Semester - III

I6MBP312 APPLICATION ORIENTED PRACTICAL – VI 4H – 2C

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9 Hours

SCOPE

This practical adds a good understanding of industrial microbiology and become qualified as microbiologist in food and beverage industries.

OBJECTIVES

This provides information on fermented food product production in food industries. To know the possible contamination of food products which may include bacteria and fungi.

1. Production of enzymes – Solid state & Submerged fermentation – GUS assay – Amylase
2. Production of protease from submerged fermentation
3. Production of sauerkraut ,yoghurt, wine
4. Enumeration of Microorganisms from Food samples
5. Detection and enumeration of Microorganisms present in lab surfaces.
6. Analysis of Milk quality by MBRT and resazurin
7. Detection of coliforms from water - MPN test
8. Isolation of plant pathogens – Bacteria and fungi
9. Acid production
10. Mushroom Cultivation
11. Immobilization technique (Sodium alginate method)

SUGGESTED READINGS

REFERENCES

1. Adams, M.R., and Moss, M.O., (2000). Food Microbiology. Royal Society of Chemistry. Cambridge, U.K.
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5. Demain, A.L., and Davies, J.E., (1999). Manual of Industrial Microbiology and Biotechnology
   (2nd ed.). ASM Press, Washington.
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9. Davies, J.E., and Demain, A.L., (2009). Manual of Industrial Microbiology and Biotechnology ASM Publisher, USA.
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APPLICATION ORIENTED PRACTICAL – VI (16MBP312)

Experiment No. 1

PRODUCTION AND ASSAY OF ENZYME AMYLASE

AIM:

To produce amylase from micro organism

BACKGROUND:

Amylase is an enzyme that breaks down starch or glycogen. Amylase is produced by a variety of living organism ranging from bacteria to plants and humans. Bacteria and fungi secrete amylase to the outside of their cells to assay out extra cellular digestion when they have broken down the insoluble starch into soluble end product such as (glucose or maltose) are absorbed into their cells.

MATERIALS REQUIRED:

Autoclave, microwave oven, nutrient agar, PDA, soluble starch, balance shaker, spectrophotometer, water bath, disposable spoons, micropipette, sterile water, petridishes, inoculation loop, descending needle or cork borer, Bunsen burner, glass spreader, 95% ethanol.

AMYLASE PRODUCTION MEDIUM:

Bacteriological peptone eg: $MGSO_4$ (0.5g), Kcl (0.5g), starch (1g), sterilize by autoclaving at 121ºC for 15 minutes.

PROCEDURE:

ISOLATION OF AMYLASE PRODUCERS:

1. Collect 100g of the top soil and transfer into a "Ziploc" bag.
2. Suspend about 10g of soil in 100ml sterile distilled water, mix properly (10^{-1}).
3. Pipette 10 ml of the above and transfer to another 90ml of water (10^{-2}).
4. Dilute further in two more 90 ml sterile water blanks(10^{-3}& 10^{-4}).
5. Spread 0.1 ml of the diluted samples (10^{-1} &10^{-2}) on NA plates (4 plates) containing 1% w/v of soluble starch and incubated at 30ºC for 24 hours.
6. Starch hydrolyzing colonies will have an area of cleaning around them. (It is confirmed by flooding plates with Grams iodine).
7. Transfer distinguishable amylase producing bacteria by streaking on a fresh plate of NA containing 1% starch.
8. Transfer the amylase producing isolates of bacteria to NA and allow to grow for 24 hours, then store in the refrigerator until needed.

AMYLASE PRODUCTION:

SEED CULTURE

- Prepare amylase production medium sterilize properly
- Inoculate loopful of amylase producing *Bacillus spp.*
- Incubate for 24 hours at 37ºC.

FERMENTATION

- Prepare 250 ml of amylase production medium in 500 ml conical flask.
- Sterile at 121ºC for 15 minute.
- Inoculate 25 ml of seed culture.
- Incubate at 37ºC for 24 hours under shaking condition.
- Withdraw culture and subject to extraction of enzyme.

EXTRACTION OF ENZYME FROM BACTERIA

- Pour the bacterial culture into centrifuge tubes and spin for 20 minutes at 5000 rpm
- Decant the supernatent and collect the crude enzyme extract in a sterilized beaker.

ENZYME ACTIVITY

- 1 ml of starch solution and 1 ml of properly diluted enzyme is pipetted in a test tube.
- It is incubated at 27ºC for 15 minutes.
- The reaction is stopped by the addition of 2 ml of dinitro salicylic acid reagent.
- The solution is then heated in a boiling water bath for 5 minutes.
- While the tubes are warm, add 1 ml of potassium sodium tartarate solution.
- Then cool it in running tap water.
- The volume is made to 10 ml by addition of 6 ml water.
- The absorbance is read at 560 nm.
- Terminates the reaction at zero time in the control tube.

Experiment No. 2

PRODUCTION OF PROTEASE FROM SUBMERGED FERMENTATION

AIM:

To produce protease from micro organism

BACKGROUND:

Protease and metaloprotease constitute one of the most important groups of industrial enzymes accounting for about 60% of the total enzyme market. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases and among bacteria, *Bacillus spp.* are specific producers of extra cellular proteases. These enzymes have wide industrial application including pharmaceutical industry, leather industry, manufacture of protein, hydrolysed food industry and waste processing industry. Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed resulting in fast reaction rates i.e., increase in the solubility of non-gaseous reactants and products & reducing incidence of microbial contamination by mesophilic organism. Protease secreted from thermophilic organisms are thus of particular interest and have become increasingly useful in range of commercial applications.

