LABORATORY MANUAL OF DOWNSTREAM PROCESSING

DEPARTMENT OF BIOTECHNOLOGY

6th Semester

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MEMBRANE BASED FILTRATION

Aim of the experiment:-

To select appropriate method of sterilization and disinfection through membrane based filtration.

Theory:-

Membrane filtration:-

Sterilization of heat sensitive substance like enzymes, antibiotics, amino acids could not be done by autoclaving because these may be denatured and rendered non-functional. hence this can be sterilized through various types of filters which may written bacteria. Millipore and Whatman membrane filter is made up of cellulose acetate and cellulose nitrate that contains small sized pores of varying diameter that is (0.2 to 0.45 and 0.5 mm).

But membrane filter 0.22 mm is generally preferred. Millipore membrane filter is placed inside the filtration assembly which is made up of autoclavable plastic materials, stainless steel is used. In such case solution to be sterilized usually are passed through membrane filters by negative pressure applied through centrifugal force. The filtrate obtained becomes microbes free.

Membrane filtration method:-

membrane filtration method is an alternative to the mpn method for the microbiological analysis of water. Sometime it also refers as millipore filter technique or MF technique. The millipore filter technique was made by two scientist “Geetz and Tsureishi” in 1951, membrane filtration method make the use of membrane filters.

A Millipore filter processes a uniform velocity are predetermined pore size of 0.45 mm. The pores in the membrane filter are sufficiently small to trap the different types of microorganisms. Membrane filter is composed of polymeric substance.

The membrane filtration method operates on four membrane filtration assembly. To carry out the water analysis, a filtration unit comprises of many components like a funnel, vacuum pump, filtration flask, filter pump etc.

Definition of membrane filtration methods:-

membrane filtration method can be defined as a process of microbiological analysis of water by making the use of special filter like membrane filter to trap the microorganisms.

It is a very effective method for the isolation and enumeration of microorganisms in the test water sample. Knowing the quantity of microbial mass the quality of water can also be determined. Therefore the membrane filtration method can check out the quality of water and quantity of microorganisms present inside the water.

Membrane filtration method was introduced in the last 1950 widely accepted by the E.P.A. membrane filtration is the best technique for water analysis as it allows the testing of large volume of water sample in very less amount of time.

The main project of membrane filtration method used to determine the presence or absence of a particular group of microbes that is commonly present in waste water and groundwater.

The water quality is analysed indirectly by counting the number of colonies through colony counter. One colony of bacteria will represent the single bacterium in the hundred ml of water sample.

Membrane filter:-

Membrane filter can be defined as the molecular biological filter which is formed by the biological inert cellulose esters. It is having a uniform or a predetermined for size which traps the different type of microorganism whose size ranges from 0.01 to 0.10 mm.

The membrane filters are widely used in laboratories for isolation and separation methods. In industries like pharmaceuticals cosmetics food and beverage membrane filters are used to monitor and preservation of bacteria and sterilizing the heat liable fluids.

More than this membrane filter are extensively used in water engineering to to analyse the quantity of water by direct microbial count.

Absorbent pad:-

An absorbent pad consists of a filter paper disc which are free of sulphide and other compounds which suppress the growth of bacteria. The MF techniqueincreases the use of absorbent paper of diameter 48mm with the thickness of 0.8 mm for stop the absorbent paper absorbs 1.8 to 2.2 ml of nutrient medium.

The absorbent pad structured with the appropriate liquid growth medium to which 1.5% of agar may be added. Over the saturated absorbent medium, a membrane filter is placed and incubated for the growth of bacteria colonies.

Membrane filtration assembly:-

1. To perform membrane filtration there is a filtration unit which commonly refers to a membrane filtration assembly.
2. Filtration unit comprises to the looking ring carbon disc, stainless base, rubber stopper, filter flask and vessel pump.
3. First the water sample passed through a funnel. A stainless funnel present at the tap below.
4. Membrane filter is placed over the carbon disc.
5. The stainless base holds the carbon disc, and the membrane filter. At the base a rubber stopper controls the flow of water to the filter flask.
6. On the one end of the filter flask there is a connector which connects to the vacuum motor. With the filtration unit of vacuum, it provides a negative pressure which is is the solution of filtrate.

