broth tube and streak a nutrient agar slant.
- Gram stains any bacteria found on the slant.
- The formation of gas in the lactose broth and the demonstration of gram-negative, nonsporing rods in the agar culture is a satisfactorily completed test revealing the presence of coliforms and indicating that the water sample was polluted.
- This is a positive completed test.

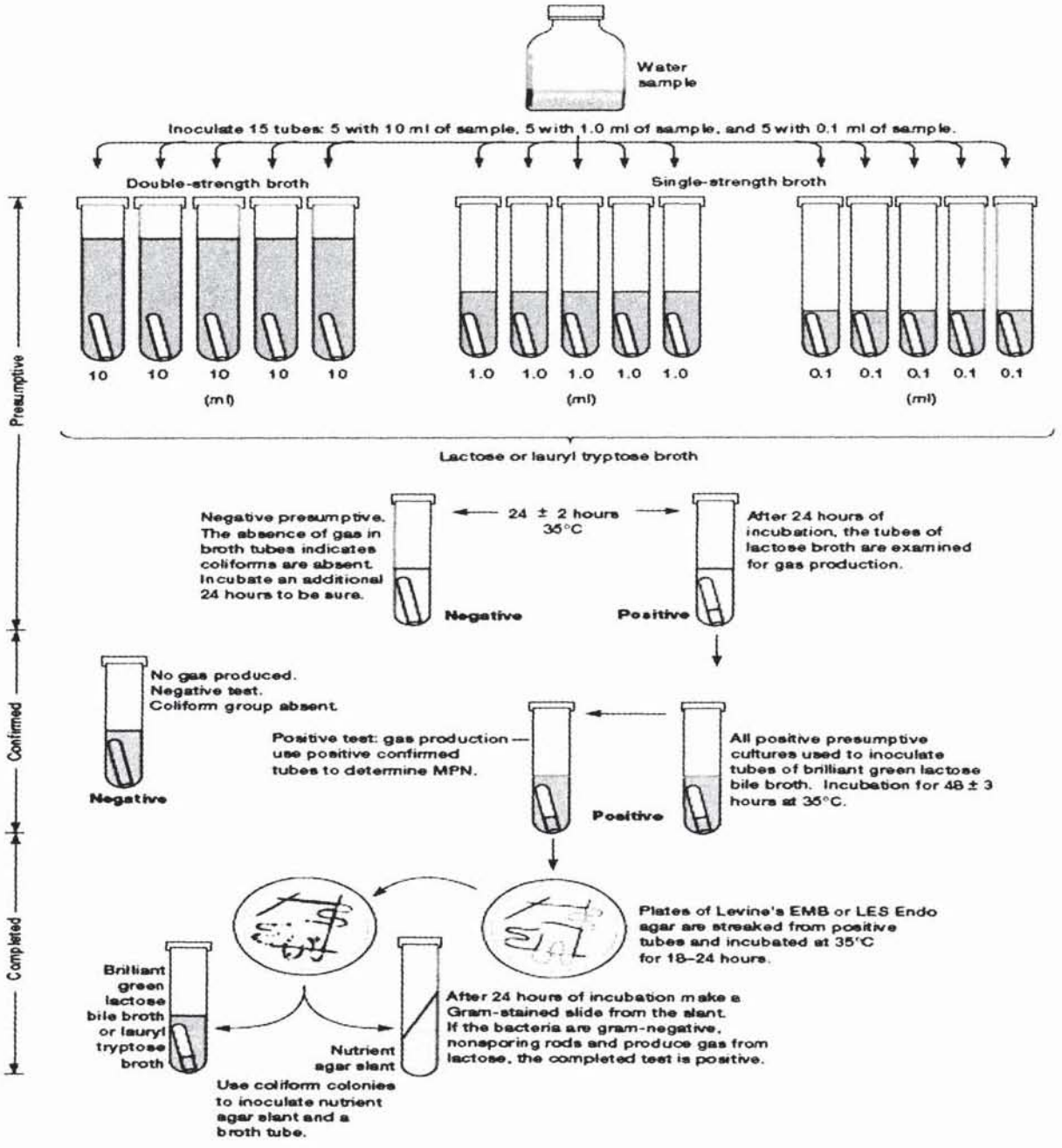


Figure1.7 Flow Diagram for presumptive, confirmed and completed coliform test

Source: Prescott et al. Microbiology, 2nd Edition, 2002

Precautions:

- *Do* not confuse the appearance of an air bubble in a clear Durham tube with actual gas production. If gas is formed as a result of fermentation, the broth medium will become *cloudy. Active fermentation may be shown by the continued appearance of small bubbles* of gas throughout the medium outside the inner vial when the fermentation tube is gently shaken.
- When taking your water sample, the upper 38 cm of most waters usually contains the greatest numbers of live bacteria.
- Use sterile containers to collect water samples.

Observation Table:

Type of sample	Dilution of sample	Number of positive tubes for 10 mL samples	Number of positive tubes for 1 mL sample	Number of positive tubes for 0.1mL samples	MPN index	MPN count/ 100 mL

Result and Interpretations:

Review Questions:

- Can you name few bacteria that are normally found in the milk?
- Differentiate food poisoning and intoxication.
- Does standard plate count method correctly reflect the total bacterial count of a sample?
- Enlist the possible ways in which food may get contaminated with the colonic microflora.
- Explain the function of the methylene blue in the reduction test for judging the milk quality?
- Explain why milk is stored refrigerated?
- For microbes, how does the milk differ from water?
- Name the tests used for testing antibiotics in milk?
- What are the methylene blue reduction times for different types of milk?
- What are the possibilities that a milk sample can gets contaminated by handlers?
- What is the basic principle of Delvo test for detecting antibiotics in milk?
- Why 30 to 300 colonies per plate are commonly used for making the calculations?
- Why an 11 g sample of fruits is blended with 99 ml of diluents to yield a 1/10 dilution?
- Why *E. coli* is selected as the indicator of water potability?
- Why it is not suitable to thaw and then refreeze the food products without cooking?
- Why milk is preferably pasteurized and not sterilized?
- Why MPN method is a qualitative one rather than quantitative?
- Why standard plate count performed on food products?

User's Notes

SECTION B:
FERMENTER IN FOOD INDUSTRY

2.1. Introduction:

The art of studying fermentation is called zymology or zymurgy. Louis Pasteur was one of the first zymologists and referred to fermentation as "the result of life without air".

A general definition of fermentation is an energy-yielding anaerobic metabolic process in which organisms convert nutrients (typically carbohydrates) to alcohols and acids (lactic acid and acetic acid). The most commonly known definition for fermentation is the conversion of sugar to alcohol, using yeast, under anaerobic conditions, as in the production of beer or wine, vinegars and cider. Fermentation is among the oldest of historical biotechnological processes that people have been using for thousands of years. However, in biotechnology, the term is used more loosely to refer to growth of microorganisms on food, under either aerobic or anaerobic conditions.

The design of fermentation equipment has evolved in a largely empirical manner. The earliest fermentations required only rudimentary standards of hygiene, either because of the nature of the substrate, or because the vigour of the desired organism exceeded that of potential competitors, or because the products of fermentation were inhibitory, or because the expected shelf life of the product was short. This applied to the fermentation processes involved in the manufacture of wine, beer, cheese, yoghurt, vinegar, sauerkraut and so on. Even with these products the transition from domestic art to commercial practice required improved standards to increase shelf-life and to maintain acceptably-consistent standards of product quality, but equipment and process control remained essentially simple. Some sophistication occurred with the development of pure-culture techniques in beer making, but the first really fastidious fermentation, the manufacture of acetone and butanol, was initiated on a large scale less than sixty years ago. In this system the maintenance of a strictly anaerobic environment was essential. This provided protection against a wide range of contaminants requiring atmospheric oxygen, and the principal hazard was contamination by bacteriophage.

The next major development was the adaptation of submerged-culture techniques for the production of penicillin, rapidly followed by processes for other antibiotics, vitamins, and amino acids, and the conversion of steroids. These processes have been developed over the past thirty years or so, and have attracted the combined attention of microbiologists, biochemists and engineers to resolve a variety of problems in order to improve both yields and process efficiency. More recently, a good deal of attention has been devoted to problems associated with the replacement of batch processes by continuous processes, and the utilization of gaseous and liquid hydrocarbons as substrates, instead of the traditional carbohydrates. The latter processes, in particular, have stimulated investigations into the use of fermenter configurations other than the agitated cylindrical vessel which has been almost universally adopted for aerobic microbial processes and for some anaerobic processes.

Among the new configurations, two in particular are the air-lift and the tower, have been exploited commercially and hence attracted the attention of research workers.

2.2. Fermenter design and requirements of the microbial system

The function of the fermenter is to provide an environment suitable for the controlled growth of a pure culture or of a defined mixture of organisms.

The materials of construction must be such that they will not adversely affect, nor be adversely affected by, the desired microbial activity, either by interaction with the fermentation medium or by harbouring unwanted organisms. They must be resistant to corrosion by the nutrient medium and products, and to the effects of sterilization temperatures. The actual construction of the equipment from suitable materials must also take account of these factors and of the stresses imposed by pressurization and the weight of the vessel contents.

There must be provision for the regulation of temperature and of the supply of air, for charging and discharging the vessel contents, for inoculation and also for sampling and for the control of pH and foaming. Frequently, even in batch wise systems, it will be necessary to provide for controlled addition of nutrients or other materials during the course of the fermentation. In continuous-culture systems additional facilities must be provided to control culture volume and medium flow-rate. Depending on the method of control, some system will be required to measure and control the cell concentration or the concentration of some rate-limiting substrate. These controls relate to the macroenvironment (the total culture) and only measure or control the conditions of the microorganisms in an indirect, empirical sense. Some control systems, such as those based on the rate of oxygen uptake, evolution of carbon dioxide or concentration of NADH, relate more directly to cell activity, but none gives a direct measure of the condition in the microenvironment, that is the environment in the immediate vicinity of t he cell.

It is an assumption implicit in all control systems that biochemically similar macroenvironments produce biochemically-similar microenvironments. Anomalies and difficulties which arise in scaling-up or in conducting a given fermentation in different types of equipment indicate that this assumption may be false. One possible source of anomalies is differences in the extent of cell aggregation, which, in biochemically-similar environments, may be strongly affected by hydrodynamic factors.

Another likely source of error lies in systems for assessing conditions in the macroenvironment. These will generally indicate a time-average value at a particular sampling or sensing point and may conceal considerable variations from one element of culture to another. Thus attempts to control conditions in the culture as a whole may have somewhat attenuated effects on the microenvironment. At the same time, the activity of the microorganism will itself have effects on the microenvironment, both directly and through

effecting changes in the macroenvironment. The direct effects on the microenvironment will result from:

(a) Consumption of substrates

(b) Output of products

(c) Production of heat

(d) Aggregation

Factors (a) and (b) will result in deficiency of substrate and accumulation of product, with the possibility of resultant inhibition, unless there is adequate interchange between the region immediately adjacent to the cell and the bulk of the fluid.

A similar interchange is also necessary to prevent significant and possibly damaging increase in temperature. Aggregation of cells will adversely affect both heat and mass transfer, partly by reducing the area available for transfer, partly by increasing the length of the transfer paths and partly by reducing the transfer coefficients.

The factors within the control of the designer and operator are:

(a) System geometry

(b) Aeration rate

(c) Intensity of agitation

(d) Temperature

(e) Pressure

(f) Nutrient supply

(g) pH and other parameters involving specific ions

(h) Dilution rate in continuous flow systems

(i) Foaming

When considering the design of vertical stirred vessels, the main variables in geometry are the height-to-diameter ratio, the number, type, dimensions and positions of impellers, the number and breadth of baffles, and the design and location of coils for heating and cooling. In relation to power input, the geometrical specification for the impeller and the degree of baffling cannot be divorced from the speed of rotation. Some account must also be taken of the rate of aeration and degree of gas hold-up in the system, since this will affect the density of the culture which is the most significant property governing overall power input under conditions of fully-developed turbulence.

Control of the other factors mentioned above will have only minor effects on vessel design arising from the introduction of facilities for sensing, sampling, addition and withdrawal. A major advantage of the stirred vessel over other designs is the degree of operational flexibility which it provides even when installed. This arises largely because mixing and mass transfer are influenced both by the action of the impeller(s) and by the rate of aeration, which can, within fairly wide limits, be varied independently, albeit at the expense of changing the impeller speed or geometry. By contrast, in the air-lift mixing and mass transfer

are both dependent in a given piece of equipment, on the rate of aeration, and cannot readily be varied independently. In the tower fermenter, unless provision is made for re-cycling, mixing and mass transfer are strongly affected by the dilution rate, which must be determined on biological grounds, whereas the dilution rate can be fixed independently of considerations of aeration and mass transfer in stirred vessels and air-lift fermenters.

The objective of fermenter design and operation is to ensure that the desired activity of the microorganisms concerned shall not be restricted by the characteristics of the equipment. It is, therefore, useful to consider the problem in terms of the physical processes which might limit microbial activity. Fermenters are constructed from materials that can withstand repeated steam sterilization and cleaning cycles. Materials contacting the fermentation medium and broth should also be non-reactive and non-absorptive. **Glass** is used to construct fermenters up to about 30 liters capacity. The advantages of glass are that it is smooth, non-toxic, corrosion-proof and transparent for easy inspection of the vessel contents. Because entry ports are required for medium, inoculum, air and instruments such as pH and temperature sensors, glass fermenters are usually equipped with stainless steel head plates containing many screw fittings. Most pilot- and large-scale fermenters are made of **corrosion- resistant 'stainless steel**, although mild steel with stainless steel cladding has also been used. Cheaper grades of stainless steel may be used for the jacket and other surfaces isolated from the broth. Interior steel surfaces are polished to a bright 'mirror' finish to facilitate cleaning and sterilization of the reactor; welds on the interior of the vessel are ground flush before polishing. Electropolishing is preferred over mechanical polishing which leaves tiny ridges and grooves in the metal to accumulate dirt and microorganisms.

The fermenter is equipped with **sparger, impeller and baffles** that determine the effectiveness of mixing and oxygen mass transfer in stirred bioreactors. Aerobic cultures are continuously sparged with air; however, most components of air are inert and leave directly through the exhaust gas line.

Baffles, which are vertical strips of metal mounted against the wall of the tank, are installed to reduce vortexing and swirling of the liquid. Baffles are attached to the tank by means of welded brackets; four equally-spaced baffles are usually sufficient to prevent vortex formation. The optimum baffle width depends on the impeller design and fluid viscosity but is of the order 1/10-1/12 the tank diameter. Efficient mixing in the fermenter can be achieved by the **impellers**. In standard designs the impeller is located about one impeller diameter, or one-third the tank diameter, above the bottom of the tank. Mixing is facilitated when circulation currents below the impeller are smaller than those above; fluid particles leaving the impeller at the same instant then take different periods of time to return and exchange material. Rate of distribution throughout the vessel is increased when upper and lower circulation loops are asynchronous. Another device for improving mixing is multiple impellers, although this requires an increase in power input.