MATERAILS REQUIRED:

Autoclave, microwave oven, NA, casein, gelatin, balance, spectrophotometer, water bath, inoculation loop, glass spreader, 95% ethanol.

PROTEASE PRODUCTION MEDIA(g/l of distilled water)

$MgSO_4$-0.5g, K_2HPO_4-2.0g, Kcl-0.3g, NH_4NO_3-10g, Trisodium citrate-10g

Adjusted pH to 6.97 with 1M NaOH&this basal medium is sterilized by autoclaving121ºc for 15 minutes. Peptone is sterilized separately and are aseptically added to the flask containing the liquid medium after cooling

PROCEDURE:

1. Suspend about 10 g of soil in 100 ml sterile distilled water, mix properly(10^{-1}).
2. Pipette 10 ml of the above and transfer to another 90 ml of water(10^{-2}).
3. Dilute ferthur in two more 90 ml sterile water blanks (10^{-3}& 10^{-4}).
4. Spread 0.1 ml of the diluted samples (10^{-1}&10^{-2}) on gelatin hydrolysis medium (4 plates) &incubate at 30ºC for 24 hours.
5. Gelatin utilizing colonies will have in area of clearimg around them.
6. It is confirmed by flooding plates with mercuric chloride solution.

7. Transfer distinguishable, protease producing bacteria, streak on a flask plate of nutrient agar containing 1% gelatin.

PROTEASE PRODUCTION:

SEED CULTURE

- Prepare protease production medium, sterilize properly.
- Inoculate loopful of protease producing *Bacillus spp.*
- Incubate for 24 hours at 37ºC

FERMENTATION

- Prepare 250 ml of protease production medium in 500 ml conical flask.
- Sterilize at 121ºC for 15 minutes.
- Inoculate the above medium with 1 ml of an overnight culture &incubated at 37ºC for 24 hours at 1500 rpm in a rotatory shaker.
- At the time of interval, the turbidity of the culture is determined by measuring the increase in optical density at 470nm with a spectrophotometer.
- Withdraw culture and subject to extraction of enzyme.

EXTRACTION OF ENZYME FROM BACTERIA

- Pour the bacterial culture into centrifuge tubes and spin for 20 minutes at 5000 rpm.
- Decant the supernatant into a sterile beaker which is the trade enzyme extract.

ENZYME ACTIVITY

- Pipette 1 ml of culture extract "enzyme" into a test tube.
- Add 1 ml of 1 % soluble casein in citrate phosphate buffer(pH 6.5).
- Incubate in a water bath at 40ºc for 30 minutes.
- Set up a blank consisting of 2 ml of the enzyme extract that has been boiled for 20 minutes was added to the casein solution and treated with the same reagent as the experimental tubes.
- Estimate protein content by using Folins method.

Enzyme activity may be defined as the amount of casein produced per ml in the reaction mixture per unit time.

FOLINS METHOD

REAGENTS:

- 2% Na_2CO_3 in 0.1 N NaOH (Reagent A)
- 0.5% CuSo4& 1% potassium tartarate (Reagent B)
- Alkaline $CuSo_4$ (Reagent C)

-1ml Reagent A+1 ml Reagent B prior to use.

- Folins phenol reagent (reagent A)
- Protein solution.
- 4% trichloroacetic acid

MATERIALS REQUIRED

Culture supernatant, casein, tris buffer, TCA

STOCK SOLUTION: 10 mg of BSA was weighed & diluted or dissolved in 10 ml of distilled water, using a standard flask.

WORKING STANDARD: 10ml of stock solution was make up to 100 ml using distilled wayter 1 ml of this solution contains 0.1 mg proteins.

PROCEDURE:

1. 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard was transferred into a series of the test tube.
2. The volume was made to 1 ml in all test tubes. The test tube witn 1 ml of distilled water serves as the blank.
3. 5 ml of reagent C was added to each test tube including blank. The solutions are mixed well, allowed to stand for 10 minutes.
4. 0.5 ml of reagent D was added, mixed well and incubated at room temoeratur in dark for 30 minutes. A blue colour develops following incubation.
5. The OD value is measured at 660 nm.
6. About 0.5 ml of the culture filtrate was obtained after centrifugation was taken in a test tube.
7. To this mixture 1 ml of 1 % casein was added and incubated for 20 minutes at 35ºC
8. The reaction was arrested by adding 3 ml of 4% TCA.
9. The resultant mixture was centrifuged &0.1ml of supernatent was taken and made upto 1 ml with distilled water.
10. About 5ml reagent C was added and incubated for 10 minutes.
11. About 0.5 ml reagent D was added, mixed well and incubated at room temperature in dark for 30 minutes.
12. Absorbance is measured at 620 nm.