Method of Membrane filteration:-

Membrane filtration method involves the following steps:-

1. **Sample collection**:- collect water samples like ground water and waste water in the sterilized sample bottles. Then dilute the water sample making a volume upto 100ml.

2. **Selection of appropriate nutrient medium**:- For the isolation and enumeration of different pathogenic microorganisms in the water use a suitable nutrient medium. After selection of an appropriate nutrient medium transfer it to the sterilization petri plate containing absorbent pad at the bottom. Stock the absorbent pad with the liquid bottom. the saturation of absorbent pad with the nutrient medium will promote microbial growth.

3. **Membrane filtration:-** for the membrane filtration of water, place a membrane filter over the carbon disc by the help of forceps sterilized with flame. Claim the opening of the sample bottles and pour the test water samples through the funnel. A suction system allows the complete separation of filtrate through the membrane filter. After completion and separation of the filtrate, rince the funnel with bottled water.

4. The placement of membrane filter:- flame the forceps and take out the membrane filter from the filtration assembly. Then place the membrane filter over the prepared petri dish containing absorbent pad saturated with liquid nutrient medium.

5. Incubation:- Incubate the petri plate for 24- 48 times at 35- 37°C temperature.

6. Quantitative analysis:- count the number of colonies directly on the colony counter.

7. Qualitative analysis:- to test the water quality isolate and identify the type of microorganisms by subtracting the isolated colonies. Then perform the staining method microscope observation and further test to identify the specific type.

Advantages:-

1. MF technique is an alternative to the most probable number method.
2. One can test a large volume of water, sample by the millipore filtration method within less time.
3. Membrane filtration method consumes less time than the MPN method as it only involves 4 to 5 steps to get the results.
4. Membrane filtration techniques give relevant and reliable results then MPN methods.
5. Number of bacteria present in the water sample are counted directly by the colony counter.

Disadvantages:-

1. Membrane filtration method is not applicable for testing of turbid water.
2. There may be a chance of bacterial overgrowth as the water contains many microorganisms.

Conclusion:-

Therefore membrane filtration method is an efficient method as it isolates and enumerates the number of microorganisms from the microbiological contaminated fluid sample.

PROTEIN PRECIPITATION AND RECOVERY

Aim of the experiment:-

To isolate casein from milk by isoelectric precipitation method.

Principle:-

Precipitation involves the conversion of soluble into insoluble solids which can be separated from the liquid by physical methods of separation such as filtration or centrifugation. Precipitation serves primarily as a method for the recovery and concentration of desired products and it is well established for the recovery of bulk protein. Proteins are the most complex biomolecules, many different methods have been devised to separate proteins from other proteins.

Similar to amino acids the complex protein in itself can be positively or negatively charged overall due to the terminal amine NH_2 and carboxyl- COH groups on the side chain.

Is positively charged at low pH and negatively charged at high pH. Similarly by changing the pH of the protein solution the ionization of the weak acetic acid basic amino acid side chain of a protein is affected releasing in a net zero charge of the protein at a certain PH value called its isoelectric point or isoelectric PH. When the pH of the protein solution at low ionic strength is adjusted to a value equal to its isoelectric point, solubility of the global protein decreases drastically over a narrow pH range and it tends to precipitate out. Isoelectric precipitation.

The effect of PH on the solubility of a typical protein such is given to show casein in a heterogeneous mixture of protein food in milk. It exists in milk as a solid calcium salt, precipitation at a PH value of 4.6 calcium is a nutritionally adequate protein.

This means that it has all the essential amino acids required for the normal growth and development. Casein is a phosphoprotein produced in a mammary tissue and is 3-5 years of Bovine and human milk.

Casein is a protein with hydrophobicity precipitation at its isoelectric point formed in large strong aggnetus.

Materials required:-

skimmed milk , acetic acid, beaker, pH meter, isopropanol, filter paper, magnetic stirrer, conical flask.