During fermentation, the fermenter vessel containing medium is heated using steam and held at the sterilization temperature for a period of time; cooling water is then used to bring the temperature back to normal operating conditions. In addition, metabolic activity of cells generates a substantial amount of heat in fermenters; this heat must be removed to avoid temperature increases. Equipment used for heat exchange in bioreactors usually takes one of the forms i.e., either fermenter may have an external jacket or coil through which steam or cooling water is circulated. Alternatively, helical or baffle coils may be located internally. External jackets on bioreactors provide sufficient heat-transfer area for laboratory and other small-scale systems; however they are likely to be inadequate for large-scale fermentations. Internal coils are frequently used in production vessels; the coil can be operated with high liquid velocity and the entire tube surface is exposed to the reactor contents providing a relatively large heat-transfer area.

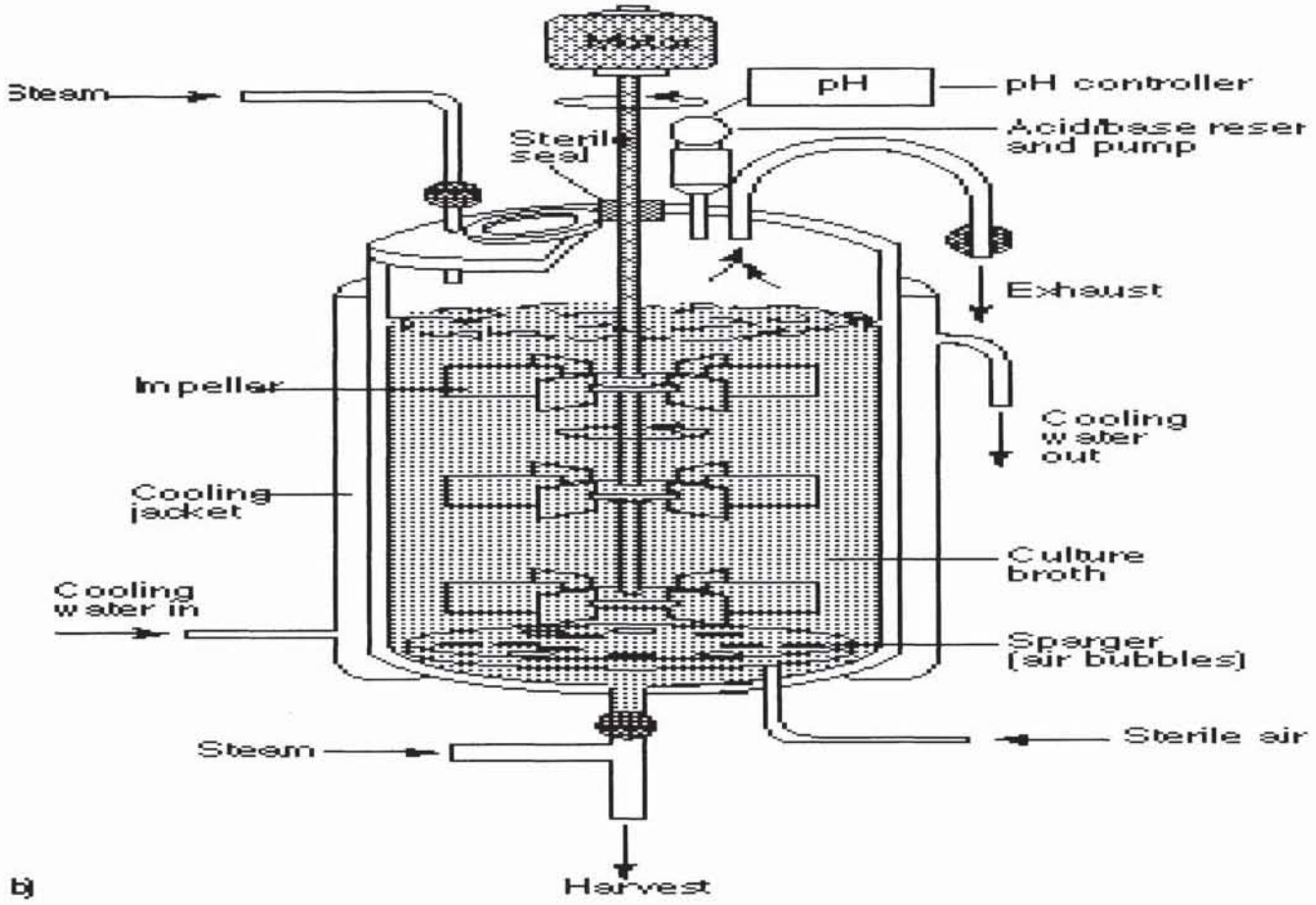


Figure 2.1 A detailed diagram showing different parts of fermenter

As in fermenter cells are provided with nutrients and very carefully controlled environment to keep them in desired growth stage. Nutrients and other materials are fed in by valve operated pipelines. Conditions in the fermenter are carefully monitored to regulate cell growth. Fermenter and all pipe work must be sterile before fermentation begins. This is usually achieved by flushing the whole system with superheated steam before the production begins.

Interior is monitored by sterilizable probes which record temp., pressure, stirrer speed, pH, oxygen and carbon dioxide levels. These are all recorded and electronic control systems with automatic valves will regulate them. E.g. if medium becomes too acidic, bases can be added from a reservoir to correct the pH Incoming air is filtered and pumped into the base of the fermenter – a valve releases the pressure from the top f the tank.

Antifoam Agents: Most cell cultures produce a variety of foam-producing and foam-stabilizing agents, such as proteins, polysaccharides and fatty acids. Foam build-up in fermenters is very common, particularly in aerobic systems. Foaming causes a range of reactor operating problems; foam control is therefore an important consideration in fermentation design. Excessive foam overflowing from the top of the fermenter provides a route for entry of contaminating organisms and causes blockage of outlet gas lines. Liquid and cells trapped in the foam represent a loss of bioreactor volume; conditions in the foam may not be favourable for metabolic activity. In addition, fragile cells can be damaged by collapsing foam. Addition of special antifoam compounds to the medium is the most common method of reducing foam build-up in fermenters.

Mechanical foam breakers, such as high-speed discs rotating at the top of the vessel and centrifugal foam destroyers, are suitable when foam development is moderate. However, some of these devices need large quantities of power to operate in commercial-scale vessels; their limited foam-destroying capacity is also a problem with highly-foaming cultures. In many cases, use of chemical antifoam agents is unavoidable.

Batch bioreactions

The majority of bioreactions are batch-wise. The first phase of batch bioreaction is commonly sterilization, after which the sterile culture medium is inoculated with microorganisms that have been cultivated to achieve a specific result.

During this dynamic reaction period, cells, substrates (including the nutrient salts and vitamins) and concentrations of the products vary with time. Proper mixing keeps the differences in composition and temperature at acceptable levels. To promote aerobic cultivation, the medium is aerated to provide a continuous flow of oxygen. Gaseous byproducts formed, such as CO2, are removed, and aeration and gas-removal processes take place semi continuously. Next, an acid or alkali is added if the pH needs to be controlled. To keep foaming to acceptable levels, antifoaming agents may be added when indicated by a foam sensor.

One of the first types of batch systems is the tray fermenter, used in the early days of commercial aerobic bioreactions for products such as citric acid and penicillin. In this system, the trays are loaded with the culture medium and the organisms, and the airflow produces the bioreaction, during which exhaust gas is discharged. When the bioreaction is complete, end product is removed from the trays. Because this method is inefficient for

producing large commercial quantities, it fell quickly to the wayside with the emergence of submerged tank systems, which are designed to handle significantly higher volumes.

Overall, batch bioreaction systems provide a number of advantages, including:

- Reduced risk of contamination or cell mutation, due to a relatively brief growth period.
- Lower capital investment when compared to continuous processes for the same bioreactor volume.
- More flexibility with varying product/biological systems.
- Higher raw material conversion levels, resulting from a controlled growth period.

The disadvantages include:

- Lower productivity levels due to time for filling, heating, sterilizing, cooling, emptying and cleaning the reactor.
- Increased focus on instrumentation due to frequent sterilization.
- Greater expense incurred in preparing several subcultures for inoculation.
- Higher costs for labor and/or process control for this non-stationary process.
- Larger industrial hygiene risks due to potential contact with pathogenic microorganisms or toxins.

Common applications for batch bioreactors include:

- Products that must be produced with minimal risk of contamination or organism mutation.
- Operations in which only small amounts of product are produced.
- Processes using one reactor to make various products.
- Processes in which batch or semi continuous product separation is adequate.

Continuous bioreactions

The defining characteristic of continuous bioreaction is a perpetual feeding process. A culture medium that is either sterile or comprised of microorganisms is continuously fed into the bioreactor to maintain the steady state. Of course, the product is also drawn continuously from the reactor. The reaction variables and control parameters remain consistent, establishing a time-constant state within the reactor. The result is continuous productivity and output.

These systems provide a number of advantages, including:

- Increased potential for automating the process.
- Reduced labor expense, due to automation.
- Less non-productive time expended in emptying, filling and sterilizing the reactor.
- Consistent product quality due to invariable operating parameters.
- Decreased toxicity risks to staff, due to automation.
- Reduced stress on instruments due to sterilization.

The disadvantages of continuous bioreactors include:

- Minimal flexibility, since only slight variations in the process are possible (throughput, medium composition, oxygen concentration and temperature).

• Mandatory uniformity of raw material quality is necessary to ensure that the process remains continuous.

• Higher investment costs in control and automation equipment, and increased expenses for continuous sterilization of the medium.

• Greater processing costs with continuous replenishment of non-soluble, solid substrates such as straw.

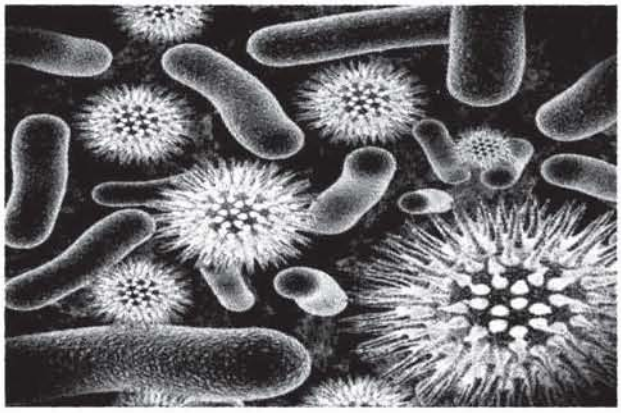
Continuous bioreaction is frequently used for processes with high-volume production; for processes using gas, liquid or soluble solid substrates; and for processes involving microorganisms with high mutation-stability.

Review Question:

- Define Bioreactor
- Give the advantage of using glass for constructing fermenter body?
- What are impellers? Why they are used in fermenters?
- What is role of baffles in fermenter?
- What is the location of sparger in fermenter?
- Give the advantages of continuous and batch fermenter.
- What are probes? How they help in fermenter operation?
- Comment on computer controlled fermentation process.
- What are antifoam agents?
- Enlist the different types of fermenter.
- Differentiate between continuous and batch fermenter.
- Discuss the importance of cooling coils in fermenter.
- How heat is generated in fermenter?
- Draw a well labeled diagram of a fermenter.

User's Notes

SECTION C:
INDUSTRIALLY IMPORTANT MICROBES



Introduction:

Industrial Microbiology - Use of microbes to obtain a product or service of economic value constitutes industrial microbiology. Any process mediated by or involving microorganisms in which a product of economic value is obtained is called fermentation. The terms industrial microbiology and fermentation are virtually synonymous in their scope, objectives and activities. The microbial product may be microbial cells (living or dead), microbial biomass, and components of microbial cells, intracellular or extracellular enzymes or chemicals produced by the microbes utilizing the medium constituents or the provided substrate.

The services generated by microorganisms range from the degradation of organic wastes, detoxification of industrial wastes and toxic compounds, to the degradation of petroleum to manage oil spills, etc. Industrial microbiology also encompasses activities like production of biocontrol agents, inoculants used as biofertilizers, etc.

Obviously, the scope and activities of industrial microbiology are too extensive to be covered in any detail in a book like this scope; therefore, the coverage in this chapter remains generalized and rather elementary.

The activities in industrial microbiology begin with the isolation of microorganisms from nature, their screening for product formation, improvement of product yields, maintenance of cultures, mass culture using bioreactors, and usually end with the recovery of products and their purification.

Microbial Products of Potential Importance -

Product / Activity	**Examples**
Products	
1. Amino acids	L-glutamic acid, L-lysine
2. Antibiotics	Streptomycin, penicillin, tetracyclines, polymyxin
3. Beverages	Wine, beer, distilled beverages
4. Biodegradable plastic	β-polyhydroxybutyrate
5. Enzymes	Amylase, proteases, pectinases, invertase, cellulase
6. Flavouring agents	Monosodium glutamate, nucleotides
7. Foods	Cheese, pickles, yoghurt, bread, vinegar
8. Gases	CO_2, H_2,CH_4
9. Organic acids	Lactic, citric, acetic, butyric, fumaric
10. Organic solvents	Acetone, ethanol, butanol, amyl alcohol
11. Others	Glycerol, fats, steroids, gibberellins
11 a. Vitamins	B12, riboflavin, A
12. Recombinant proteins	Insulin, interferon, subunit vaccines
13. Substrates	A wide range of compounds used for chemical syntheses of

	valuable products.
Cells/Biomass	
14. Biomass	Food and feed yeast, other organisms used as single cell protein (SCP)
15. Cells	Biofertilizers, biocontrol agents, bacterial insecticides, mycorrhyzae
16. Vaccines	A variety of viral and bacterial vaccines
Activities	
Biotransformation	Steroids, antibiotics D-sorbitol
Degradation	Disposal of biological and industrial wastes, detoxification of toxic compounds, petroleum
Solubilization/accumulation	Improved recovery of oil and metals, discovery of new oil reserves, removal of toxic metals

The microorganisms of industrial importance are, generally, bacteria, actinomycetes, fungi and algae. These organisms occur virtually everywhere, e.g., in air, water, soil, surfaces of plants and animals, and plant and animals tissues. But most common sources of industrial microorganisms are soils, and lake and river mud.

Often the ecological habitat from which a desired microorganism is more likely to be isolated will depend on the characteristics of the product desired from it, and of process development. For example, if the objective is to isolate a source of enzymes, which can withstand high temperatures, the obvious place to look will be hot water springs.

A variety of complex isolation procedures have been developed, but no single method can reveal all the microorganisms present in a sample. Many different microorganisms can be isolated by using specialized enrichment techniques, e.g., soil treatment (UV irradiation, air drying or heating at 70-120°C, filtration or continuous percolation, washings from root systems, treatment with detergents or alcohols, preinoculation with toxic agents), selective inhibitors (antimetabolites, antibiotics, etc.), nutritional (specific C and N sources), variations in pH, temperature, aeration, etc.

The enrichment techniques are designed for selective multiplication of only some of the microorganisms present in a sample. These approaches however take a long time (20-40 days), and require considerable labour and money.

The main isolation methods used routinely for isolation from soil samples are: sponging (soil directly), dilution, gradient plate, aerosol dilution, flotation, and differential centrifugation. Often these methods are used in conjunction with an enrichment technique.