Experiment No. 3

PRODUCTION OF SAUERKRAUT

AIM:

To become acquainted with the microbiological production of sauerkraut

PRINCIPLE:

Sauerkraut is a classic examples of a food of plant origin produced by microbiological fermentation. Its preparation requires the fermentation activities of a mixed microbial flora, including *Leuconostocmesenteroides, Lactobacillus plantarum, L.brevis&Enterococcus feacalis.*

In the production of sauerkraut, shredded cabbage is treated with Nacl, which creates an osmotic environment in which plasmolysis occurs, extracting the juice from the cabbage tissue. The resultant brine solution favour the growth of lactic acid producing microorganism and inhibits the growth of other microorganisms. The lactic acid is responsible for the characters; sauerkraut flavor and also act as a preservative that cause food spoilage.

Production of the lactic acid is initiated by *L.mesenteroides,* which are cocci and sustained by *L.plantarum*which are bacilli when the acid concentration reaches a level of 0.7% to 1 %. The fermentative activities of *L.mesenteroides*cause and the final stages are carried out by *L.plantarum, L.brevis&E.fecalis.* This finished product contains a total acidity of 1.5 % to 2 % of which lactic acid represents 1% to 1.5%.

CHARACTERISTICS

1. **Odour:** acid earthy, spicy or patrid
2. **Colour:**brown pink, straw yellow, pale yellow or colourless
3. **Texture:**
    a. Soft => fermentation irritated by *L. plantarum* rather than *L. mesentreroides*
    b. Slimy => rapid growth of *L. cuccumeris*at elevated temperatures
    c. Rotted => spoilage by bacteria, yeast or mold
4. **pH:**The pH of the finished product should be in the range of 3.1 to 3.7
5. Place 10 ml of the fermentation juice and 10 ml distilled water into an Erlenmeyer flask. Boil to clear of the CO_2.
    a. Cool & add 5 drops of 1% phenol to the diluted juice.
    b. Titrate to the first persistent sample with pink colour with 0.1 N NaOH.
    c. Calculate % lactic acid as follows

        % lactic acid =ml of alkaline * normality of alkaline * 9/ weight of sample in g

        1ml = 1 g
6. Microscopic appearance of the microbial flora.

MATERIALS REQUIRED:

- ⇨ **Reagents:** 1% phenolphthalein, 0.1 N NaOH, methylene blue and uniodized table salt.
- ⇨ **Equipment:** Two wide mouthed jars with covers, two wooden boards to fit into jars, two heavy weights, cheese cloth, pH meter or indicator paper, pan balance microscope, Bunsen burner, inoculating loop, glass slides, cover slips, 10 ml disposable pipettes, mechanical pipetting device, knife &erlenmeyer flask.

Procedure:

1. Remove the outer leaves and all bruised tissues from each of the cabbage heads.
2. Thoroughly wash the heads in the tap water.
3. Shed the cabbage.
4. Weight the shredded cabbage on a balance and separate into two equal portions.
5. Weight out the table salt in amounts equal to 3% of the weight of each of the portions of shredded cabbage.
6. Place the shredded cabbage and salt in alter native layers in the two wide mouthed jars.
7. Place a wooden boards over each of the mixture and press gently to squeeze out a layer of juice from the cabbage.
8. Place a weight on each of the boarders and cover the jars with cheese cloths.
9. Incubate the jars for 14 days at 30ºC.

Experiment No. 4

PRODUCTION OF WINE

Aim:

To become acquainted with wine production by the fermentative activities of yeast cells.

Principle:

Wine is a product of natural fermentation of the juice of grapes & other fruits, such as peaches pears, plums and apples by the action of yeast cells. The biochemical conversion of juice to wine occurs when the yeast cell enzymatically degrade the fruit, sugar, fructose & glucose first to acetaldehyde & then to alcohol.

Grapes containing 20% to 30% sugar concentration will yield wines with an alcohol content of apprx., 10% to 15% for red wine, the crushed grapes must be fermented with their skin to allow extraction of their colours into the juice. White wine is produced from the juice of white grapes.

The commercial production of wine is a long & extracting process. First, the grapes are crushed or pressed to express the juice, which is called must. Potassium metabisulfite is added to the must to retard the growth of acetic acid bacteria, molds & wild yeast that are endogeneous to grapes in the vineyard. A wine producing strain of yeast,*S.cervisciae*varellipsoideces is used to inoculate the must, which is then incubated 3 to 5 days under aerobic conditions at 21ºC to 32ºC. This is followed by an anaerobic incubation period. The wine is then aged for a period of 1 to 5 years in aging tanks or wooden barrels. During this times the wine is clarified. If any turbidity there by producing volatile esters that are responsible for characteristics flavours. The clarified product is then filtered, pasteurized at 60ºC for 30 minutes & bottled.

⇨ Production of white wine from white grape juice:

1. Total acidity (expressed as % tartaric acid). To a 10 ml aliquot of the fermenting wine, add 10 ml od distilled water and 5 drops of 1 % phenolphthalein solution. Mix & titrate to the first persistent pink colour with 0.1 N NaOH.