Procedure:-

Around 200 ml of skimmed milk is taken in a 500 ml beaker and warmed 200 degree Celsius. Acetic acid is prepared by dissolving 7.0 ml of glacial acetic acid in 50 ml water is added drop wise to the beaker containing milk with constant stirring till the procedure of precipitation is complete. A mass of Casein is precipitated because it is not an instantaneous process and a few minutes was given between acid additions to allow for sterilization. The precipitation was allowed for decantation of upper water layer. Most of the precipitate is washed with 200 ml of distilled water there to remove the salt and then filtered using Buchner funnel where a mass of Casein that is obtained is busted with isopropanol or ethanol and dried.

Finally the amount of casein isolated was calculated.

Confirmation test:-

The isolated case is dissolved in water and allowed to settle for a few minutes. Few drops of Collins reagent is added to the above solution and heated. The appearance of blue colour indicates the presence of protein Casein.

Observation:-

Weight of watch glass (W1) = 36.8 grams

Weight of watch glass (w2) = 41.2 grams

Weight of dry casein= (w2 - w1) = 41.2 - 36.8 = 4.4grams

Result:-

Amount of casein obtained is 4.4 grams.

ION EXCHANGE CHROMATOGRAPHY

Aim of the experiment:-

To observe ion exchange chromatography technique.

Objective:-

Isolation of an enzyme lysozyme from chicken egg while using cation exchange chromatography in 1903, the Russian botanist Mikhail Tswelt, developed chromatography to separate a mixture of plant pigments using a column of calcium carbonate. He became the first scientist to recognise that chlorophyll was not a single chemical compound. Therefore, chromatography techniques that explored various physical and chemical properties of biomolecules have been developed for example, ion exchange chromatography, molecular sieve chromatography etc..

The way of distribution of compounds between the two immiscible phases is described by the partition or distribution coefficient k, this value different for different kind of biomolecules. These features are used for separation of biomolecules from each other. It should be noted that a stationary phase is present in all the chromatographic systems which maybe solid, liquid or a solid-liquid mixture which is immobilized. The mobile phase maybe liquid or gas phase.

On the basis of their net charge, ion exchange chromatography is used to separate charged molecules. Basically, there are two types of ion exchangers; cation exchanges (that exchange cation that is positively charged protein) and anion exchanger (that exchange anion on the negatively charged protein). If a protein has a net charge (positive) at pH 7.0, it will bind to a cation exchanger, but not be negatively charged protein. Similarly, if a protein has a net charge (negative) at pH 7.0, it will combine to an anion exchanger, where is positively charged protein will not bind to it. The bound protein can be elluted by increasing the salt concentration for changing the pH of the ellution buffer.

Principle:-

At pH 7.0 lysosome acts as cation that is positively charged ions. Hence, it binds to the cation exchanger that is carboxylic methyl cellulose (CMC). This helps to get separated from the other negatively charged proteins. You can ellute lysosome by replacing it with more strong cation that is (Na+ in NaCl).

Requirements:-

1. Carboxy-methyl cellulose (CMC)
2. Cation exchange column
3. 5 ml gel
4. Column stand, empty column
5. Bacterium, micrococcus
6. Standard of lysosome, chicken egg, spectrophotometer, centrifuge
7. 1 m NaCl
8. Neutralizing agent (0.1N HCL)
9. Prepare fresh buffers as below;
   Equilibrium buffer:- 0.067M phosphate buffer, pH 7.0
   Washing buffer:- 0.067M phosphate buffer pH 7.0
   Elution Buffer:- 0.067M phosphate buffer containing 0.5 m NaCl

Procedure:-

1. Procure stand, fix the column on it and pack with 2.5 ml of CMC.
2. Add 25 ml of equilibrium buffer to equilibrate the column.
3. Collect5 ml of egg white after breaking and egg. Add an equal volume of distilled water and mix thoroughly.
4. With the help of either a pH metre aur pH paper strip slowly at the neutralising solution (This result has slight turbidity of egg white).
5. Centrifuge the egg white at 7000 RPM for 10 minutes if possible at 4 degree Celsius and collect the supernatant.
6. Add the remaining supernatant to the equilibrated CMC column.
7. With intermittent mixing incubator for 30 minutes at room temperature.
8. after 30 minutes, allow the CMC to get settled. Without disturbing the gel slowly dick and the rest of the supernatant.
9. Using 30 ml of washing buffer, wash the column completely.
10. Using 7.5 ml of elution buffer elute the lysozyme of the column.
11. Before storing for next use wash the column with 10 ml of 1 m NaCl solution.
    (column may be reused 2-3 times).
12. Calculate protein concentration in load.
13. Elute it by using burette method of protein estimation. (see experiment number 10)
14. Measure lysozyme activity in load and elute it.

