Semester -II

18BTP211 RECOMBINANT DNA, FERMENTATION AND BIOPROCESS TECHNOLOGY - PRACTICAL III **4H-2C**

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

Course Objectives:

To make the students to understand the various techniques involved in genetic engineering, characterization of the microorganism in fermentation and downstream processing.

Course Outcomes:

Students will able to perform the DNA and RNA isolation and manipulation techniques.
Students will able to perform the production of industrially important metabolites from microbial source using fermentation and bioprocess techniques.

Recombinant DNA Technology

1. Isolation and analysis of total DNA from Microbes (*E. coli*), plant
2. Isolation and analysis of plasmid DNA
3. Isolation and analysis of total RNA
4. Restriction digestion of DNA, Ligation of DNA
5. Transformation of plasmid DNA using calcium chloride
6. Amplification by PCR
7. Southern blotting (Demonstration)
8. Northern blotting (Demonstration)
9. Western blotting (Demonstration)

Fermentation Technology

1. Isolation and secondary screening of industrially important microorganisms
2. Production of amylase or protease, Enzyme immobilization
3. Wine Production an alcohol determination by chromic acid method
4. Down stream processing by Solvent extraction

Suggested Readings

Glover, D.M., & Hames, B.D. (2000). *DNA Cloning- a Practical Approach.* (2 nd ed.) Oxford: IRL Press.

James, J.G., & Rao, V.B. (2001). *Recombinant DNA Principles and Methodologies.* (2 nd ed.) New York: Marcel Dekker Publications.

Maliga, P. (2000). *Methods in Plant Molecular Biology. A Laboratory Course Manual.* (3 rd ed.) New York: Cold Spring Harbour Laboratory Press.

Brook, J.S., Fritsch, E.F., & Maniatis, T. (2000). *Molecular Cloning: A Laboratory Manual*. (2 nd ed.) New York: Cold Spring Harbor Laboratory Press.

Lab manual

Recombinant DNA Technology Practicals

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Recombinant DNA Technology

1. Isolation and analysis of total DNA from Microbes (*E. coli*), plant
2. Isolation and analysis of plasmid DNA
3. Isolation and analysis of total RNA
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5. Transformation of plasmid DNA using calcium chloride
6. Amplification by PCR
7. Southern blotting (Demonstration)
8. Northern blotting (Demonstration)
9. Western blotting (Demonstration)

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EX. NO :1 a. Isolation and analysis of total DNA from Microbes (*E. coli*)

DATE :

Aim: To isolate total genomic DNA from the bacterial cells and visualizing the same DNA by gel electrophoresis.

Principle:

Genomic DNA preparation differs from the plasmid DNA preparation. Genomic DNA is extracted from bacterial cells by immediate and complete lysis whereas plasmid DNA is isolated by slow-cell lysis to form a sphaeroplast.

The procedure of genomic DNA extraction can be divided into 4 stages:

1. A culture of bacterial cell is grown and harvested.
2. The cells are broken open to release their contents.
3. The cells extracted are treated to remove all components except the DNA.
4. The resulting DNA is then purified.

Materials required:

1. TE buffer (pH 8.0)
2. 10% (w/v) sodium dodecyl sulfate (SDS)
3. 20 mg/mL proteinase K
4. Phenol/chloroform (50:50)
5. Isopropanol
6. 70% ethanol
7. 3M sodium acetate pH 5.2

Procedure:

1) *E. coli* culture was grown overnight in Luria broth.
2) The overnight culture was transfered into 1.5 mL to a microcentrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was decanted and repeated with another 1.5 mL of cells.
3) The bacterial pellet was resuspended in 467 µL TE buffer by repeated pipetting. 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K was added, mixed, and incubated for 1 hr at 37°C.