Total acidity:

i) % tartaric acid = ml alkali * normality * 7.5/weight of sample in g

1 ml = 1 g

ii) Volatile acidity (expressed as 1 % acetic acid): following titration, calculate volatile acidity using the formula
% tartaric acid = ml alkali * normality * 6.0/weight of sample in g

iii) Alcohol (expressed as volume %)

iv) Aroma : fruity, yeast like,sweet

v) Clearity : clear turbid

MATERIALS REQUIRED:

- Cultures : 50 ml of white grape juice broth culture of *S.cervisciae*varellipsoideces incubated for 48 hours at 25ºC
- Media: 500 ml of pasteurized commercial white grape juice.
- Reagents : 1% phenolphthalein solution, 0.1 N NaOH sucrose.
- Equipment: 1l Erlenmeyer flask, one holded rubber stopper containing a 2-inch glass table plugged with cotton, pan balance, spatula, glassine paper and burette or pipette for titration

PROCEDURE:

1) Pour 500 ml of the white grape juice into the 1l Erlenmeyer flask. Add 20 g of sucrose and 50 ml of *S.Cervisiae* grape juice broth culture (10% starter culture). Close the flask with the stopper containing a cotton prugged air vent.
2) After 2 days & 4 days of incubation, add 20 g of sucrose to the fermenting wine.
3) Incubate the fermenting wine for 21 days at 25ºC

Experiment No. 5

YOGHURT PRODUCTION

AIM:

To prepare yoghurt from milk using *Lactobacillus & streptococcus.*

INTRODUCTION:

Yoghurt is produced by the fermentation of warm milk by *L. vulgaricus& S. thermophillus.* These teo bacteria are able to grow at 40-45ºC during which they produce lactic acid and various other by products that give its unique flavour

MATERIALS REQUIRED:

Cow milk, concentrated skim milk nonfat dry milk, whey lactose.

- ⇨ SWEETNESS: glucose or sucrose, high intensity sweetness (eg: aspartame).
- ⇨ STABILIZERS: gelatin,carboxy methyl cellulose, lowest bean guar, alginates, carrage means whey protein concentrate, starter culture, *S. salivarius* , sub spp*thermophillus*(ST) and *Lactobacillus delbreukii*, sub spp. *vulgaricus*(LB).

PROCEDURE:

1. 100 ml of whole milk was taken in 50 ml sterile beaker & boiled.
2. 4g of powdered milk was added and sterilized constantly using sterile glass rod.
3. Required quantity of sweetness, stabilizer and flavouring agent was added.
4. Milk sample was cooled to 45ºC and inoculated with 1 teaspoon of commercial yoghurt or 10 ml of culture.
5. The inoculated sample was wrapped and incubated at 45ºC for 24 hours.

Experiment No. 6

ENUMERATION OF MICROROGANISM FROM FOOD SAMPLE

AIM:

To analyse and distinguish the types of spoilage caused by microorganisms to bread and vegetables.

PRINCIPLE

Foods are especially susceptible to microbial decomposition because they are rich in nutrients that support the growth of microbes contaminated foods are often unedible and may also be the source of human disease if the contaminating organism are pathogens or toxin produces some of the important foods poisons caused by bacteria are botulism(caused due to clostridium botulism), staphylococcal(staphylococcus aureus) and enteric(E-coli) and salmonella typhymenium. There food preserving are serious and sometimes fatal.

MICROBIAL SPOILAGE OF FOOD:

Moulds are the most common cause for spoilage of food. The temperature attained in the banking procedure usually are high enough to kill the air during cooking from handling or from wrapper and usually initiate growth in the crease of the leaf and between the slice of the bread. Chief moulds involved in the spoilage of bread are the bread moulds, Rhizopus, stolonifer, other moulds are penicillium expansum or penicillium stoloniferum, Aspergillus niger etc. Ropiness of bread is common in home baked breads, especially during hot weather. It may be cause by bacterial species namely Bacillus or Bacillus litheniforms.

MICROBIAL SPOILAGE OF VEGETABLES:

Microbial spoilage of vegetables may be due to plant pathogens saprophyte. Organisms, which may be secondary invaders and may enter a healthy vegetables in the case of various rots and grows on its surface Bacterial spoilage is commonly encountered in most piled vegetables.

MATERIALS REQUIRED:

Sample: Spoiled Bread and Vegetables.

Medium: Nutrient agar medium for bacterial isolation, peptone broth, malt extract agar for fungal and mold isolation.

Equipments: Sterile petridishes, inoculation loop, Bunsen burner, s sterile pipettes and pestles etc.

PROCEDURE:

1. About 20gm of food samples to be tested was taken and placed in a clean mortor.

2. The sample was then ground to a fine pulp with the help of a pestle.
3. The sample was discharged into 20ml of sterile buffered peptone broth to obtain 10^-1 dilution, the sample was serially diluted upto 10^-2 dilution.
4. Then the pour plate technique and spread plate technique was performed using nutrient agar for technical isolation and malt agar for fungal and mold isolation.
5. After 24 hours of incubation of the plats at 37degree Celsius in the case of bacteria and 3-5 days of incubation at about 25degree Celsius incase of fungi. The colonies were counted and the sample of spoiled bread and vegetables can be used to judge the microbial quantity of the food sample.

RESULT:

Analysis of spoiled Bread samples:

The chief types of microbial spoilage of baked bread was analysed microscopically.

Microscopic observation of fungal methods:

The chief moulds involved in the spoilage of bread moulds, Rhizopus sps, with its white cottony mycelium and black dots of sporangia.