Principle of measuring lysozyme activity:-

Lysozyme lyses the bacterial cell wall and hydrolyses the peptidoglycan layer of the wall, when a suspension of m.leutius (a gram positive bacterium) is treated with lysozyme, the turbid suspension is clarified due to lysis of cell wall. The cell lysis can be measured by estimating the absorbance of suspension at 450nm before and after cell lysis. Initially the suspension of bacterial cells scatter more light, hence absorbed is higher.

The lysed cells scatter less light, hence absorbance get decreased. Lysozyme activity is compared with a standard solution of lysozyme which contains known amount of protein.

It is done as below:-

1. Prepare lysozyme standard solution by dissolving one milligram lysozyme in 3 ml of 0.07M phosphate buffer (solution consists of 0.3 milligram lysozyme per ml).
2. Dilute the suspension of m.luteus by adding phosphate buffer (pH 7.0; 0.07 M) so as to obtain A450 between (0.5 and 0.7).
3. Pour 3 ml of dilute m. Luteus suspension in a burette and measure OD at 450 minutes.
4. It gives a value of OD at 0 seconds.
5. To this Burette add 50 ml of standard lysozyme and record time.
6. Gently mix the contents of burette for 15 seconds.
7. Then keep the Burette in a spectrophotometer and measure OD at 450NM after 60 seconds of adding the lysozyme.
8. Repeat the same process for elute and load for 2 to 3 times.

Conclusion:-

The ion exchange chromatography technique has been carried out successfully.

ELECTROPHORESIS

Aim of the experiment:-

To separate given mixture of protein sample obtained from crude E.coli cell extract sodium dodecyl sulphate. Poly acrylamide gel electrophoresis (SDS- PAGE) and to visualise the protein band pattern by co-massive brilliant blue staining.

Principle:-

Electrophoresis is the migration of charged molecule in solution in response to electric field. Their rate of migration depends on the strength of the field, on the net charge, size and shape of the molecule and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool electrophoresis is simple, rapid and highly sensitive. It is used for analytical study of the properties of a single charged species and as a separation technique where protein are amphoteric compounds than net charge therefore is determined by the pH of the medium in which a pH above its isoelectric point, a protein has a net negative charge and migrates towards the anode in an electrical field.

Below its isoelectric point, the point is positively charged and migrates towards the cathode. The net charge carried by protein is in addition independent of its size that is the charge carried per unit mass of molecules different from protein to protein. At a given pH therefore and under non denaturing condition, the electrophoretic separation of proteins is determined by both size and charge of the molecules. Electrophoresis of proteins is generally carried out in jewels made up of cross linked polymers polyacrylamide. The polyacrylamide gel acts as a molecule size, slowing the migration of proteins approximately in proportion to their charge to mass ratio. In (SDS- PAGE) the an ionic detergent SDS is used.

Sodium dedyl-sulphate (SDS) binds to most amino acid in amount roughly proportional to the molecular weight of the protein that is bound. SDS contributes a large net negative charge rendering the intrinsic charge of the charge of protein in significant and conferring on each protein, a similar charge to mass ratio.

In SDS-PAGE the an ionic detergent SDS is used. Sodium didyl-sulphate (SDS) binds to most amino acid in Mount roughly proportional to the molecular weight of the protein. The bound SDS contributes a large net negative charge rendering the intrinsic change of the charge in proteins. This is insignificant and confurring on each proteins as a similar change aaj to mass ratio. It is also usually necessary to reduce the disulphate bridge in proteins before they adopt the random configuration necessary for separation by size. This is done with 2- MER up to

Ethanol or Dithiotheither. There are two types of buffer system in vertical gel electrophoresis that is continuous and non continuous. A continuous system is only a single separation gel and uses the same buffer that is in the tank and the gel.

In non continuous system on non restrictive large pore gel called stacking gel is layered on top of a separating gel called a resolving gel. SDS-PAGE is a non continuous buffer system.