3.1 Aim: Isolation of Amylase producers from the environment.

Theory:

Amylases are enzymes that break down starch or glycogen. Amylases are produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to the outside of their cells to carry out extra-cellular digestion. When they have broken down the insoluble starch, the soluble end products such as (glucose or maltose) are absorbed into their cells. Amylases are classified based on how they break down starch molecules:

i. α-amylase (alpha-amylase) - Reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied sized chains of glucose.
ii. ß-amylase (Beta-amylase) - Breaks the glucose-glucose bonds down by removing two glucose units at a time, thereby producing maltose.
iii. Amyloglucosidase (AMG) - Breaks successive bonds from the non-reducing end of the straight chain, producing glucose Many microbial amylases usually contain a mixture of these amylases.

Humans exploit microbial amylases for the following purposes:

1. High Fructose Corn syrup preparation
2. Additives to detergents for removing stains
3. Saccharification of starch for alcohol production
4. Brewing.

The soil contains a rich deposit of both bacteria and fungi, which produce amylases. Starch hydrolyzing fungi or bacteria could be isolated from the soil, foods or could be purchased. Buying saves time and ensures a high yielding strain. However, isolating could be fun, and constitutes an additional lab. Although many microorganisms produce this enzyme, the ones most commonly used for their industrial production are *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquifaciens* and *Aspergillus niger.*

Figure 3.1 Plate showing zone of starch hydrolysis

Source: homepages.wmich.edu/~rossbach/bios312/LabProc...

Materials Required:

Autoclave or pressure cooker, Hot Plate or Microwave oven, Nutrient Agar, Potato Dextrose Agar, Soluble starch (1%), Gram's Iodine. Hand trowel or disposable spoons. Sterile pipettes (One each of 10 mL, 5 mL and 1 mL), Pipette pumps, Six bottles of sterile water containing 90 mL each, Sterile Glass Petri dishes or Pre-sterilized disposable Petri-dishes, Bunsen burner and matches, Glass spreader

Procedure:

- Suspend about 10 grams of either soil or rotten potato in 90 mL sterile distilled water and mix properly
- Pipette 10 mL of the above and transfer to another 90 mL of water
- Dilute further in two more 90 mL sterile water blanks

For Fungi: Spread 0.1 mL from the dilutions on Potato Dextrose Agar plates (fortified with 0.1 mg/mL streptomycin sulfate) with a glass spreader. (The glass spreader is quickly sterilized by dipping in 95% ethanol and putting in the flame, so that the alcohol burns off) Incubate at room temperature for about 3 days

For Bacteria: Spread 0.1 mL of the diluted samples on Nutrient Agar plates containing 1 % w/v soluble starch and incubate at 30°C for 24 hours

- Starch-producing colonies will have an area of clearing around them.
- Confirm by flooding plates with Gram's iodine.
- Transfer distinguishable, amylase-producing fungi to fresh plates of Potato Dextrose agar
- Containing 1 % starch, using a sterilized dissecting needle. For bacteria, streak on a fresh
- Plate of Nutrient Agar containing 1 % starch.
- Transfer your isolated amylase-producing fungi to Potato Dextrose Agar slants, and the bacteria to Nutrient Agar. Allow bacteria to grow for 24 hours and fungi to grow for 72 hours, then store in the refrigerator until needed.

Observation Table:

Sample	Isolate	Amylolytic/ Non-amylolytic	Morphological/ Biochemical characteristics of isolates

Results and Interpretation:

3.2 Aim: Production and assaying of microbial proteases.

Theory:

Proteases are the key enzyme in the industrial application. Microbial proteases play important role in biotechnological process with worldwide sale representing about 60% of the total enzyme marked. A number of bacteria, fungi and yeast have been reported for protease production. Many of the organisms produce more than one kind of protease. The type of proteolytic enzyme formed may depend on the composition of the medium. A protease (also termed peptidase or proteinase) breaks down proteins. A protease is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases work best in acidic conditions except alkaline proteases. It's optimal activity shown in alkaline (basic) pH.

The alkaline proteases are highly exploited enzymes in food processing, leather, detergent, pharmaceutical, diagnostics, waste management, silver recovery medical purposes as well as feeds and chemical industries. The protease enzyme constitutes two thirds of total enzymes used in various industries. Of all proteases, alkaline proteases produced by Bacillus species are of great importance in detergent industry due to their high thermostability and pH stability.

Figure 3.2 Plate showing caseinolytic activities of *Bacillus subtilis*

Source: http://academic.pgcc.edu/~kroberts/web/exoenzymes/exoenzymes.htm

For production of enzyme for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. The microbial proteases of Aspergillus species, in particular, have been studied in detail since they are known for their capacity to secrete high levels of enzymes in their growth environment. Several of these secreted enzymes, produced in a large-scale submerged fermentation, have been widely used in the food and beverage industry for decades.

Materials Required:

Aspergillus niger or *Bacillus subtilis,* Potato Dextrose Agar Medium, Nutrient Agar, Petri plate sterilized, Saline tubes, Incubator, Inoculating loop, Laminar air flow, Enzyme production medium

Procedure:

Preparation of enzyme production medium:

A. Production medium for *Bacillus subtilis:*

Ingredients	Composition (g/ L)
Peptone	1
NaCl	5
Skim Milk	10
Agar	20
pH 7.0-7.2 at 25°C	

- Sterilize peptone separately and add aseptically to the flask containing the liquid medium, after cooling.
- Inoculate the medium (50m1 in 250m1 conical flask) with 1ml of an overnight culture of *Bacillus subtilis.*
- Incubate at 50°C in a rotary shaker at 150 rpm for 12hr.
- At time intervals determine the turbidity of the culture measuring the increase in optical density at 450 nm with a spectrophotometer.

B. Production medium for *Aspergillus niger*

Ingredients	Composition (g/ L)
Ammonium sulphate	1.0
Magnesium Sulphate heptahydrate	5.0
Potassium di-Hydrogen Phosphate	5.0
Ferrous Sulphate heptahydrate	0.005
Glucose	10.0
Jowar seeds	
pH 5.0 at 25°C	

- Inoculate the liquid medium with overnight grown Aspergillus culture.
- Examine fermentation duration for 24 to 120 hrs.
- Kept the culture flask on rotary shaker at 300 rpm at 28°C.

Product recovery and purification:

Protease is an extracellular enzyme so its recovery is quite easy. After incubation centrifuge the production medium at 12,200 rpm for 15 min to separate the cells. Collect the supernatant as it will contain the crude enzyme and store at 4°C till further use.

Enzyme assay:

Casein solution of 2% (1 ml) was incubated with 0.1 ml of enzyme solution and 0.9 ml of sodium phosphate buffer (pH 7) for 10 minutes at 40°C. The reaction was stopped using 10% TCA solution. After 20 minutes the mixture was centrifuged 10,000 rpm for 5 minutes. The colour intensity of supernatant was read at 280 nm.

The enzyme activity was calculated from standard curve of L-Tyrosine. The serial increase in concentration of tyrosine (10-100tg/ml) was read at 280 nm for the standard graph. Enzyme activity was depends on the temperature, particular pH, substrate concentration and the active site of enzyme.

Observation Table:

Sample	Isolate	Proteolytic/ Non-proteolytic	Morphological/ Biochemical characteristics of isolates

Results and Interpretations:

3.3 Aim: Production and assaying of microbial lipases.

Theory:

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, in the oleochemical industry, dairy industry, agrochemical industry, bakery (flavour improvement), beverages (improved aroma), paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes. One limiting factor is a shortage of lipases having the specific required processing characteristics. An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases. The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year. Most of the industrial microbial lipases are derived from fungi and bacteria.

Some commercially available fungal lipases are *Candida rugosa*, *Candida antartica*, *Thermomyces lanuginosus*, *Rhizomucor miehei* and Bacterial lipases are *Burkholderia cepacia*, *Pseudomonas alcaligenes*, *Pseudomaonas mendocina* and *Chromobacter viscosum*.

Figure 3.3 Plate showing lipidolysis by *Staphylococcus epidermidis* in spirit blue agar

Source: http://academic.pgcc.edu/~kroberts/web/exoenzymes/exoenzymes.htm

Materials Required:

Media, Petriplates, Normal Saline (9 mL), Erlenmeyer flask (250 mL), wax marking pencil, sterile pipettes (1 and 10 mL), mechanical pippetor.

Medium A:

Ingredients	Composition (g/ L)
Olive oil	20.0
Gall powder	10.0
Ammonium Sulphate	5.0
Magnesium Sulphate	1.0
Agar	20.0

* Heat and emulsify the mixture, and adjust the pH to 8.7 with Na2CO3.

Medium B

Ingredients	Composition (g/ L)
Roasted soybean meal	20.0
Corn steep liquor (CSL)	10.0
Wheat starch	10.0
Di Potassium hydrogen phosphate	5.0
Adjust the pH to 8.7 with NaOH	

Procedure:

i. Isolation and cultivation:

- Suspend the soil samples in water and spread on plates of medium A
- Incubate the plates at 30°C for 72hr.
- Transfer the microorganisms which formed clear zones around the colonies on the plates to stock culture slants of medium A and nutrient agar.
- In order to examine lipase production, take 7ml of medium B in a test tube (18 x 180 mm) and inoculate with a loopful of an isolated microorganism and culture at 30°C for 40hr with shaking.
- Use culture broth for the assay of lipase activity.

ii. Assay method for lipase activity:

- Emulsify 10 ml of olive oil 90ml of a 10% gum aerobic solution.
- Make a reaction mixture, consisting of 2.5 ml of olive oil emulsion, 2.0ml of glycine-NaOH buffer (1 m, pH 8.7), 0.5ml of distilled water and 0.1 ml of enzyme solution.
- Place reaction mixture in a 25ml glass stoppered test tube and incubate for 10 min at 37°C.
- Add 15ml of a heptane-isopropanol mixture (11:4) to the reaction mixture.
- Shake vigorously for 30 sec, and allow to stand for more than 30 min.
- Pipette 5 ml of the upper layer into a test tube (20 x 130) and titrate under a nitrogen gas stream against 0.01 N ethanolic potassium hydroxide solution with thymol blue as an indicator.

- Terminate the reaction by the addition of 1 ml of 2N sulphuric acid.
- As a blank test, add 2N sulphuric acid solution to the reaction mixture before the addition of enzyme solution.
- Subtract the titration value of the blank test from the previous titration value.
- Calculate the amount of liberated fatty acid from the standard line prepared using a known amount of palmitic acid. One lipase unit is defined as the amount of enzyme which liberates 1 mmol of fatty acid in one minute under the assay conditions described above.

Observation Table:

Sample	Isolate	Lipidolytic/ Non-lipidolytic	Morphological/ Biochemical characteristics of isolates

Results and Interpretations:

3.4 Aim: Isolation of Antibiotic Producing Microbes from Soil.

Theory:

Although pharmaceutical companies currently do much of their drug discovery using computer modeling, traditionally, antibiotics were discovered by screening. In this approach, a large number of isolates of possible antibiotic producing microorganisms are obtained from nature in pure culture, and these isolates are then tested for antibiotic production.

Many soil microorganisms produce anti-bacterial or anti-fungal chemicals, compounds which are termed antibiotics. It is relatively simple to identify antibiotic producing microorganisms from isolated soil bacteria or fungi by testing them against standard microbial strains on Petri dishes containing a nutrient medium. In this activity antibiotic microorganisms will be identified by their ability to produce compounds which inhibit the growth of a common bacterium, non pathogenic *staphylococcus*.

Soil bacteria known as Streptomyces produce many clinically useful antibiotics. More than 500 Streptomyces species are recognized, and nearly half of them produce antibiotics, according to some studies. Here is a method for isolating and screening antibiotic producers.

This activity is intended to demonstrate that common microorganisms isolated from soils are sources of antibiotics. A simple growth method can demonstrate how the effect of diffusible compounds from different microorganisms can be used to observe the inhibition of growth of a common bacterium associated with humans. This same activity scaled up many times is how the pharmaceutical industry discovers new antibiotics for human use.

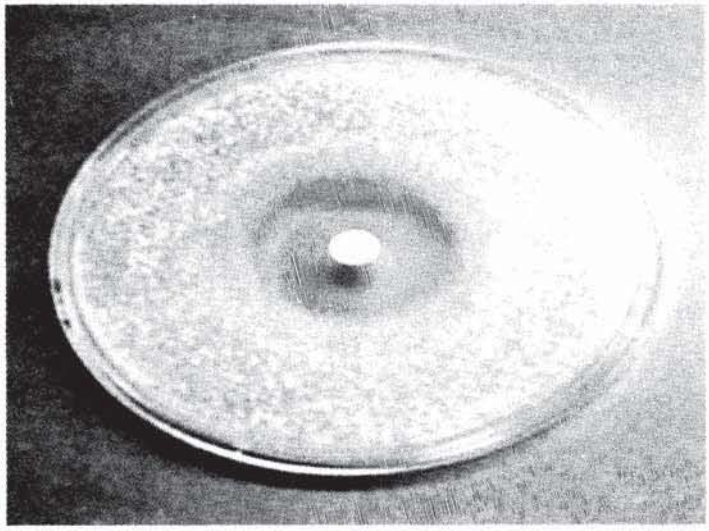


Figure 3.4 Plate showing antibiotic producing *Rhodococcus fascians*

Source: www.technologyreview.com/biomedicine/20425/

Material Required:

Sterile Petri dishes, sterile soft agar and agar (Muller Hinton agar), inoculating loop, Bunsen burner, Laminar-air flow. Disposable inoculating loops, Petri dishes containing Mueller-Hinton agar, microbial culture of non pathogenic *Staphylococcus* spp., soil sample

Procedure:

- Take a soil sample and serially dilute it upto 10^{-8} dilutions.
- Transfer one ml of diluted sample and pour plate with the agar
- Incubate the plate at 35°C in incubator for 24-48 hours
- After 24-48 hours incubation inspect the plate for the growth of bacteria.
- Make replica copy of the standard plate by touching the loop to specific colonies and mark them on two replica plates
- On one of the plate, indicator organism seeded in soft agar will be poured (20 ml soft agar with 50µl of culture)
- Incubate the plate at 35°C in incubator for 18 hours and look for the zone of clearance.
- Observe the colonies with zone of clearance around them and cultivate the respective colonies from second replica plate for further checking the anti microbial activity towards other indicator microorganisms.

Observation Table:

Sample	Isolate	Antibiotic Producers (inhibition zone in dia)	Morphological/ Biochemical characteristics of isolates

Results and Interpretations:

3.5 Aim: Isolation and screening of *Streptomyces* species as antibiotic producers.

Material required:

Streptomyces-selective media, soil sample, dilution blanks (9 mL), Sterile Petri plates, Incubator (at 35-37°C), Wax marking pencil, sterile pipettes (1mL, 10 mL) and mechanical pipettor, Soft agar seeded with fresh *Staphylococcus epidermidis* culture, other non pathogenic cultures, Muller-Hinton agar, sterile inoculating loop.