- Green spread penicillium sp., was observed.
- Aspergillus sp., was identified with its greenish or purplish brown to black conidial heads and yellow pigment diffusing into the blood.

Interpretation:

As the bacterial growth of 10^-2 is 58*10^2 CFU/g of the spoiled bread sample shows the presence of numerous micro organisms.

The fungal growth is 10^-2 dilution in 17 plaques/ml shows that the bread is spoiled by most of the fungal species also.

Analysis of spoiled vegetables:

Microscopic vegetables of fungal moulds:

Rhizopus sps., was observed with its white mycelium and black dots of sporangia Aspergillus sps., was observed within its spore becoming heads which are long, tightly packed and rough coloured from tuft to grey to blackish. The blue green bread mould was observed to be penicillium sp., which causes soft rot of the vegetables.

Geotrichum sps., was observed with its white yellowish growth appearing as a fir felt like mars and appears as a soft and creamy on the vegetables.

Trichodema sp., was observed on the vegetables as the bright green conidia glued to together and tuft of white hyphae stick up well above the conidiospore.

Microscopic observation of the fungal:

Moulds:

The fungal mycelia growth was teased off with the teasing needle and lactophenol cotton blue staining method wasperformed to be observed under microscopic for characteristic morphology.

Microscopic observation of bacterial species:

The greenish tinge produced on the surface of the vegetables was deduced to be pseudomonas sps., staphylococcus aureus was observed on the sliminess was caused by saprophyte bacteria in the vegetable sample.

Interpretation:

As the bacterial growth in 10^-2 dilution is 69*10^2 CFU/g of the spoiled vegetable sample shows the presence of numerous micro organisms. The fungal growth in 10^-2 dilution is 33 propagulated ml shows that the vegetables has been spoiled by most of the fungal species also.

Discussion:

As microorganisms are ubiquitous their presence in food is unavoidable and complete sterility in food products is inevitable. But when the number of microorganisms in the food crosses a limit, it is considered as unfit for consumption, when pathogenic microorganisms are involved its consumption may lead to a number of complication ranging from food poisoning like salmonellosia, many organisations like world health organisms has set the standard of food Quality.

Experiment No. 7

Detection and enumeration of microorganisms present in utensils

Aim

To isolate the pathogenic microorganisms from utensils

Background

Various types of surface such as plastic, stainless steel, glass and wood are used today in the food industry. These surfaces are subject to contamination by microorganisms, some of which are able to form biofilms. Contamination of surfaces depends on their characteristics, such as smooth, rough, porous, or irregular, and their state, for example before or after the cleaning process, new or old, dry or wet. The kitchen is probably the most crucial area that harbors and transmits infection. Utensils can contribute to the spread of bacteria such as *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus, Salmonella* spp. and *Campylobacter* spp.

Materials

Utensils - knife, spoon and fork, nutrient agar, potato dextrose agar, petriplates, sterile cotton swabs.

Procedure

Swabbing method is commonly used in plastic, stainless steel and wood to detect the pathogenic bacteria.

Swabbing is done by using a sterile cotton swab with an applicator stick for detecting microorganism from surfaces.

The cotton swab bud applied on a material surface to recover the bacterial and fungal spores by direct plating method.

The direct plating was done by rubbing the sample on agar plate of nutrient medium for bacteria and potato dextrose agar for fungi and incubated at 37 °C for 24 h.

The bacterial isolates are counted and the results were tabulated.

Experiment No. 8

ANALYSIS OF MILK BY METHYLENE BLUE REDUCTION TEST (MBRT)

AIM:

To determine the methylene blue reduction time of the given milk sample to check the quantity of milk.

PRINCIPLE:

Milk is good medium for the growth of microorganisms. A variety of microorganisms can be found in both raw milk and pasteurized milk. These are actively growing microorganisms. Normally the milk is contaminated with microorganism such as *S.aureus, S. pyogens, .aeuroginosa, Enterobacter spp., Bacillus spp., etc,*. Contaminated milk is one of the important sources for transmission of diseases from animals to humans. The main reason for this contamination is the unproper handling of milk. Normally milk is contaminated during the milking process by the animal, such as udder and adjacent areas unsterilized diary utensils such as milking machines. Milk is also a good source of contamination of microorganisms.

The principle of methylene blue reduction test depends on the fact that the colour imparted to the milk by adding a dye such as methylene will disappear more or less quickly, which depends on the quality of the milk sample to be examined. Methylene blue is a redox indicator, that lose its colour under the absence of oxygen and is though to be reduced. The depletion of oxygen in the milk is due to the expanded rate of bacterial metabolism. The dye reduction time refers to the microbial load, in the milk and the total metabolic reactions of the microorganisms.

MATERIALS REQUIRED:

- ⇨ SAMPLE: milk
- ⇨ REAGENTS: methylene blue indicator.
- ⇨ SOLUTION: 0.1 mg of methylene blue solution was atken and mixed in 250 ml distilled water 1:25000 dilution.
- ⇨ GLASSWARE AND EQUIPMENT: screw capped tubes, beaker, pipette, water bath, autoclave, hot air oven.