The Stacking gel buffer has different PH (6.8), than buffer composition from the resolving gel in the stacking gel chloride ionized and run faster followed by the zwitter ionic glycinate in between CL and migrate in the same fashion.

SDS-PAGE is used to separate proteins based on molecular mass with smaller polypeptides migrating more rapidly. It is also used to estimate the molecular weight of a protein by plotting the log of the marker proteins versus relative migration of known samples during electrophoresis. A linear relationship exist between the logarithm of the molecular weight of an SDS denatured polypeptide or negative nuclic acid and its RF. The RF is calculated as the ratio of the distance migrated by the molecules, that to migrated by the marker dye front.

Reagents required:-

1. 30% acrylamide- Bisacrylamide solution, 30 grams of acrylamide and 0.8 grams of bisacrylamide is dissolved in hundred ml of distilled water.
2. 10% ammonium
3. TEMED
4. Stacking gel buffer : 0.5m tris HCL, PH 6.8
5. Resolving gel buffer: 1.5 tris HCL, pH 8.8
6. Resolving buffer: 3 grams of tris, 14.4 grams glycine and 1 gram SDS is dissolved in one litre of distilled water of PH 8.3
7. Staining solution 6.25% ko massive brilliant blue (CBB) in methanol, acetic acid and water weather ratio of 5 : 1 : 4.
8. De-staining solution : methanol : acetic acid : water (5 : 1 : 4)
9. Samples solubilizing buffer : Tris 362 mg, SDS- 600grams, Glycerol 2-5ml, BPA- 30mg, Beta mercupto ethanol 0.6ml.
10. E.coli cell extract.

Materials required:-

1. Vertical gel electrophoresis apparatus

2. Cooling centrifuge
3. Eppendorf tubes
4. Micropipettes
5. Other standard labwares

Procedure:-

1. 10% of resolving gel is prepared and processed in between the glass plates.
2. The gel is allowed to polymerize.
3. Polymerized resolving gel is over wide with 5% stacking gel.
4. The comb is inserted without any air bubbles in between the plates containing the stacking gel solution.
5. When the cone is polymerized, it is used for loading the samples.
6. 10ml of sample is mixed with 3 ml of the loading dye and loaded into the Wells.
7. Electrophoresis is carried out at hundred volts till the die forms front migrated out of the gel.
8. After completion of the electrophoretic run the gel is removed from the plates and placed in the staining solution for several hours.
9. The acceptance is removed buy washing in the destaining solution tell a clean background is obtained and the gel is documented.

Observation:-

The protein from the crude cell extract obtained from e coli cell is observed as different bands according to their molecular masses. The lower molecular weight proteins migrated faster than the higher molecular weight proteins.

Result:-

Protein present in the crude e.coli cell extract separated by SDS-PAGE and visualised by comassive brilliant blue colour.

CENTRIFUGATION

INTRODUCTION

The centrifuge is ubiquitous in biomedical laboratories and a basic knowledge of the theory of centrifugation is more than useful. Centrifuge performance can be classified as low-speed, high-speed and ultra-speed. Usual applications include the separation of serum or plasma from red blood cells, the separation of precipitated solids from the liquid phase

of a mixture, or the separation of liquids of varying density.

PRINCIPLES OF CENTRIFUGATION

Particles suspended in a fluid move, under the influence of gravity, towards the bottom of a vessel at a rate that depends, in general, on their size and density. Centrifugation is

a technique designed to utilise centrifugal forces, which are greater than the force of gravity, to speed up the sedimentation rate of particles. This is achieved by spinning the vessel containing the fluid and particles

about an axis of rotation so that the particles experience a force acting away from the axis. The force is measured in multiples of the

Table 1. Calculations used to convert rpm to RCF, and *vice versa*.

$RCF = 11.18 \times r \, (rpm/1000)^2$ $rpm = 299.07 \sqrt{RCF/r}$
r: radius (cm)

Earth's gravitational force and is known as the relative centrifugal field (RCF) or, more commonly, the '***g***' force.