Procedure:

i. Isolation of Streptomyces species from soil

- Add a gram of soil to 9 mL sterile water and mix thoroughly.
- Dilute the soil mixture further by transferring 1 mL down a series of dilution tubes.
- Select the most dilute mixtures and add 1 mL of the liquid to plates containing Streptomyces-selective media.
- Spread the sample evenly, and then incubate for 5-7 days at room temperature.

ii. Testing for antibiotic production

- To test for antibiotic production, overlay the plates with an indicator organism, such as the non pathogenic bacterium *Staphylococcus epidermidis*.
- Incubate the plated at 37°C for 24 hours.
- Check the plates for zone of inhibition surrounding potential antibiotic-producing organisms.

iii. Test to check the antibiotic spectrum of a suspected antibiotic producer

- Streak the suspected antibiotic producer across a fresh Muller-Hinton agar plate.
- Incubate the plate to permit bacterial growth and antibiotic production.
- Cross-streak test organisms along the plate.
- Incubate the plates at 37°C for overnight. And check for growth inhibition
- Observe the growth of the test organisms.

Observation Table:

Sample	Isolate	Antibiotic producer/ non- antibiotic producer	Antibiotic spectrum

Results and Interpretations:

3.6 Aim: Production of Nisin from *Lactococcus lactis*.

Theory:

Nisin is a naturally occurring antimicrobial peptide and was discovered in 1928. Nisin is a polycyclic peptide antibacterial with 34 amino acid residues used as a food preservative. It contains the uncommon amino acids lanthionine, methyllanthionine, didehydroalanine and di-dehydroaminobutyric acid. These unusual amino acids are introduced by posttranslational modification of the precursor peptide.

Nisin is produced by fermentation using the bacterium Lactococcus lactis. Commercially, it is obtained from the culturing of Lactoccus lactis on natural substrates, such as milk or dextrose, and is not chemically synthesized. It is used in processed cheese, meats, beverages, etc. during production to extend shelf life by suppressing Gram-positive spoilage and pathogenic bacteria. While most bacteriocins generally inhibit only closely related species, Nisin is a rare example of a "broad-spectrum" bacteriocin effective against many Gram-positive organisms, including lactic acid bacteria (commonly associated with spoilage), *Listeria monocytogenes* (a known pathogen), etc. However, when coupled with the chelator EDTA, Nisin has also been known to inhibit Gram-negative bacteria, as well. Nisin is soluble in water and can be effective at levels nearing the parts per billion ranges. In foods, it is common to use

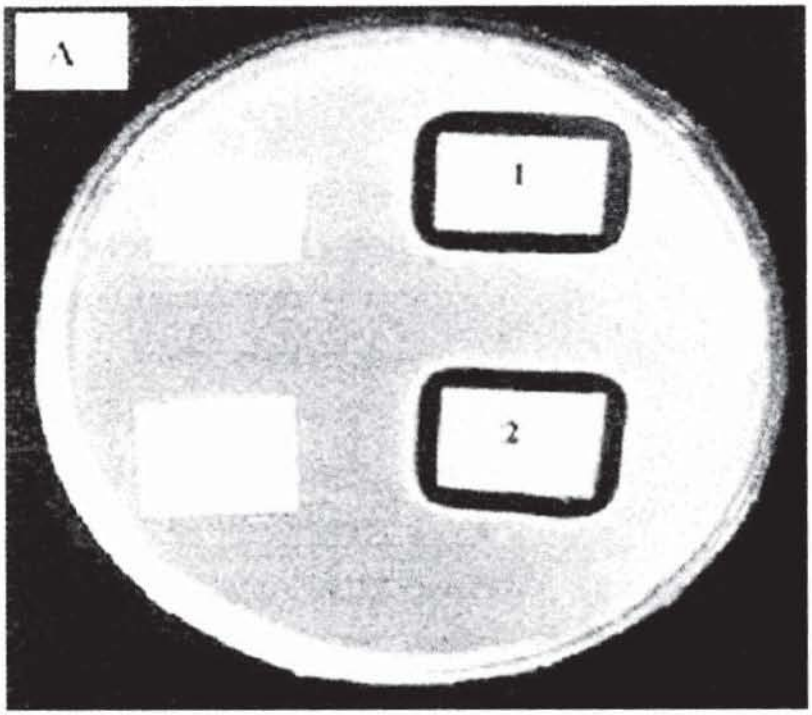


Figure 3.6 Plate showing antibacterial activity of Nisin

Source: www.teagasc.ie/.../4541/eopr-4541.asp

Nisin at levels ranging from ~1-25ppm, depending on the food type and regulatory approval. Due to its naturally selective spectrum of activity, it is also employed as a selective agent in microbiological media for the isolation of gram-negative bacteria, yeast, and moulds. Subtilin and Epidermin are related to Nisin. All are members of a class of molecules known as lantibiotics. Nisin solubility and stability increases substantially with increase in acidity.

Nisin is stable at pH 2 and can be autoclaved at 121°C. Under alkaline pH there is an increasing loss of activity, with complete inactivation after 30 min at 63°C at pH 11.

Materials required:

Lactococcus lactis, Pediococcus 34 (Indicator Strain), MRS broth, M17 broth, MRS agar, Nisin standard, Petriplates, Erlenmeyer flask (500 mL), wax marking pencil, waterbath, incubator, centrifuge, filters (0.22-0.45μ), sterile well borer, sterile tips (200 μL and 1.0 mL), autopipette (200 μL and 1.0 mL).

Procedure:

- Inoculate the culture in MRS broth and M17 broth.
- Prepare the nisin standard with a solution of 0.02 M HCl.
- Correlate several concentrations of a nisin standard (100 to 105 AU ml^{-1}) with the diameter of the inhibition halo (H diameter in mm).
- Determine the activity of nisin from sample cultures based on the calibration curves.
- For nisin activity detection, centrifuge the cell suspension at 12,000xg for 10 min at 25°C.
- Collect the supernatant and filter through a 0.22 mm membrane filter.
- Express the titers of nisin and quantify the release in culture media
- Express in arbitrary units (AU. mL-1 of medium) by the agar diffusion assay utilizing *Pediococcus 34* as a sensitive indicator microorganism.
- Grow *Pediococcus 34* in MRS broth and incubate at 100 rpm/37°C/24 h).
- Transfer a 1.5 ml aliquot of the suspension (OD660 = 0.7) and mix with 250 ml of soft agar (MRS broth with 0.8% of bacteriologic grade agar).
- Transfer each 20 ml of inoculated medium to Petri plates (100 mm diameter).
- After the solidification of agar, cut ~3 mm wells with a sterile metal pipe with 5 mm total diameter.
- Determine the activity of nisin from sample cultures based on the calibration curves.

Observations Table:

Media	Activity (AU/ mL)	Nisin (m/ L)	Specific production (mg/ DCWg)	Productivity (mg/ DCWg .h-1)
MRS				
M17				

Results and Interpretations:

3.7 Aim: Production of antimicrobial substances from lactic acid bacteria.

Theory:

Bacteria produce many inhibitory compounds which can inhibit the growth of potential spoilage or pathogenic microorganisms. These substances include organic acids (lactic acid, propionic acid, acetic acid etc.), hydrogen peroxide and diacetyl, bacteriocins like Nisin, Acidophilin, and Diplococcin etc. if these anti microbial compounds are produced in solid media. Then clear zones are developed around the test organisms growing in particular solid media.

Material required:

Lactic acid bacteria, Indicator Strain, MRS broth, MRS agar, Petriplates, Erlenmeyer flask (500 mL), wax marking pencil, waterbath, incubator, centrifuge, filters (0.22-0.45µ), sterile well borer, sterile tips (200 µL and 1.0 mL), autopipette (200 µL and 1.0 mL), Gram staining kit, glass slides

Procedure:

- Check the purity of cultures gram staining and check for their purity
- Inoculate the pure cultures obtained in above steps in MRS broth
- Incubate at 37°C for 48 hr.
- After 48 hours of growth centrifuge the culture at 7000 rpm for 10-15 minutes
- Collect the supernatant and sterilize using 0.22 mm membrane filters
- Use *S.aureus, E. Coli, B. subtilis* as test organisms.

Production of antimicrobial substances can be determined by 3 methods:

- Well Assay Method
- Spot/ Streak or Lawn Method
- Disk Method

i. Well Assay Method:

- Add 0.1 ml of test cultures in soft agar test tube
- Pour these test tubes on the solidified nutrient agar and MRS medium plate
- Incubate the plates at 37°C for 24 hrs.
- Cut wells on media using borer
- Add 0.1 ml of sterilized supernatant i.e. antimicrobial substance to each well and allow for diffusion under refrigeration conditions
- Incubate the plates at 37°C for 24 hr and observe for the zone of inhibition

ii. Streak plate method:

- Prepare MRS agar and nutrient agar plates
- Streak the culture to be tested for antimicrobial activity on the media

- Allow to grow at 37°C for 24 hrs
- Mix 0.1 ml of test culture in soft agar test tube and pour over the above streaked plates
- Again incubate at 37°C for 24 hrs
- Observe for a zone of clearance

iii. Disk Method:

- Prepare MRS agar and nutrient agar plates
- Mix 0.1 ml of test culture in soft agar test tube and pour on the above plates
- Soak filter paper disk (sterilized) in the supernatant obtained from different lactic acid bacteria cultures
- Impregnate these disk on the above plates
- Incubate at 37°C for 24 hrs
- Observe for a zone of clearance and measure the diameter

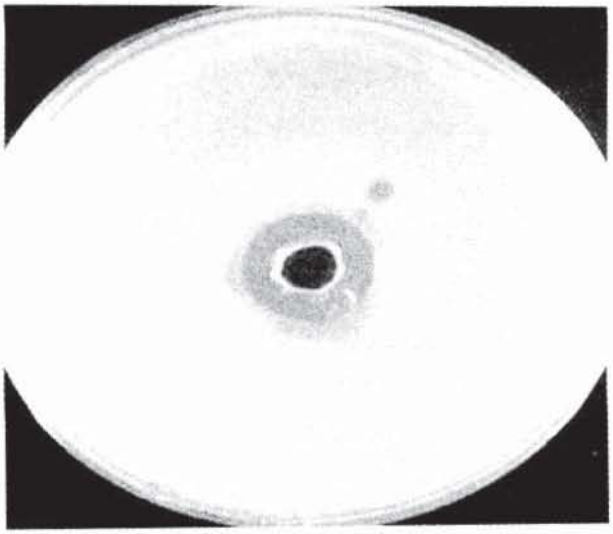


Figure 3.7 Agar well assays

Source: www.comcen.com.au/.../mastitis2006.htm

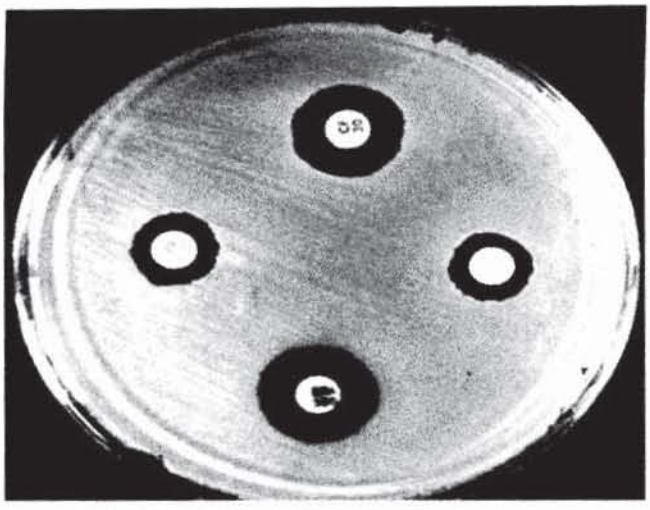


Figure 3.8 Disk diffusion methods

Source: faculty.mc3.edu/jearl/ML/ml-15.htm

Observation Table:

Lactic acid bacterial Isolates	Test organism 1 (zone of inhibition in diameter)	Test organism 2 (zone of inhibition in diameter)	Test organism 3 (zone of inhibition in diameter)

Results and Interpretations:

3.8 Aim: Isolation of psychrophiles from milk samples.

Theory:

Psychrophilic bacteria have been defined as bacteria that grow well at 0°C within 2 weeks and based partly on a standard method for determining psychrophilic bacteria, as bacteria that grow at a relatively rapid rate at 7.2°C, i.e., that form visible colonies on plates at this temperature in 10 days. Foster defines them as bacteria that grow relatively rapidly at 1.7 to 10°C, the temperature normally used in commercial holding and distribution channels. Some investigators prefer to describe such bacteria as psychrotrophic rather than psychrophilic, and there seems to be merit in using some term that indicates that they merely are able to grow at low temperatures rather than that they are cold-loving. Spoilage of pasteurized milk and milk products often results from the growth of heat-sensitive, gram-negative, nonsporeforming bacteria that enter products after pasteurization. Obviously, it would be a mistake to relax efforts to prevent nonsporeforming psychrophilic bacteria from getting into milk and dairy products after pasteurization. However, as attempts are made to extend the shelf-life of fluid dairy products, psychrophilic spore forming bacilli will become a greater potential problem. This is also important in regard to the use of dairy products in other foods, the development of aseptic filling, and the probability of a shift toward "sterilization" of milk and fluid milk products.

Material required:

Refrigerated Spoiled milk sample, Trypticase soya agar, Petriplates, dilution blanks (9 mL), wax marking pencil, Refrigerator, sterile pipettes, mechanical pipettor, Gram staining reagents, Schaeffer-Fulton staining reagents.

Procedure:

- Take 1 mL of the milk sample and dilute in 9 mL of dilution buffer upto 1000 to 10,000 times.
- Pour portion (1 mL) of the diluted sample from appropriate dilution to the sterile Petri plate under aseptic conditions and finally pour TSA on it.
- Incubate the plates at 4°C for 2 to 3 days.
- Transfer the colonies to TSA slants, incubated at 4°C, and store in a refrigerator for identification.
- Perform Gram staining and Spore detection test.

Observation Table:

Isolate	Gram reaction	Spore former	Other observations

Results and Interpretation:

3.9 Isolation of salt tolerant microorganisms from food samples.

Theory:

Halotolerant or halophilic microorganisms, able to live in saline environments, offer a multitude of actual or potential applications in various fields of biotechnology. The technical applications of bacteriorhodopsin comprise holography, spatial light modulators, optical computing, and optical memories. Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery. Other useful biosubstances are enzymes, such as new isomerases and hydrolases that are active and stable at high salt contents. Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented food and food supplements. The degradation or transformation of a range of organic pollutants and the production of alternative energy are other fields of applications of these groups of extremophiles.

Material required: Food/ milk sample, Nutrient agar with 2, 5 and 10% Sodium Chloride, Potato Dextrose agar with 2, 5 and 10% Sodium Chloride, Petriplates, Phosphate buffer saline (pH 7.0), wax marking pencil, sterile pipettes, mechanical pipettor, Gram staining reagents, Schaeffer-Fulton staining reagents, Lactophenol cotton blue stain, slides.

Procedure:

- Take 10 g of the food sample and dilute in 90 mL of Phosphate buffer saline (pH 7.0), make further dilutions upto 1000 to 10,000 times.
- Pour portion (1 mL) of the diluted sample from appropriate dilution to the sterile Petri plate under aseptic conditions and finally pour Nutrient agar (salt concentration 2, 5 and 10%) and Potato Dextrose agar (salt concentration 2, 5 and 10%) on it.
- Incubate the plates at 37°C for 2 to 3 days for bacteria and at 25-30°C for 5-7 days for fungi.
- Transfer the colonies to NA and PDA slants, incubated at respective temperatures, and finally store in a refrigerator for identification.
- Perform Gram staining, Spore detection test and Biochemical test for the identification according to Bergey's Manual.