PROCEDURE:

1. Sterilized screw capped tubes, were taken and 10 ml of the given milk sample was transferred into each test tube and to that 1 ml of methylene blue dye was added.
2. A thin foil was placed and gently inverted to mix (avoid air bubbles).
3. The tubes were placed in the water bath at 37ºC and checked at intervals of every 5 minutes.

4. The time for the dye reduction to occur gives a clear picture about the number of organism present in the sample which is assumed with the help of a standard chart.

Experiment No. 9

BACTERIOLOGICAL EXAMINATION OF WATER BY MPN TEST

INTRODUCTION

The three basic test to detect coliform bacteria in water are presumptive. Confirmed and Complete. The tests are performed sequentially on each sample under analysis. They detect the presence of coliform bacteria (indicator and fecal contamination). The gram –ve, non spore forming Bacilli that ferment lactose with the production of acid, gas that is detectable following a 24hours incubation period at 31 degree Celsius.

PRESUMPTIVE TEST:

Determination of the most probable Number of Coliform bacteria :

PURPOSE:

To determine the presence of Coliform bacteria in water sample

To obtain some index as to the possible number of Organisms present in the sample under analysis.

PRINCIPLE:

The presumptive test is specific foe detection of Coliform bacteria measured adequate of the water to be tested are added to a lactose fermentation both containing on incubated gas vial. Because these bacteria are capable of using lactose as a carbon source(the other enteric organism are not) their detection facilitates by the use of this medium. In this experiment the lactose fermentation broth also contain a surface tension depressant, bile salt which is used to suppress the growth of organisms other than the Coliform bacteria.

Tubes of the lactose medium are inoculated with 10ml,1ml,0.1ml aliquots of the water sample. The series consist of atleast 3 groups. Each composed of a 5 tubes of the specified medium. The tubes in each groups are then inoculated with the designated volume of water sample as described under ‘procedure’. The greater the number of tubes per group. The greater the sensitivity of test development if gas in any of the tube is presumptive evidence of tge presence of Coliform in the sample. The presumptive test also enables the microbiological to obtain some ideas of the number of coliform organism by means of MPN test.

MATERIALS REQUIRED:

Culture: Water Sample

Media: 15 double strength lactose fermentation broth and 30 single strength lactose fermentation broth.

Equipment: Bunsen burner, 45 test tuber, test tube rack, sterile 10ml pipette, sterile 1ml pipette and 0.1ml mechanical pipetting device and glassware marking pencil.

PROCEUDRE:

1. Set up 3 separate series consisting of 3 groups, a total of 15 tubes per series in a test tubes rack; for each tube labels the water source and volume of sample inoculates as illustrated
2. Mix the sewage plant water sample by shaking thoroughly. Excessive case handling sewage waste water sample because enteric pathogen may be present.
3. Flame bottle then using a10ml, pipette transfer to 10ml, aliquote of sample to 5 tubes labelled 1.32*10ml.
4. Flame bottle then using 1ml pipette transfer 1ml aliquote of water sample to 5 tubes labelled LB1*0.1ml.
5. Flame bottle then using o.1ml pipette transfer 0.1ml aliquote of water sample to 5 tubes labelled lB1*0.1ml
6. Repeat step 2 through 5 for the tap and pond water sample.
7. Incubate all tubes for 48 hours at 37degree celsius.

CONFIRMED TEST:

Purpose: To confirm the presence of Coliform bacteria in a water sample for which the presumption test was positive.

Principle: The presence of positive doubtful presumptive test immediately suggest that the water sample is non-portable confirmation of these results is necessary since positive presumptive tests may be the result of organisms of non-coliforms origin that are not recognised as indicator of focal pollution. The confirmed test required that selective and differential media such as eosin methylene blue(eMB) or endo agar be streaked from a positive lactose broth obtained from presumptive test. The nature of differential and selective media was discussed Eosio-methylene blue contains the dye methylene blue, which inhibits the growth of gram +ve organisms. In the presence of an acid environment. EMB forms a complex that precipitates out onto the coliform colonies producing dark centre and a green metalistic sheen. The reaction is characteristic of e-coli, the major indictor of fecal pollution.The endo-agar is a nutrient medium containing the dyr

fuschin which is present in the decolourized state. In the presence of acid produced by coliform bacteria fuschin form a dark pinkcolour complex that turns the E-coli colonies and surrounding medium pink

MATERIALS REQUIRED:

Culture: One 24 hrs old positive lactose broth culture from each of the 3 series from the presumptive test.

Media: Three each per designated student group. Eosin methylene blue agar plate or endo agar plate.

Equipment: Bunsen burner, glassware, marking pencil, inoculation loop.

PROCEDURE:

1. Label the covers of the 3 EMB plates or 3 endo-agar plates with the source of water sample.
2. Using a +ve 24 hours lactose broth culture from the sewage water series from the presumptive test, streak the surface of one EMB or Endo agar plate.
3. Repeat step using a +ve lactose broth culture from the pond and tap water series to inoculate the remaining plates.
4. Inoculate all plate cultures in an inverted position for 24 hours at 31degree Celsius .

COMPLETED TEST:

Purpose: To confirm the presence of Coliform bacteria in a water sample, or if necessary, to confirm a suspicious but doubtful result of previous test.