INSTRUMENT COMPONENTS

Rotor

The design of most centrifuges allows the drive system to accept rotors of different sizes and capacities, although most instrument rotors are now capable of accepting a large range of tube sizes through the use of adaptors. Rotors have three basic designs: horizontal, in which the tubes are carried

in buckets that can swing outwards to a horizontal position and can operate at speeds to about 3000 rpm; fixed angle, in which the sample tubes are held at a fixed angle to the vertical position and can attain much higher speeds (approximately 7000 rpm) because

of the aerodynamic construction of the rotor; and vertical, in which the tubes are fixed

in the vertical position. In general, the horizontal rotor offers advantages to the clinical laboratory because sedimentation of large particles (eg red blood cells) is efficient at low force and because a flat sediment is produced.

The load on the rotor should always be balanced before operating the centrifuge, particularly when using high-speed instruments in which the buckets and caps are often numbered so that they can be matched on opposite sides of the rotor.

The load must be balanced both by equal mass and by centres of gravity across the centre of rotation. Thus, it is important not to run the centrifuge with buckets, carriers or shields missing from the unit, and not to exceed the maximum rated speed of the rotor in use. Most modern rotors have microprocessor-controlled automatic rotor identification so that it is impossible to set the speed beyond the safety limit for that rotor.

Motor

In general, centrifuge motors are high-torque, series-wound DC motors, the rotation of which increases as the voltage is increased. The rotor shaft is driven directly or through a gyro, although occasionally a pulley system is used. Electrical contact to the commutator is provided by graphite brushes, which gradually wear down as they press against the commutator turning

at high speed, and thus should be replaced at specified intervals. Modern centrifuges have induction drive motors that have no brushes to change. The shaft of the motor turns through sleeve bearings located at the top and bottom of the motor. Most instruments contain sealed bearings that are permanently lubricated, while others require periodic application of oil or grease.

The speed of the centrifuge is controlled by a potentiometer that raises and lowers the voltage supplied to the motor. The calibrations on the speed control are often only relative voltage increments and should never be taken as accurate indicators of speed. Therefore, periodic recalibration is required.

Imbalance detector

Some instruments have an internal imbalance detector that monitors the rotor during operation, causing automatic shutdown if rotor loads are severely out of balance.

Tachometer

A tachometer indicates the speed in rpm. Most modern centrifuges use electronic tachometers, in which a magnet rotates around a coil to produce a current that can be measured.

Safety lid

Modern centrifuges must have a door-locking mechanism to prevent the lid from being opened while the instrument is running. If there is a power failure or the safety latch fails for some reason it may be necessary to trip the door-locking mechanism manually to retrieve the samples. Manufacturers' instructions should be checked for the exact procedure required.

Refrigerator

A centrifuge generates heat as it rotates and if samples are

temperature labile then a refrigerated centrifuge should be used. Some centrifuges enable the rotor and chamber to be precooled before a run.

Braking system

Braking devices are incorporated to provide rapid rotor deceleration. Modern instruments have an electrical braking system that functions by reversing the polarity of the electrical current to the motor. Other machines may have a mechanical brake.

CENTRIFUGE TUBES

It is advisable to use a conical-bottomed tube in a swing-out bucket rotor for the sedimentation of cells. This tube type will

retain the pellet of cells more effectively as the supernatant is removed. All tubes for use with high-speed rotors are round-bottomed. Pyrex glass tubes can withstand forces of around 2000 x***g***, while Corex tubes can be used up to 12,000 x***g***. Polycarbonate or polyallomer are the most common plastic tubes in use but great care must be taken when using organic solvents. Manufacturers usually provide extensive information about solvent, salt

and pH resistance, as well as sterilisation procedures.

PREVENTIVE MAINTENANCE

If the bearings on the upper and lower ends of the motor shaft are not of the sealed type then they should be lubricated as per the manufacturer's instructions.

Brushes should be removed regularly and checked for wear; they should be replaced if they are worn to more than one-half of their original length. When reinserting used brushes, replace them in the same orientation. New brushes should be broken in by slowly accelerating the unloaded unit to mid-speed and then allowing it to run for a period of time.

The rotor, buckets and shields or carriers should be examined for signs of mechanical stress (eg cracks, corrosion).

Some manufacturers etch the expiry date on the rotor and this should be checked periodically.

Regularly lubricate the contact areas between the centrifuge buckets and the pins.

Regularly check the condition of the

O-ring on the tie-down nut on top of the rotor, and replace it if worn or damaged.