Observation Table:

Isolate	Growth at salt concentration (in %)	Morphological and Biochemical analysis

Results and Interpretations:

3.10 Aim: Isolation of Sugar tolerant microorganisms from food samples.

Theory:

Osmotic concentrations of substrates where upon the micro-organisms grow help classifying them as under:

(i) Osmophobic: Those micro-organisms that die of dehydration if subjected to substrates of high osmotic concentrations.

(ii) Osmophilic: Those microorganism that best grown on substrates of high osmotic concentrations.

(iii) Halophilic: Halophiles represent those microbes that preferably grow in high osmotic concentrations produced by dissolved salts.

(iv) Osmoduric: These are those microbes that grow normally on substrates of moderate osmotic concentrations but, prove to be resistant to wide osmotic changes in their substratum.

It has been reported that high-sugar foods are sometimes spoiled by sugar tolerant or osmophilic yeasts. It has been proposed that the yeasts which can grow in the presence of 40-70 % (w/w) sugar be called sugar-tolerant yeasts or osmophilic yeasts. These yeasts grow very slowly near the minimal water activity (Aw) for growth, and very few species can grow in foods containing 65-70 % (w/w) sugar such as neriyokan (sweet bean curd). However, sudden changes in atmospheric temperature may increase the Aw of food by condensing moisture on the surface, thus accelerating microbial growth.

Most yeast grows best under aerobic conditions, but a large number of yeasts can also grow under low oxygen tension. Thus, yeasts may cause fermentative spoilage of products under low oxygen conditions, such as gas-exchange packaged foods and vacuum-packaged foods. This is an important problem in preserving high-sugar foods.

Material required:

Food sample, YM agar, Petriplates, wax marking pencil, Inoculating loop.

Procedure:

- **Isolate yeasts by direct streaking on medium containing 25 or 40% (w/w) glucose, 0.5% polypeptone, 0.3 % malt extract, 0.3 % yeast extract, and 2.5% agar.**
- **Purify isolated yeast strains by conventional streaking technique using the same media as used for isolation.**

i. Test of sugar-tolerance of the isolates:

- **Use the four kinds of agar media shown in Table 1 for testing sugar-tolerance. 2. Inoculate actively growing cultures on YM agar on to agar plates.**

Media
YM agar
25% (w/w) glucose-poly-peptone-yeast ext.-malt ext. agar

40% (w/w) glucose-poly-peptone-yeast ext.-malt ext. agar
50% (w/w) glucose-poly-peptone-yeast ext.-malt ext. agar

- **Tightly close the plates with rubber bands and incubate at 26°C for 2 weeks, and measure the diameter of each colony.**

ii. Selection of yeast strains for identification:

- Investigate all of the strains for fermentation of glucose, assimilation of maltose, galactose, sucrose, raffinose, lactose, and nitrate, growth in vitamin free medium, and growth at 30, 37, and 42°C.

Observations:

Isolate	% of sugar tolerated	Glucose fermentation	Maltose fermentation	Nitrate reduction	Growth in vitamin free media	Growth at different temperatures

Results and Interpretation:

Review Questions:

- What are secondary metabolites?
- Explain the role of bacteriocin in food industry?
- Although antibiotics are secondary metabolites but industrially they are produced in continuous phase fermenter. Explain.
- In principle, how do bacteriocins such as nisin function? What bacterial genus produces this important polypeptide?
- Approximately how many new antibiotics are being discovered per year? What portion of these is derived from Actinomycetes?
- Give some important specific compounds that are produced by the use of microorganisms.
- What is the importance of crowded plate technique?
- What is the function of lipases?
- How can one determine whether a bacterium is lipolytic?
- What are two functions of lipids in bacterial cells?
- Give some examples of foods that might be spoiled by lipolytic bacteria.
- How is the ability of certain bacteria to attack phospholipids related to pathogenicity?
- What is the difference between a triglyceride (triacylglycerol) and a phospholipid?
- What are several pathways that bacteria use to metabolize lipids?
- Describe the function of hydrolases.

- Describe the chemistry of starch hydrolysis.

- The chemical used to detect microbial starch hydrolysis on starch plates is _____.

- What does starch hydrolysis by a bacterium indicate?

- Amylase is an enzyme that attacks starch. The smallest product of this hydrolysis is called _____.

- How is it possible that bacteria may grow heavily on starch agar but not necessarily produce α-amylase?

- What are the ingredients of starch agar?

- Define the following terms:
  - amino acid
  - casein
  - hydrolysis
  - peptide bond
  - protease
  - protein
  - proteolysis

- How can plate count agar that contains milk be used to demonstrate proteolysis?

- Why are some bacteria able to grow on plate count agar that contains milk even though they do not produce any proteases?

- Draw the chemical reaction for proteolytic hydrolysis.
- Why was sterile skim milk used in this experiment?

- Why is milk white?

- What is the significance of psychrophiles in dairy industry?

- Explain the physiological adaptations of halophiles?

- Give the economic importance of sugar tolerant bacteria in food industry?

User's Notes

SECTION D:
MICROBIAL PRODUCTION

4.1 Aim: Production of lactic acid from whey.

Theory:

Whey is a by-product of the cheese industry which is often disposed as a waste in the past, causing high environmental contamination. Considerable efforts have been made over the past years to find new outlets for whey utilization and reduce environmental pollution. Liquid whey is composed of lactose (5%), water (93%), proteins (0.85%), minerals (0.53%) and a minimum amount of fat (0.36%). The main whey proteins are β-lactoglobulin (BLG) (58%) and α-lactalbumin (ALA) (13%) while immunoglobulins, serum albumins and proteose peptones are present in lesser extent.

Lactic acid bacteria (LAB) have been extensively used as starter cultures in the fermented food industry due to their metabolic activity on proteins, sugars and lipids, thus contributing to food digestibility and preservation as well as the improvement of texture and sensory profile of the end product. These microorganisms have complex nutritional requirements. The concentration of free amino acids in milk and whey are very limited, thus the sustained growth of LAB depends on the production of proteinases, peptidases and specific peptide and amino acid transport systems.

Whey fermentation by LAB could decrease the high lactose content in whey, producing mainly lactic acid and other metabolites such as aroma compounds contributing to the flavor and texture and increasing carbohydrate solubility and sweetness of the end product.

The homo-fermentative lactic acid bacteria catabolize 'glucose via the Embden-Meyerhof pathway. Two lactic acid molecules are produced from each molecule of glucose, typically with a yield of better than 90 g per 100 g glucose. Pentose sugars are also metabolized by some homo-fermentative species sterospecific and lactic acid are the products of this metabolism. Organisms may produce D (-), L (+) or DL-lactic acid.

$$C_6H_{12}0_6 \rightarrow 2CH_3CHOHCOOH$$

Method of Lactic Acid Commercial Production:

Pasteurized whey is inoculated with a starter culture containing lactobacilli, e.g., Lactobacillus *bulgaricus* and *L. delbrueckii*. To prepare a sufficient amount of inoculum for addition to main fermentation tank, the culture is successively transferred in sterile skin milk, pasteurized skin milk, and finally whey. The inoculum from whey is now added to the main fermentation tank containing large amount of whey. The temperature of the fermentation taken is maintained at 43-50^{0}C to prevent the growth of many extraneous microorganisms. During the fermentation, slurry of lime [calcium hydroxide; $Ca(OH)_2$] is added intermittently to prevent the accumulation of acid otherwise the latter would retard fermentation. When the fermentation is completed (in about 2-4 days), the fermented liquid is boiled at about 82^{0}C to allow the coagulation of protein, the lactalbumin, which is then filtered and processed for use as animal-feed supplement. The filtrate containing the calcium lactate is spray dried after

treating with sodium sulfide. To obtain lactic acid, the calcium lactate is treated with sulphuric acid and the lactic acid thus obtained is further purified.

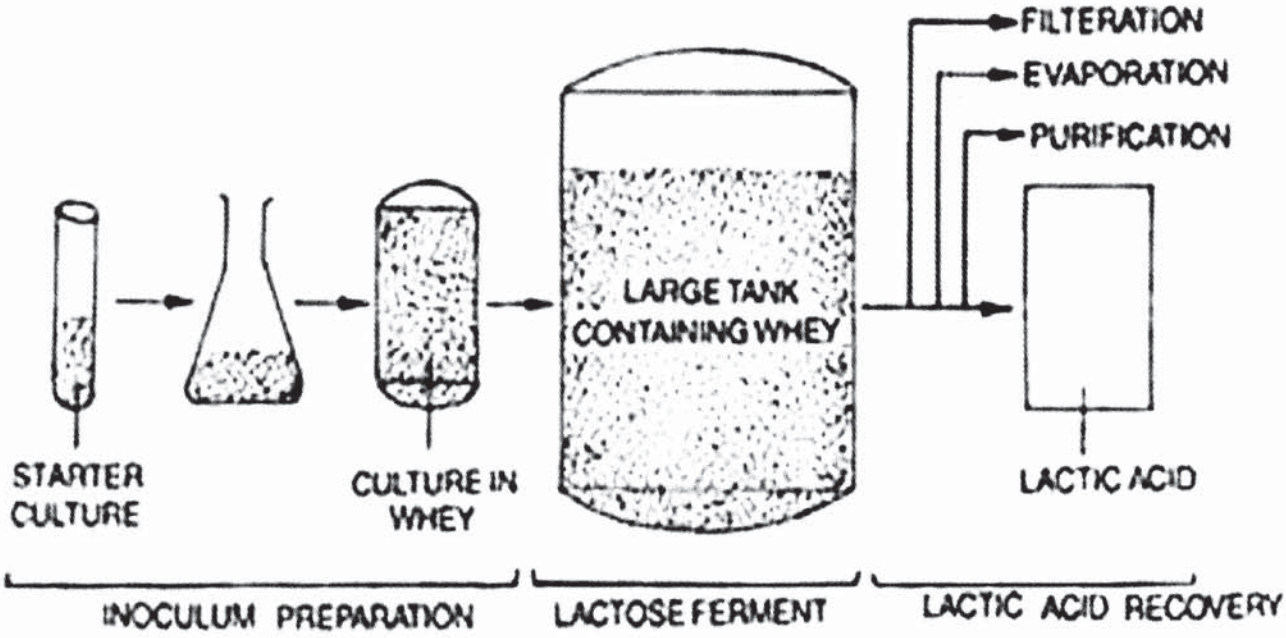


Figure 4.1 Lactic acid productions from lab scale to Industrial scale

Uses:

(i) It is used as an acidulant in fruit juices, confectionery and essences.
(ii) Derivative of lactic acid such as calcium lactate and iron lactate are used in the treatment of calcium deficiency and anemia respectively.
(iii) Lactic acid production from whey also helps removal of pollution of our environment because untreated whey disposed off in our waterways would cause dangerous consequences. It is so because whey is very high in Biological Oxygen Demand (BOD).

Materials Required:

Whey, whey with 5% lactose, lactose broth, MRS broth, lactic acid culture, Erlenmeyer flask (500 mL), waterbath (60°C), wax marking pencil, sterile pipette (10 mL)

Procedure:

- Transfer 100 ml of each media (whey, whey with 5% lactose, lactose broth, MRS broth) in three conical flasks (triplicate) and either autoclave at 121° C/ 15sec or pasteurize at 60°C in waterbath for 30 minutes.
- Initially check the acidity and pH of all the media.
- Inoculate 0.1ml of activated *lactobacillus acidophilus* in each flask containing media
- Incubate all the flasks at 37° C for 24 hrs
- After incubation, record pH of all media and titrate the sample against 0.1N NaOH

- Compare the acidity and pH with the initial values

Observation Table:

$$\text{Initial acidity} = \frac{\text{Volume of NaOH used} \times \text{Normality of NaOH} \times 9}{\text{Volume of sample}}$$

Parameter	Whey	Whey with 5% lactose	Lactose broth	MRs broth
pH				
Volume of NaOH used (ml)				
Acidity in % lactic acid				

Results and Interpretations:

4.2 Aim: Application of microbial consortia in food fermentations.

Theory:

Microorganisms necessary in food fermentations may be added as pure culture of mixed culture or in some instances a number of cultures may be added if the desired microorganism is known to be present in sufficient numbers in original raw material. Known mixtures of pure cultures sometimes are prepared; either they are grown together or grown separately and mixed at the time of use. A number of lactic acid bacteria are used in the form of mixtures in dairy industry and are a good example of bacteria consortia. The most common microbial consortia used as dairy starters usually consist of a mixture of strains of *Lactococcus lactis* ssp. Lactis and *Leuconostoc mesenteriodes* ssp *cremoris* for the production of lactic acid and *Leuconostoc cremoris* and *Lactococcus lactis ssp. diacetylactis* for the production of flavour and aroma. The typical yoghurt starter is a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

Materials required:

Skim milk, Erlenmeyer flask (500 mL), wax marking pencil, sterile pipette (10 mL)

Procedure:

- Take 100 ml of skim milk in 250 ml conical flask and keep for autoclaving.
- Use both pure and mixed cultures.
- Inoculate cultures separately into different conical flasks containing sterilized skim milk and incubated at 37°C for 24 hrs.
- After incubation analyze the sample for following parameters
- Titrable acidity
- Total bacterial count
- Direct microscopic counts

i. Titrable acidity:

- Add 1ml of phenolphthalein to 10 ml of fermented milk and titre against N/9 NaOH till pink colour appears.
- Record the volume of NaOH used and calculate % Lactic Acid by using the following formula:

$$\textbf{\% lactic acid} = 9 \times \frac{\text{Normality of NaOH} \times \text{volume of NaOH used}}{\text{Volume of milk}}$$

ii. Total Bacterial Count:

- Serially dilute 1 ml of the fermented milk sample upto 10^{-5}
- Plate 10^{-4}- 10^{-5} samples on nutrient agar medium
- Incubate at 37°C for 24 hr in an inverted position.

iii. Direct Microscopic Count:

- Clean DMC slide with alcohol
- Pipette out 0.01 ml of fermented milk with breeds pipette and spread it in 1 cm^2 area
- Allow the slide to dir dry
- Add few drops of methylene blue or Newmann's Stain on the slide
- Observe under 100X objective of compound microscope
- Calculate number of microbes using microscopic factor:
  $MF = 100 X100/ \pi r^2$
- Calculate the average number of microorganism using this formula

Observation Table:

Inoculumn No.	Titrable Acidity	Total count		Direct microscopic count
		10^{-4}	10^{-5}	
1				
2				
3				
4				
5				

Results and Interpretations:

4.3 Aim: Production of ethyl alcohol from molasses and whey by yeasts.

Theory:

Ethyl alcohol is among most common solvents and raw material used in a variety of chemical industries. It is also used as a germicide, a beverage, antifreeze, a fuel, a depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals. Ethanol is produced both as a petrochemical, through the hydration of ethylene, and biologically by microbial fermentation of cheap sugary substrates such as molasses. However, the microorganisms used must be tolerant to high sugars and high concentration of alcohol and must grow vigorously to produce a large quantity of alcohol. Yeasts, particularly *Saccharomyces cerevisiae*, represent the best known microorganisms used in the production of ethyl alcohol. In addition, *Saccharomyces uvarum*, *Kluyveromyces fragilis* and *Kluyveromyces lactis* have also been used for alcohol production. Several bacteria like *Escherichia coli, Klebsiella oxytoca*, and *Zymomonas mobilis* have been genetically engineered to produce ethanol. The chemical reaction that results in the microbial fermentation of carbohydrate into alcohol is:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

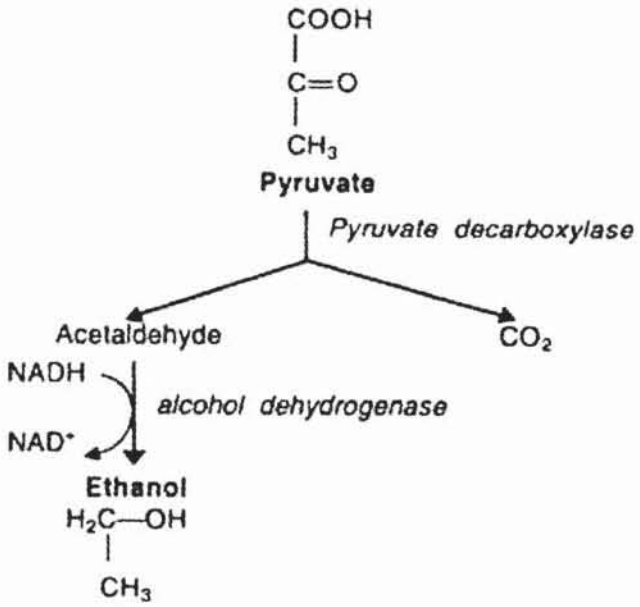


Figure 4.1 Steps in the Biosynthesis of Ethanol

Some of the inexpensive substrates used in alcohol industry are either crude cane molasses or best molasses which contain about 50 per cent fermentable sugars. Waste sulphite liquor from paper industries, whey from milk, starch yielding grams (corn), potatoes and grapes may also be used as substrate. Some countries used sugar beet for the purpose. The production process involves the dilution of molasses to a suitable sugar concentration (15-16 per cent sugars), addition of a small quantity of nitrogen source (urea, ammonium sulfate or ammonium phosphate), adjustment of pH to about 5.0, and the, addition of an actively growing yeast culture. The fermentation is carried out in big deep tanks of steel or stainless

steel. The fermentation is allowed to continue for about 24-36 hours at 25°C-30°C after which the cells are allowed to settle. The fermented mash is then distilled and passed through rectifying columns to recover ethyl alcohol.

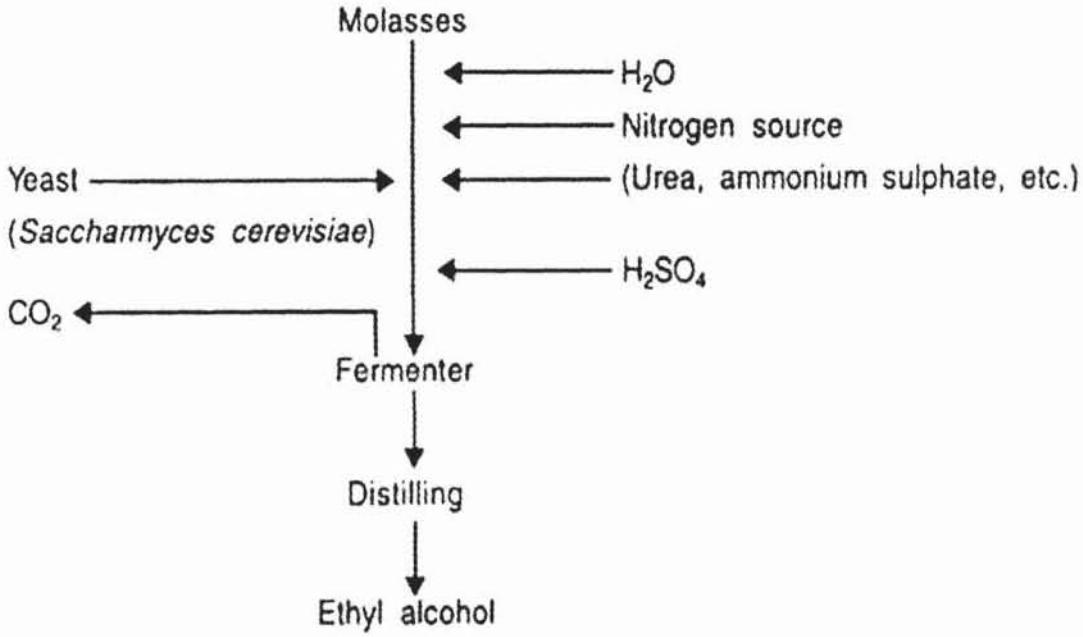


Figure 4.2 Steps in the Manufacture of Ethyl Alcohol using Molasses as Fermentation

Source: http://www.studentsguide.in/microbiology/industrial-microbiology/

Substrate:

Whey is another important waste material from which ethanol can be produced. The disposal of whey is a worldwide problem. Large quantities of whey are produced as a by-product during the manufacture of cheese and casein, and this must be disposed of or processed in an environmentally acceptable way. Since most of the components are of small molecular weight and soluble, they can quickly deplete oxygen levels in natural water systems: the COD (Chemical Oxygen Demand) of raw whey is about 60 kg m^{-3}. The key to the utilization of this resource has been changing the perception of whey from a 'waste material' to an 'opportunity' for further processing. The yeast used is lactose fermenting organism called *Kluveromyces fragilis*. This yeast produces β-galactosidase which breaks down lactose (a disaccharide) into its component sugars which are glucose and galactose.

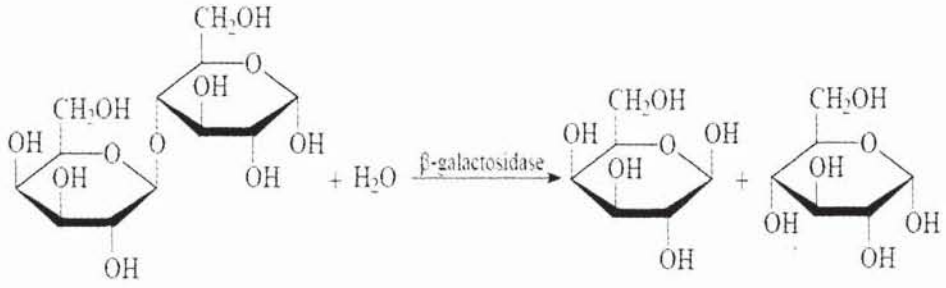


Figure 4.3 Chemical reaction showing breakdown of lactose

Materials Required:

Molasses, Whey, Culture of *Saccharomyces cerevisiae* (for molasses) and *Kluveromyces fragilis* (for whey), Sterile water, Urea, Ammonium sulfate or Ammonium phosphate, Erlenmeyer flask (500 mL), wax marking pencil, sterile pipettes (10 mL), water bath(60°C), Incubator

Procedure:

- Take 500 ml of pasteurized molasses and dilute to a suitable sugar concentration (15-16 per cent sugars).
- Add a small quantity of nitrogen source (urea, ammonium sulfate or ammonium phosphate).
- Take 500 ml of pasteurized whey.
- Adjust the pH to about 5.0
- Inoculate 1% actively growing culture of *Saccharomyces cerevisiae* in pasteurized molasses and *Kluveromyces fragilis* in pasteurized whey.
- Incubate at 30°C for 3-7 days.

Observation Table:

1. Smell the signs of fermentation (alcohol smell).
2. Check for alcohol production.

Substrate	**Alcohol concentration (in percent)**
Molasses	
Whey	

Results and Interpretations:

4.4 Aim: Citric acid production from whey with sugars and additives by *Aspergillus niger.*

Theory: Citric acid, a carboxylic organic acid, soluble in water with a pleasant taste, is the most important acid used in the food industries. Until about 1920, all commercial citric acid was produced from lemon and lime juices. Later on it was reported that citric acid can be produced by fermentation process using species of microorganisms namely *Aspergillus niger*, a fungus which was used commercially for the first time in 1923. They also indicated that factors affecting the production of citric acid by fermentation include the nutritional composition of the media, environmental conditions, deficiency of manganese and other metals, pH, and dissolved oxygen tension.

At present time citric acid is produced commercially using mutant strains of *Aspergillus niger*, and with a significant amount by *Saccharomycopsis lipolytica*, *Pencillium simplicissimum* and *Aspergillus foeitidus*. Other carbohydrates and wastes that have been considered, experimentally, to produce citric acid by *Aspergillus niger* includes inulin, date fruit syrup, sugar cane molasses, soya whey, Carob pod and cheese whey.

Large amounts of whey are produced world wide as a by-product of cheese and other dairy products manufacturing. Whey in the Middle Eastern region is generally considered a waste and disposed in the sewage system leaving a small amount for drinking for domestic animals. The aim of this study was to produce citric acid by *Aspergillus niger* from cheese whey fortified with different sucrose, tricalcium phosphates and riboflavin in a liquid surface culture process.

Materials required:

Pateurized cheese whey, Sucrose, tricalcium phosphate, riboflavin, 500 ml Erlemyer flask, Fresh *Aspergillus niger* culture (approx 10^3 spore suspension), sterile pipette (10 mL), wax marking pencil, incubator

Procedure:

- Take 100 mL cheese whey in 500 mL Erlemyer flask and add sucrose (15g) and tricalcium phosphate (1g).
- Pateurized cheese whey at 60°C for 30 minutes and add filter sterilized riboflavin (10 mg/L) to fortify the media. Adjust the initial pH of the fermentation media to 3.0 using 1 N of HCl and/or NaOH.
- Carry out surface liquid culture fermentation process by inoculating the media with the fungal culture (approx 10^3 spore suspension) and incubate at 30°C for up to 20 days.
- Determine citric acid concentration titrating with 0.1 N NaOH and phenolphthalein as indicator and calculated as % according to the following formula:
- **Citric acid (in percent)** = Normality X volume of NaOH X Equiv. wt. of CA/ Weight of sample (g) X 10

- For determining biomass, take the whole fungal culture growth and filter with Whatman filter paper No.4, Wash with distilled water (250 ml) and dry at 105°C to constant weight.
- Measure culture pH by pH meter.

Observation Table:

Parameters	**Citric acid**
Citric acid (in percentage)	
Biomass (in g/ L)	
pH	

Results and Interpretation:

4.5 Aim: Production of sauerkraut by microorganisms.

Theory:

Sauerkraut is defined as the clean soured product of characteristic flavour, obtained by full fermentation of properly prepared and shreaded cabbage in presence of not less than 2-3% salt. Finally containing not less than 1-1.5% acid expresses as lactic acid.

The basic process involves fermentation of shredded cabbage by the mixed activity of *Leuconostoc mesenteroides*, *Lactobacillus brevis*, and *Lactobacillus plantarum* in the presence of 2.2-2.8% w/v NaCl, the latter play 3 important functions.

- It inhibits the growth of initial spoilage organisms like pseudomonas
- It extracts moisture from the shredded cabbage by osmosis to form the brine in which fermentation takes place.
- It helps to maintain the crisp texture of the cabbage by conditioning water and inhibit endogenous pectinolytic enzymes which cause the product to soften.

The lactic acid produced during fermentation performs two important functions

- It imprarts characteristic flavour to the product
- Act as a preservative by inhibiting the growth of food spoilage microorganisms.

Material required:

Sterile Glass beaker (500 ml), Cabbage, uniodized table salt, Clean Knife, weighing pan, wooden boards, cheese cloth, rubber bands/ thread, pH strips or pH meter, plate count agar, N/10 NaOH, phenolphthalein (1%)

Procedure:

- Trim the spotted and damaged outer leaves from all the cabbage heads.
- Divide each head into two halves with sterile knife and remove the core
- Wash these halves with clean running water
- Weigh the shredded cabbage and divide into two parts of 250 gm each
- Weigh sodium chloride accordingly and divide into two parts of equal weight
- Place the shredded cabbage and slat in alternating layer in a wide mouthed jar
- Place a wooden board over each mixture and gently press to squeeze out the juice.
- Place a weight over the wooden boards
- Cover the jar with cheese cloth by using rubber bands.
- Incubate the jars at room temperature for 28 days for the fermentation of substrate
- Record the following results at an interval of 7 and 14 days for the chemical and microbiological changes during sauerkraut production
- Odour: Acidic, Earthy, Spicy Or Putrid
- Colour: Colourless, Brown, Pink, Straw, Yellow And Pale
- Taste: Sour, Salty, Sweet And Bitter
- Texture: Soft, Slimy And Rotten

- pH: Determine by pH paper
- Total Acidity: Expressed as % lactic acid by titration
- Total bacterial count: By using selective media

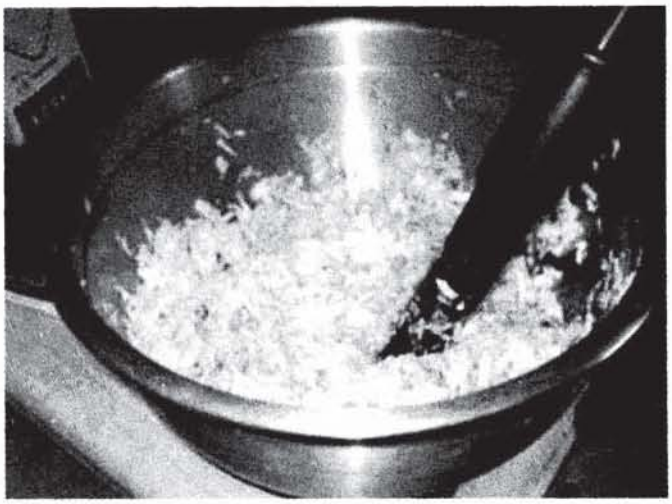


Figure 4.4: Sauerkraut preparation

Source: www.learningherbs.com/sauerkraut_recipe.html

Observation Table:

Parameters	7 days	14 days
pH		
Acidity (in percent)		
Total bacterial count		
Odour		
Colour		
Taste		
Texture		

Results and Interpretation:

4.6 Aim: Production of single cell proteins.

Theory:

Increasing concern about pollution occurring from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. Additionally, there is a demand for the formulation of innovative and alternative proteinaceous food sources due to an insufficient supply from the traditional protein sources such as meat, fish or eggs.

Much interest has been focused on the potential of converting soy milk wastes, potato effluents, sugarcane bagasse, orange peels, shrimp-shell wastes, kimchi production wastes or forestry wastes (e.g. wood hydrolysates) to single cell protein (SCP) Technically, SCP is the manufacture of cell mass using microorganisms by culturing on abundantly available wastes. Algae, fungi and bacteria are the chief sources of microbial protein that can be utilized as SCP.

The production of the microbial biomass is done either by a submerged or solid state fermentation process. After fermentation, biomass is harvested and may be used as a protein source or be subjected to processing steps like washing, cell disruption, protein extraction and purification. In general, high production rates and protein yields as well as ease of production control makes SCP more attractive as a protein source compared with conventional plant and animal sources.