Principle:

The completed test is the final analyse of the water sample. It is used to examine the coliform colonies that appeared on the EMB or Endo agar plates used in the confirmed test. An isolated colony is picked from the confirmatory test plate and inoculated into tube of lactose both and streaked on a nutrient agar slant to perform a gram stain following inoculation and incubation tubes showing acid and gas in the lactose broth and the presence of gram negative bacilli on microscopic examination are further confirmation of the presence of E-coli and they are indicative of the completed test.

MATERIALS REQUIRED:

Cultures: 24 hours Coliform +ve EMB or endo agar culture from each of the three series of the confirmed test.

Media: Nutrient agar slants and lactose fermentation broth.

Reagent: Crystal violet, Grams iodine, 95% ethyl alcohol and safranin.

Equipment: Bunsen burner, staining tray, inoculating loop, lens paper, bibulous paper, microscopic and glassware, marking pencil.

PROCEDURE:

1. Label each tube with the source of its water sample.
2. Inoculate one lactose broth and one nutrient agar slant from the same isolated E-coli colony obtained from an EMB or endo agar plate from each of the experiment water sample.
3. Incubate all tubes for 24 hours at 31degree Celsius.

RESULT:

PRESUMPTIVE TEST: Examine all tubes after 24 hour and 48 hours of incubation, the result were tabulated.

Experiment No. 10

ISOLATION OF PLANT PATHOGEN

The term "pathogen" means the organisms that cause diseases on living beings. It may be fungus, bacteria, virus etc,. The pathogen can easily be isolated in artificial culture media for identification and subsequent characterization.

MATERIALS & METHODS:

- ⇨ Infected plant parts/ plants.
- ⇨ Petridishes.
- ⇨ Forceps and knife.
- ⇨ $HgCl_2$ solution(1:1000)

PROCEDURE:

1) An infected plant or leaf of rice was brought to the laboratory from the field for isolation of pathogen.
2) The infected bisionic part was removed by a knife and quickly transferred to the sterilizing solution.
3) Surface sterilization of the infected plant was done by transferring the infected leaf segment into a petridish containing $HgCL_2$ solution (1:1000) & kept for 2-3 min.
4) Then the leaf segment was transferred to a series of petridishes containing $HgCL_2$.
5) Finally the excised leaf segment was placed aseptically into the slant for culture of pathogen.
6) The slant was then incubated at a required temperature for 3-5 days after proper leveling. Finally the culture thus developed was examined microscopically.

Experiment No. 11

CITRIC ACID PRODUCTION

INTRODUCTION:

Citric acid is a weak organic acid with the formula $C_6H_8O_7$. It is a natural preservative, conservative and is also used to add an acidic or sour taste to foods & drinks. The conjugate base of citric acid, citrate, is as important as an intermediate in the citric acid cycle, which occurs in the metabolism of all aerobic organism. It consists of 3 carboxyl (R-COOH) groups. The most commercially used method for the production of citric acid is bu*A.niger* using cane molasses as an example of fungal over flow metabolism. Many microorganisms, such as fungal & bacteria can produce citric acid but *A.niger* remained the organism of choice for the production of citric acid due to its genetic stability, high yields [apartly of using cheaper raw materials like cane molasses] & absence of undesirable reactions by recycling & reusing waste material from cane molasses citric acid production can be easily achieved by using microorganisms that have the ability to produce citric acid efficiency such as *A.niger*.

MATERIALS & METHOD:

- Physical characteristics of the sugarcane molasses:
  - ⇨ The physical characteristics of sugar cane molasses, such as moisture content, ash measurement and pH were analyzed.
- Moisture content and ash measurement:
  - ⇨ The moisture content and ash measurement of molasses was performed by taking 10g of molasses sample & oven dried in at 104ºC for 30 minutes. Then the results were calculated using the following equation

    Moisture content (%) = (A-X/A) * 100

    Ash unit = weight of molasses before burning (A) – wt of molasses after burning(X)
- The pH values
  - ⇨ The pH values was measured before & inoculation of molasses sample using pH meter device.
- Production of citric acid from raw sugarcane molasses:

  A.niger transferred to the flask containing raw sugar cane molasses media with different concentration (20%, 30%, 40%, 50% & 60%) by taking 100 ml, 150 ml, 200 ml,250 ml & 300 ml of sugarcane molasses and the volume was completed to 500 ml using sterile distilled water. The flasks were autoclaved at 115ºC for 10 minutes. An amount of 50 ml distilled water was added to the fungal pure culture to make a fungal suspension & then 10 ml from this suspension was transferred to

the sugarcane molasses media. All flasks were incubated at 28ºC for 144 hrs. to10 days. After incubation the suspension was distilled to monitor the growth and observe the result.

=> Detection of citric acid:

The detection of citric acid was done chemically by the addition of 3 drops of bromocresol green indicator to the 10 ml of distilled yield.

⇨ Determination of citric acid concentration:
Citric acid was determined by titration using 0.1 N NaOH& phenolphthalein as indicator and calculated as percentage according to the following formula.

- Normality of citric acid = Normality of NaOH * NaOH volume/V. of citric acid
- Concentration of citric acid = Citric acid normality * equivalence * 100 /V. of distillation
- Equivalent = 96, volume of distillation : 10.

Experiment No. 12

MUSHROOM CULTIVATION

AIM:

To know cultivation technology of edible mushroom cultivation of oyster mushroom spawn production.

BACKGROUND INFORMATION:

Mushroom are fresh fungi, which constitute a major group of lower plant kingdom. The mushroom is a common fungal fruit body that produces basidiopores at the tip of the basil. The mushroom consisting of short stem and a cap which being to open like umbrella. India is the second most popular country of the world with a population of over 100 crores mushroom provide a rich addition to the diet in the form of proteins, carbohydrates, minerals and vitamins.

Nutrient value of mushroom include 91.1% moisture 29.9%, proteins 4.4%, carbohydrate 0.3, fat and 16K calories.

Presently about a dozen fungi are cultivated in over 100 countries with a production of 2-2 millions. *Agaricusbasidiospores*(56%), white button colonies, edodes(14%) spilt take *Volvaridlyvolvneay(8%)* paddy straw, *Pleurotus spp., Oyster flammulina(5%), Mushroom timmula spp.,(4.6%), Selvercarphiloter(1.1%)* are the mushroom varieties cultivated or human usage.

Species of the genus *Pleurotus* called oyster mushroom or Dhingri or wood fungus is ranked as the 4^{th} important cultivated mushroom with a productin of 15,000 tons per annum. The genus contains over 50 spp of these *S.sapidus, P.fossulates, P.sequarresules*and*P.florida*have been cultivated on India.

OYSTER MUSHROOM CULTIVATION:

The oyster mushroom are rich in protein mineral contents, devoid of starch and low calories and CHO. These are ideal food for diabetic and heart patiently and those who do not want to put on weight. The various substrates utilized for the cultivation of *Pluerotus* are banana, psuedolims wheat straw, paddy straw, raggi straw, compressed rice husk and karad hay. However the highest yields are obtained on rich straw. These can be grown in any container. Eg: earthern pot, cane gasket, polythene bags, iron baskets or in wooden trays.

SPAWN PRODUCTION:

Thatched hut/polyethylene chamber, mud/ pure house, dry paddy, straw (chopped) or other agrowaste 100kg horse grain powder, 4 kg spawn bottom of *Pluerotus spp.,* polyethylene bags 1 kg , water sprayer.

PROCEDURE:

1. Take dried paddy straw.
2. Chop the straw in to 1 to 2 cm bits.
3. Soak the chopped straw into water overnight.
4. Add horse grain powder at the rate of 8g/ kg.
5. Add spawn at the rate of 30 g /kg.
6. Mix all the constituents.
7. Fill the mixture into polyethylene bags with holes.
8. Incubate the fluid bags in a room at 21 to 35ºC with sufficient light and ventilation for 15-16 days for spawn running.

Experiment No. 13

IMMOBILISATION OF YEAST CELL BY USING SODIUM ALGINATE

AIM:

To entrap the live cells in a solid matrix and analyze its activity.

PRINCIPLE:

The technique involves a reaction between sodium alginate and calcium alginate with the optimum concentrations of the reactants, the product particles coagulates resulting in the formation of beads. This property could be used as a suitable technique to immobilize microbial cells in the form of beads. Entrapped in insoluble alginate gel is recognized as a rapid non-toxic, inexpensive, versatile and the most often used method for immobilization of cells. More than 80% of cell immobilizationprocess are still carried out using alginate. Immobilization offers many potential advantages over free cell system such as higher cell densities and cell loads, increased volumetric productivity short overall reaction times. Smaller fermenter size which may lower capital.The reverse of the same biocatalyst for prolonged period oftime due to constant cell regeneration.a continuous process which may be performed beyond the nomical washout rate, improved substrate utilization reduced risk for microbial contamination. Process design, constant rate, improved substrate utilization, constant product quality and improved or protection of cells from substrate and end product inhibition.

MATERIALS REQUIRED:

Sodium alginate 5.5 gm

Culture: any one type of seed culture for fermentation

Medium: PDA, growth medium, fermentation medium

PROCEDURE:

I) PREPARATION OF SEED CULTURE:
   1) Prepare growth medium and sterilize at 110ºC for 10 minutes.
   2) Inoculate a loopful of yeast culture from slant or 1 ml of culture from broth.
   3) Incubate at 37ºC for 18 hours

II) PREPARATION OF IMMOBILIZED CELLS:
   4) Prepare sodium alginate slurry by adding 5.5 g sodium alginate to 150 ml of 0.1% NaCl solution with continuous slurring.
   5) Allow the slurry to stand for 6 to 8 hours and add 15 ml of seed culture.
   6) Prepare the beads by controlled drop wise addition of slurry to 4% $CaCl_2$ solution.
   7) Keep the beads in $CaCl_2$ solution about 30 minutes for gelatin.

III) ACTIVATION OF IMMOBILIZED BEADS:

8) perform activation of immobilized beads with help of growth medium.
9) Inoculate required volume of beads into the rich medium and kept with for 30 minutes.
10) Note the movement of beads.

IV) FERMENTATION OF ALCOHOL USING IMMOBILIZED BEADS:

11) Add 25 ml of beads to 250 ml of fermentation medium and carry out the fermentation under static condition at room temperature.
12) Estimate the alcohol content through chromic acid method.
13) Measure volume of beads using water displacement method.