Cheese whey, a by-product of the dairy industry, is the liquid effluent remaining following the precipitation and removal of milk casein during cheese making. It represents about 85–95% of the milk volume and retains a significant amount (~55%) of milk nutrients. Among the most abundant of these nutrients is lactose (4.5–5% w/v) which is a suitable substrate for the production of value-added products using biochemical conversion processes.

Whey also contains soluble proteins (0.6–0.8% w/v), lipids (0.4–0.5% w/v) and mineral salts (8–10% of dried extract), as well as appreciable quantities of other constituents, such as lactic and citric acids, non-protein nitrogen compounds, B group vitamins, etc.

Yeasts that ferment lactose are known for the production of ethanol and SCP. Cheese whey is a cheap and largely available raw material for microbial biomass of SCP production by yeasts.

It is well known that the proteins are one of the main constituents of foods. In addition to their nutritional function, proteins contribute significantly to the expression of sensory attributes of foods. The functional properties of proteins are important in determining their usefulness in food systems.

There is limited information about single cell protein functionality and more knowledge is needed in order to assess their potential uses in foods.

Considering that in exploring single cell protein, isolated from cultivation of yeasts on cheese whey, as a new source of food protein, its functional properties need to be determined.

Materials Required:

Cheese whey, *Kluyveromyces,* Refined oil, Defatted soy flour*, *prepare by extracting soy flour (100 g) with petroleum ether (1000 ml) under continuous stirring for 2.5 h, filtering, washing with 200 ml petroleum ether and, finally, drying at 55 °C under vacuum for 3 h

Procedure:

- First cultivate and proliferate *Kluyveromyces* yeast in agar slants containing cheese whey (2% w/v).
- Successively subculture the cultures in 5, 50, 500 ml and 2 l liquid substrate [cheese whey enriched with 2.4 g/1.5 l KH_2PO_4 and 11.3 g/1.5 l $(NH_4)_2SO_4$] at optimal growth conditions of 30°C and pH 5.5.
- Centrifuge the culture broth at 4000×*g* for 40 min.
- Ferment the cheese whey by *Kluyveromyces* under aerobic conditions at 30 °C and pH 5.5 with an air supply of 0.3 bar through a sterile filter of 0.45 μm for 3-4 days.
- Obtain the biomass by centrifugation at 4000×*g* for 10 min, wash with distilled water and freeze dry.

Observations:

Substrate	Biomass (mg/ L)

Results and Interpretations:

Review Questions:

- How are bread, sauerkraut, and pickles produced?
- What microorganisms are most important in bread and pickles fermentations?
- Discuss the importance of the specific sequential activity of the microflora responsible for sauerkraut production?
- What is the function of the salt in sauerkraut production?
- Why is uniodized salt used in this process?
- What are SCPs?
- Give the advantage of Single cell protein.
- Enlist some SCP available in the market.
- Define liquid surface culture process.
- Define mother culture and seed culture.
- What is the importance of BOD?
- What is the symbiotic relationship between yoghurt cultures?
- Give the therapeutic value of fermented products.
- What would be the effect, if we dispose the whey in the environment?

User's Notes

APPENDIX-1

MEDIA COMPOSITION

BACILLUS CEREUS AGAR

Ingredients	**Grams / lit**
Enzymatic Digest of Casein	1 g
Mannitol	10 g
Sodium Chloride	2 g
Magnesium Sulfate	0.1 g
Disodium Phosphate	2.5 g
Monopotassium Phosphate	0.25 g
Bromthymol Blue	0.10 g
Sodium Pyruvate	10 g
Agar	15 g
Sterile Egg Yolk Suspension	50 mL
Polymyxin B (100,000 units) (filtered sterilized aqueous)	2 mL
Final pH: 7.2 ± 0.2 at 25°C	

BRILLIANT GREEN LACTOSE BILE BROTH

Ingredients	**Grams / lit**
Peptic digest of animal tissue	5.0
Pancreatic digest of casein	5.0
Lactose	10.0
Sucrose	10.0
NaCl	5.0
Ox bile	20.0
Brilliant green	0.0025
Phenol Red	0.08
Final pH: 6.8 ±0.2 at 25°C	

deMANN ROGOSA SHARPE (MRS) AGAR

Ingredients	**Grams / lit**
Proteose Peptone No. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0

Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium Sulfate	0.1
Manganese Sulfate	0.05
Dipotassium Phosphate	2.0
Agar	15.0
Final pH: 6.5 ±0.2 at 25°C	

deMANN ROGOSA SHARPE (MRS) BROTH

Ingredients	**Grams / lit**
Proteose Peptone No. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium Sulfate	0.1
Manganese Sulfate	0.05
Dipotassium Phosphate	2.0
Final pH: 6.5±0.2 at 25°C	

DOUBLE-STRENGTH LACTOSE BROTH (DSLB)

Ingredients	**Grams / lit**
Pancreatic Digest of Gelatin	10.0
Beef Extract	6.0
Lactose	**10.0**
Final pH: 6.5 ±0.2 at 25°C	

GLUCOSE-POLY-PEPTONE-YEAST EXT.-MALT EXT. AGAR

Ingredients	**Grams / lit**
Glucose	10.0
Polypeptone	5.0
Malt extract	3.0
Yeast extract	3.0
Final pH: 5.5 ±0.2 at 25°C	

LACTOSE BROTH

Ingredients	Grams / lit
Pancreatic Digest of Gelatin	10.0
Beef Extract	6.0
Lactose	10.0
Final pH: 7.0±0.2 at 25°C	

LAURYL TRYPTOSE BROTH

Ingredients	Grams / lit
Tryptose	20.0
Lactose	5.0
Dipotassium Phosphate	2.75
Monopotassium Phosphate	2.75
Sodium Chloride	5.0
Sodium Lauryl Sulfate	0.1
Final pH: 7.0±0.2 at 25°C	

LEVINE'S EOSIN METHYLENE BLUE AGAR

Ingredients	Grams / lit
Pancreatic digest of casein	10.0
Lactose	5.0
Sucrose	5.0
K_2HPO_4	2.0
Methylene Blue	0.065
Eosin Y	0.4
Agar	13.5
Final pH: 7.2±0.2 at 25°C	

M17 BROTH

Ingredients	Grams / lit
Casein enzyme hydrolysate	5.0
Papaic digest of soybean meal	5.0
Yeast extract	2.5
Malt extract	5.0
Ascorbic acid	0.5
Magnesium sulphate	0.25
Disodium -β-glycerophosphate	19.0

Agar	11.0
Final pH: 6.9±0.2 at 25°C	

MALT EXTRACT AGAR (MEA)

Ingredients	**Grams / lit**
Dextrin	2.75
Glycerol	2.35
Peptone	0.78
Agar	15.0
Final pH: 4.7±0.2 at 25°C	

MULLER HINTON AGAR

Ingredients	**Grams / lit**
Beef Extract	2
Acid Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH: 7.3±0.1 at 25°C	

NUTRIENT AGAR (NA)

Ingredients	**Grams / lit**
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Agar	20.0
Final pH: 7.4±0.2 at 25°C	

OXFORD PERFRINGENS AGAR

Ingredients	**Grams / lit**
Enzymatic Digest of Casein	15.0
Enzymatic Digest of Soybean Meal	5.0
Yeast Extract	5.0
Sodium Metabisulfite	1.0
Ferric Ammonium Citrate	1.0
Agar	15.0
Final pH: 7.6 ± 0.2 at 25°C	

PLATE COUNT AGAR (PCA)

Ingredients	Grams / lit
Casein enzyme hydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
Final pH: 7.0±0.2 at 25°C	

POTATO DEXTROSE AGAR (PDA)

Ingredients	Grams/ lit
Peeled Potato	200.0
Dextrose	20.0
Agar	15.0
Final pH 5.6 ± 0.2 at 25 °C	

SINGLE-STRENGTH LACTOSE BROTH (SSLB)

Ingredients	Grams / lit
Pancreatic Digest of Gelatin	**5.0**
Beef Extract	**3.0**
Lactose	**5.0**
Final pH: 7.0 ±0.2 at 25°C	

STREPTOMYCES-SELECTIVE AGAR (SSA)

Ingredients	Grams / lit
Beef Heart Infusion, Solids	10.0
Tryptose	10.0
Casein enzyme hydrolysate	4.0
Yeast extract	5.0
Dextrose	5.0
L-cysteine hydrochloride	1.0
Starch, soluble	1.0
Sodium Chloride	5.0
Monopotassium phosphate	15.0
Ammonium Sulphate	1.0
Magnesium Sulphate	0.2
Calcium Chloride	0.02
Agar	20.0

Final pH: 6.9±0.2 at 25°C

TRYPTIC AGAR

Ingredients	**Grams / lit**
Pancreatic Digest of Casein	15.0 g
Enzymatic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Final pH: 7.3±0.2 at 25°C	

TRYPTICASE SOYA AGAR (TSA)

Ingredients	**Grams/ lit**
Soy Peptone	5.0
Casein Triptic Digest	15.0
Sodium Chloride	5.0
Bacteriological Agar	15.0
Final pH 7.2 ± 0.1 at 25°C	

VIOLET RED BILE AGAR (VRBA)

Ingredients	**Grams/ lit**
Peptone from meat	7.0
Yeast extract	3.0
Sodium chloride	5.0
Lactose	10.0
Neutral red	0.03
Bile salt mixture	1.5
Crystal violet	0.002
Agar	15.0
Final pH: 7.4 ± 0.2 at 25 °C	

VIOLET RED BLUE GLUCOSE AGAR (VRBGA)

Ingredients	**Grams/ lit**
Glucose monohydrate	10.00
Pancreatic digest of Gelatin	7.00
Sodium Chloride	5.00
Yeast Extract	3.00
Bile Salts	1.50

Neutral Red	0.03
Crystal Violet	0.002
Bacteriological Agar	15.00
Final pH: 7.4 ± 0.2 at 25°C	

YEAST MALTOSE (YM) AGAR

Ingredients	**Grams/ lit**
Yeast Extract	3.0
Malt extract	3.0
Bacteriological Agar	15.0
Final pH: 5.5 ± 0.2 at 25°C	

APPENDIX-2

REAGENTS AND BUFFERS

CASEIN SOLUTION (2%)

Ingredients	Grams
Casein	2.0
Borate buffer (0.1M)	80 mL

Adjust the pH 7.6 and completely dissolved by heating on a steam bath for 15 min. Cool this solution, adjust the pH to 7.6 and make the volume up to 100 mL by the borate buffer.

GRAM-STAINING REAGENTS

(A) Crystal Violet

	Ingredients	Grams
a	Crystal violet (85%)	2.0 g
	ethyl alcohol (95%)	20.0 mL
b	Ammonium oxalate	0.8 g
	Distilled water	80.0 mL

Add solution a to solution b. Let stand for a day, and then filter. If the crystal violet is too concentrated, solution A may be diluted as much as 10 times.

(B) Gram's Iodine Solution (mordant)

Ingredients	Grams
Iodine crystals	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 mL

Store in an amber bottle; discard when the color begins to fade.

(C) Safranin (counterstain) Solution

Ingredients	Grams
Safranin	2.5 g
Ethyl alcohol (95%)	100 mL

For a working solution, dilute stock solution 1/10 (10ml of stock safranin to 90 ml of distilled water).

(D) Decolorizer

95% Acetone of Ethyl alcohol

GUM ARABIC (10%)

Ingredients	Grams
Gum Arabic	10.0 g
Make the Volume upto 100 mL with distilled water	

METHYLENE BLUE SOLUTION (1:250,000)

The Methylene blue concentration used in 1 part of dye in 3,00,000 parts of milk. Presently tablet forms are available. One tablet dissolved in 200 ml of hot, distilled water produces the stock dye solution for addition to the milk. Although this solution is stable when it is refrigerated and protected from light, it is safer to prepare the solution weekly.

NAOH (N/10)

Ingredients	**Grams**
NaOH	0.4
Distilled water	100 mL

NEWMANN'S STAIN

Ingredients	**Grams**
Methylene blue chloride	0.6 g
Ethyl alcohol (95%)	52 ml
Tetrachlorethane	44 ml
Glacial acetic acid	4 ml

PHENOPTHALEIN (1%)

Ingredients	**Concentration**
Phenolphthalein	0.1
Ethyl alcohol	89-91
Methanol	4-6
Deionized water	4-6

SODIUM PHOSPHATE BUFFER (pH 7)

Solutions	**Ingredients**	**Grams/ Lit**
Stock solution A	Monobasic sodium phosphate, monohydrate (2 M)	276.0
Stock solution B	Dibasic sodium phosphate (2 M)	284.0
Final pH: 7.0±0.2 at 25°C		

Mixing an 39.0 (**mL**) of **A** and 61.0 (mL) of **B** as diluting to a total volume of 200 ml, a 1 M phosphate buffer of the required pH at room temperature.

SOLUBLE STARCH (1%)

Ingredients	Grams
Soluble starch	1.0
Heated Water	100.0

SULFURIC ACID (2N)

Ingredients	mL
Sulfuric acid	55.0 mL
Distilled Water	45.0 mL
Make final upto 1000 ml	

TCA SOLUTION (10%)

Ingredients	Grams
Trichloroacetic acid	10.0
Anhydrous sodium acetate	10.0
Glacial acetic acid	2 mL
Make volume upto 100 mL	

Most Probable Number (MPN) Index for Various Combinations of Positive and Negative Results When Five 10ml, Five 1 ml and five 0.1 ml portions are used

No of tubes showing indication of positive reaction out of			MPN index per 100 ml
5 of 10 ml sample each	5 of 1 ml sample each	5 of 0.1 ml sample each	
0	0	0	<2
0	0	1	2
0	1	0	2
0	2	0	4
1	0	0	2
1	0	1	4
1	1	0	4
1	1	1	6
1	2	0	6
2	0	0	4
2	0	1	7
2	1	0	7
2	1	1	9
2	2	0	9
2	3	0	12
3	0	0	8
3	0	1	11
3	1	0	11
3	1	1	14
3	2	0	14
3	2	1	17
3	3	0	17
4	0	0	13
4	0	1	17
4	1	0	17
4	1	1	21
4	1	2	26

4	2	0	22
4	2	1	26
4	3	0	27
4	3	1	33
4	4	0	34
5	0	0	23
5	0	1	30
5	0	2	40
5	1	0	30
5	1	1	50
5	1	2	60
5	2	0	50
5	2	1	70
5	2	2	90
5	3	0	80
5	3	1	110
5	3	2	140
5	3	3	170
5	4	0	130
5	4	1	170
5	4	2	220
5	4	3	280
5	4	4	350
5	5	0	240
5	5	1	300
5	5	2	500
5	5	3	900
5	5	4	1600
5	5	5	>1600

Source: *Standard Methods for Examination of Water and Wastewater*, 18th Edition, American Public Health Association, New York, 1998

Glossary of Related Terms

Acidophile:

A microorganism that has its growth optimum between about pH 0 and 5.5.

Actinomycete:

An aerobic, Gram positive bacterium that forms branching filaments and asexual spores.

Aerobe:

An organism that grows in the presence of atmospheric oxygen.

Aerobic respiration:

A metabolic process in which molecules, often organic, is oxidized with oxygen as the final electron acceptor.

Agar:

A complex polysaccharide extracted from red algae and used as a solidifying agent in culture media preparation.

Alcoholic fermentation:

A fermentation process that produces ethanol and CO_2 from sugars.

Aliquot: Dispense an amount of liquid using a pipette.

Alkalophile:

A microorganism that grows best at pHs from about 8.5 to 11.5.

Anabolism:

The synthesis of complex molecules from simpler molecules with the input of energy.

Anaerobe:

An organism that grows in the absence of free oxygen.

Antibiotic:

A microbial product or its derivative that kills susceptible microorganisms or inhibits their growth.

Antibody (immunoglobulin):

A glycoprotein produced in response to the introduction of an antigen; it has the ability to combine with the antigen that stimulated its production. Also known as an immunoglobulin (Ig).

Antimicrobial agent:

An agent that kills microorganisms or inhibits their growth.

Archaea:

The domain that contains prokaryotes with isoprenoid glycerol diether or diglycerol

Aseptic technique:

Procedure to guarantee sterility and to reduce contamination

Autoclave:

An apparatus for sterilizing objects by the use of steam under pressure. Its development tremendously stimulated the growth of microbiology.

Autotroph:

An organism that uses CO_2 as its sole or principal source of carbon.

Bacillus:

A rod-shaped bacterium.

Bacteria: The domain that contains procaryotic cells with primarily diacyl glycerol diesters in their membranes and with bacterial rRNA.

Bacteriocin:

A protein produced by a bacterial strain that kills other closely related strains.

Bacteriophages: viruses that infect bacterial host cells; they usually consist of a nucleic acid molecule enclosed by a protein coat.

Batch culture:

A culture of microorganisms produced by inoculating a closed culture vessel containing a single batch of medium.

Biochemical oxygen demand (BOD):

The amount of oxygen used by organisms in water under certain standard conditions; it provides an index of the amount of microbially oxidizable organic matter present.

Biodegradation:

Metabolism of a substance by microorganisms that yield mineralized end products.

Biofilm:

Matrix-enclosed bacterial populations' adherent to each other and/ or to surface or interfaces.

Biological safety cabinet:

Cabinet use to protect personnel, product and the environment from exposure to biohazards and cross contamination during routine procedures.

Bioremediation:

The use of biologically mediated processes to remove or degrade pollutants from specific environments. Bioremediation can be carried out by modification of the environment to accelerate biological processes, either with or without the addition of specific microorganisms.

Biosensor:

The coupling of a biological process with production of an electrical signal or light to

Biotransformation or **microbial transformation:**

The use of living organisms to modify substances that are not normally used for growth.

BOD incubator:

Incubator meet different bio chemical oxygen demand test in various fields including medical, agricultural, industrial, research laboratories, storage sensitive culture, vaccines,

culture of bacteria, microorganism, serum incubation, seed germination, various industries and more.

Broth:

Culture medium without agar.

Budding:

A vegetative outgrowth of yeast and some bacteria as a means of asexual reproduction.

CFU:

Colony-forming units, i.e. colonies

Chemical oxygen demand (COD):

The amount of chemical oxidation required to convert organic matter in water and waste water to CO2.

Chemostat:

A continuous culture apparatus that feeds medium into the culture vessel at the same rate as medium containing microorganisms is removed; the medium in a chemostat contains one essential nutrient in a limiting quantity.

Coccus:

A roughly spherical bacterial cell.

Coliform:

A gram-negative, nonsporing, facultative rod that ferments lactose with gas formation within 48 hours at 35°C.

Colony:

A cluster or assemblage of microorganisms growing on a solid surface such as the surface of an agar culture medium; the assemblage often is directly visible, but also may be seen only microscopically.

Colony forming units (CFU):

The numbers of microorganisms that can form colonies when cultured using spread plates or pour plates, an indication of the number of viable microorganisms

Complex medium:

Medium with some unknown ingredients or amounts, i.e. blood agar

Consortium:

A physical association of two different organisms, usually beneficial to both organisms.

Continuous culture system:

A culture system with constant environmental conditions maintained

Culture medium:

A liquid or gel, containing nutrients, that is used to cultivate microorganisms.

Defined medium: Culture medium made with components of known composition.

Deionised water:

Water that has had the ions removed.

Diarrhoea:

An increase in the frequency of bowel movements and loss of body fluid.

Differential media:

Culture media that distinguish between groups of microorganisms based on differences in their growth and metabolic products e.g. - MacConkey agar.

Diluents:

An inert substance used to dilute.

Dilution factor:

It is equal to the final volume divided by the initial volume of solution.

Disease:

A deviation or interruption of the normal structure or function of any part of the body that is manifested by a characteristic set of symptoms and signs.

Disinfectant:

An agent, usually chemical, that disinfects inanimate objects.

Distilled water:

Water from which both ionic and non-ionic components are removed.

Durham tube:

A small, inverted, liquid-filled test tube for collecting gas formed by microbial metabolism in broth cultures. The Durham tube is used to test for the presence of organisms capable or metabolizing the broth nutrients under given conditions thorough observation for gas present in the tube.

Embden-Meyerhof pathway:

A pathway that degrades glucose to pyruvate; the six-carbon stage converts glucose to fructose 1,6- bisphosphate, and the three-carbon stage produces ATP while changing glyceraldehyde 3-phosphate to pyruvate.

Endospore:

An extremely heat- and chemical-resistant, dormant, thick-walled spore formed by bacteria for its survival in adverse conditions.

Endotoxin:

Component of the cell wall of gram-negative bacteria that can cause adverse health effects.

Enriched media:

Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some of the more fastidious ones.

Enteric bacteria:

Members of the family *Enterobacteriaceae* (Gram negative, peritrichous or nonmotile, facultatively anaerobic, straight rods with simple nutritional requirements); also used for bacteria that live in the intestinal tract.

Enterotoxin:

A toxin specifically affecting the cells of the intestinal mucosa, causing vomiting and diarrhea.

Enzyme:

A protein catalyst with specificity for both the reaction catalyzed and its substrates.

Exoenzymes:

Enzymes that are secreted by cells.

Exotoxin:

A heat-labile, toxic protein produced by a bacterium as a result of its normal metabolism or because of the acquisition of a plasmid or prophage that redirects its metabolism.

Exponential phase: The phase of the growth curve during which the microbial gowth

Extremophiles:

Microorganisms that grow under harsh or extreme environmental conditions such as very high temperatures or low pHs.

Facultative anaerobes: Microorganisms that do not require oxygen for growth, but do grow better in its presence.

Faecal coliform: Coliform bacteria with ability to ferment lactose with the production of acid and gas at 44.5°C within 24 – 48 h.

Fastidious:

Hard-to-grow bacteria, requiring growth factors or particular nutrients

Fecal coliform:

Coliforms whose normal habitat is the intestinal tract and that can grow at 44.5°C.

Fecal enterococci:

Enterococci found in the intestine of humans and other warm-blooded animals. They are used as indicators of the fecal pollution of water

Fermentation:

An energy yielding process in which an energy substrate is oxidized without an exogenous electron acceptor. Usually organic molecules serve as both electron donors and acceptors.

Food intoxication:

Food poisoning caused by microbial toxins produced in a food prior to consumption. The presence of living bacteria is not required.

Food poisoning:

A general term usually referring to a gastrointestinal disease caused by the ingestion of food contaminated by pathogens or their toxins.

Food-borne infection:

Gastrointestinal illness caused by ingestion of microorganisms, followed by their growth within the host. Symptoms arise from tissue invasion and/or toxin production.

Fungus:

Achlorophyllous, heterotrophic, spore-bearing eukaryotes with absorptive nutrition; usually, they have a walled thallus.

Gastroenteritis:

An acute inflammation of the lining of the stomach and intestines, characterized by anorexia, nausea, diarrhea, abdominal pain, and weakness. It has various causes including food poisoning due to such organisms as *E. coli, S. aureus, Campylobacter* (campylobacteriosis), and *Salmonella* species; consumption of irritating food or drink; or psychological factors such as anger, stress, and fear. Also called enterogastritis.

Generation time:

The time required for a microbial population to double in number.

Genus:

Category of organisms with like features and closely related, divided into species

Glycolysis:

The anaerobic conversion of glucose to lactic acid by use of the Embden- Meyerhof pathway.

Gram stain:

A differential staining procedure that divides bacteria into gram-positive and gram negative groups based on their ability to retain crystal violet when decolorized with an organic solvent such as ethanol.

Growth factors:

Organic compounds that must be supplied in the diet for growth because they are essential cell components or precursors of such components and cannot be synthesized by the organism.

Halobacteria or **extreme halophiles:**

A group of archaea that have an absolute dependence on high NaCl concentrations for growth and will not survive at a concentration below about 1.5 M NaCl.

Halophile:

A microorganism that requires high levels of sodium chloride for growth.

Heterolactic fermenters:

Microorganisms that ferment sugars to form lactate, and also other products such as ethanol and CO_2.

Homolactic fermenters:

Organisms that ferment sugars almost completely to lactic acid.

Identification:

The process of determining that a particular isolate or organism belongs to a recognized taxon.

Incubation period:

The period after pathogen entry into a host and before signs and symptoms

Indicator organism:

An organism whose presence indicates the condition of a substance or

Infection:

The invasion of a host by a microorganism with subsequent establishment and multiplication of the agent. An infection may or may not lead to overt disease.

Intoxication:

A disease that results from the entrance of a specific toxin into the body of a host. The toxin can induce the disease in the absence of the toxin-producing organism.

Intrinsic factors:

Food-related factors such as moisture, pH, and available nutrients that influence microbial growth.

Lactic acid fermentation:

A fermentation that produces lactic acid as the sole or primary product.

Microbiology:

The study of organisms that is usually too small to be seen with the naked eye.

Microorganism: An organism that is too small to be seen clearly with the naked eye.

Microscope:

Instrument used for viewing magnified image of the microorganisms, which are not visible with aided eyes. It is of two main types: Light and Electron microscope.

Mold:

Any of a large group of fungi that cause mold or moldiness and that exists as multicellular

Most probable number (MPN):

The statistical estimation of the probable population in a liquid by diluting and determining end points for microbial growth.

Mould:

Any of a large group of fungi that cause mould or mouldiness and that exists as multicellular filamentous colonies; also the deposit or growth caused by such fungi. Moulds typically do not produce macroscopic fruiting bodies.

Narrow-spectrum drugs:

Chemotherapeutic agents that is effective only against a limited

Nicotinamide adenine dinucleotide phosphate:

An electron-carrying coenzyme that most often participates as an electron carrier in biosynthetic metabolism.

Normal saline:

Diluents used for enumeration, consisting of 0.85% NaCl concentration. Maintain osmotic balance of cell.

Nutrient:

A substance that supports growth and reproduction.

Osmophilic microorganisms:

Microorganisms that grows best in or on media of high solute concentration.

Osmotolerant:

Organisms that grow over a fairly wide range of water activity or solute concentration.

Oxidation-reduction (redox) reactions:

Reactions involving electron transfers; the reductant donates electrons to an oxidant.

Pasteurization:

The process of heating milk and other liquids to destroy microorganisms that can cause spoilage or disease.

Pathogen:

Any virus, bacterium, or other agent that causes disease.

PCA:

Plate count agar medium, general all-purpose enrichment

Penicillins:

A group of antibiotics containing a β-lactam ring, which are active against gram-positive bacteria.

Peptones:

Water-soluble digests or hydrolysates of proteins that are used in the preparation of culture media.

Petri dish:

A shallow dish consisting of two round, overlapping halves that is used to grow microorganisms on solid culture medium; the top is larger than the bottom of the dish to prevent contamination of the culture.

pH:

A measure of the acidity or alkalinity of a solution, numerically equal to 7 for neutral solutions, increasing with increasing alkalinity.

Plate count agar:

Variation of nutrient agar, for optimizing counts of bacteria in samples population is growing at a constant and maximum rate, dividing and doubling at regular intervals.

Pour plate:

Procedure where liquified agar has been poured into a Petri dish after being mixed with bacteria

Psychrophile:

A microorganism that grows well at 0°C and has an optimum growth temperature of 15°C or lower and a temperature maximum around 20°C.

Psychrotroph:

A microorganism that grows at 0°C, but has a growth optimum between 20 and 30°C, and a maximum of about 35°C.

Pure culture:

A population of cells that is identical because they arise from a single cell.

Reducing agent or **reductant:**

The electron donor in an oxidation-reduction

Secondary metabolites:

Products of metabolism that are synthesized after growth has been

Selective media:

Culture media that favor the growth of specific microorganisms; this may be accomplished by inhibiting the growth of undesired microorganisms.

Simple stain:

Single type of nucleic acid, lacking independent metabolism, and reproducing only within Living host cells.

Smear:

A uniform thin film of bacteria/ other suspension on glass slide.

Source:

The location or object from which a pathogen is immediately transmitted to the host, either directly or through an intermediate agent.

Species:

Species of higher organisms are groups of interbreeding or potentially interbreeding natural populations that are reproductively isolated. Bacterial species are collections of strains that have many stable properties in common and differ significantly from other groups of strains.

Spore:

A differentiated, specialized form that can be used for dissemination, for survival of adverse conditions because of its heat and dessication resistance, and/or for reproduction. Spores are usually unicellular and may develop into vegetative organisms or gametes. They may be produced asexually or sexually and are of many types.

Spread plate:

Procedure where pre-made agar plates have a sample of bacterium placed on top of the agar and spread via a glass rod

Starter culture:

An inoculum, consisting of a mixture of carefully selected microorganisms, used

Stationary phase:

The phase of microbial growth in a batch culture when population growth ceases and the growth curve levels off.

Sterilization:

The process by which all living cells, viable spores, viruses, and viroids are either destroyed or removed from an object or habitat.

Strain:

A population of organisms that descends from a single organism or pure culture isolate.

Streak plate:

Procedure where a bacterial specimen is placed on a pre-made plate and diluted out using flame and multiple sections.

Streptomycin:

A bactericidal aminoglycoside antibiotic produced by *Streptomyces griseus.*

Symbiosis:

The living together or close association of two dissimilar organisms, each of these organisms being known as a symbiont.

Thermophile:

A microorganism that can grow at temperatures of 55°C or higher; the minimum is usually around 45°C.

Toxin:

A microbial product or component that can injure another cell or organism at low concentrations. Often the term refers to a poisonous protein, but toxins may be lipids and other substances.

Turbidostat:

A continuous culture system equipped with a photocell that adjusts the flow of medium through the culture vessel so as to maintain a constant cell density or turbidity.

Virus:

An infectious agent having a simple acellular organization with a protein coat and a single type of nucleic acid, lacking independent metabolism, and reproducing only within living host cells.

Vitamin:

An organic compound required by organisms in minute quantities for growth and reproduction because it cannot be synthesized by the organism; vitamins often serve as enzyme cofactors or parts of cofactors.

Water activity (aw):

A quantitative measure of water availability in the habitat; the water activity of a solution is one-hundredth its relative humidity.

Yeast:

A unicellular fungus that has a single nucleus and reproduces either asexually by budding or sexually.

Zone of inhibition:

Area of no bacterial growth around a chemical on a disc, indicates sensitivity