4) Equal volume of buffer saturated phenol : chloroform : isoamyl alcohol (25:24:1) or chloroform:Isoamyl alcohol (24:1) was added and mixed well by inverting the tube until the phases are completely mixed. (Caution: Phenol causes severe burns. Wear gloves, goggles, and a lab coat, and keep tubes capped tightly)
5) Centrifuged at 12000 rpm for 5 min.
6) The upper aqueous layer phase was transferred to a new 1.5 ml eppendorf tube and equal volume of chloroform:Isoamyl alcohol (24:1) was added, Again mixed well by inverting and transfered to a new tube and centrifuged at 12000 rpm for 5 minutes.
7) The upper aqueous phase was again transferred to a new 1.5 ml eppendorf tube.
8) To the aqueous phase, 1/10 volume of 3M sodium acetate (pH 5.2) was added.
9) Then 0.6 volume of isopropanol was added and mixed gently until the DNA gets precipitates.
10) Centrifuged at 12000 rpm for 10 min and the supernatant was discarded.
11) The DNA pellet was washed by adding 200 µL of 70% ethanol (Centrifuge at 10000 rpm for 3 min)
12) Finally the DNA pellet was suspended in a 100–200 µL TE buffer. Complete resuspension may take several days.
13) After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.
14) 0.8 % agarose gel was prepared using 1X TAE buffer.
15) Isolated DNA samples were loaded into the wells, recording which samples are loaded into which wells as lane 1, 2, etc. The power supply was started after sample loading, with the voltage set to 50 V.
16) Ran the gel until the second dye from the well has reached 3/4 th of the gel.
17) After the run was completed, the gel was observed under UV transilluminater.
18) Then DNA was stored at 4°C short term, –20°C or –80°C long term

Result and Observations:

EX. NO :1b Isolation and analysis of total DNA from plant
DATE :

Aim: To isolate total genomic DNA from the given plant tissue sample

Principle:

High molecular weight DNA free from protein and RNA should be the basic technique for DNA isolation protocol. The isolated high molecular weight genomic DNA is essential for all molecular biology experiments. The cell wall of plant cell must be broken or digested to release cellular components, which generally performed by grinding with dry ice or liquid nitrogen. The plasma membrane of cell must be disrupted using a detergents (SDS or CTAB). The release DNA must be protected from endogenous nucleases using EDTA. The released cellular components generally emulsified with either chloroform or phenol to denature the proteins. The released DNA must be precipitated using isopropanol. The precipitated DNA then washed with alcohol to remove the salt present in the DNA.

Materials required:

1. 2X CTAB Lysis Buffer - containing
   - 0.1 M Tris HCl (pH 8.0)
   - 20 mM Na EDTA (pH 8.0)
   - 1.4 M NaCl
   - 2% CTAB (W/V)
   - 2% PVP (W/V)
   - 0.3% β-mercaptoethanol or $Na_2S_2O_5$ (add when used).
2. 1X CTAB lysis buffer – dilute 2X CTAB lysis buffer using distilled water (1:1 ratio)
3. Chloroform:isoamyl alcohol (24:1)
4. Ice cold isopropanol
5. 3M sodium acetate (pH 5.2)
6. TE buffer (pH 8.0)

Procedure:

1. 0.1 g of plant tissue was ground using liquid nitrogen to get fine powder
2. The ground fine powder was the transferred into 2 ml eppendorf tube. (Do not allow the sample to thaw)

3. Immediately, 0.9 ml of 2X CTAB lysis buffer was added and vortexed vigorously.

4. Then incubated at 65ºC for 30 min. The contents were mixed frequently during the incubation.

5. Then centrifuged at 12000 rpm for 5 min, the supernatant was transferred into new 2 ml eppendorf tube.

6. To the pellet, 0.3 ml of 1X CTAB lysis buffer was added, mixed well by vortexing and again incubated at 65ºC for 10 min.

7. The contents were centrifuged at 12000 rpm for 10 min and the supernatant was transferred into the 1st supernatant sample.

8. To the supernatant, equal volume of chloroform: isoamyl alcohol (24:1) was added and invert mixed for 15 min, centrifuged at 10000 rpm for 10 min at room temperature.

10. The aqueous layer was transferred into a new 1.5 ml eppendorf tube.

11. 0.7 volume of ice cold isopropanol and 10 µL of 3M sodium acetate (pH 5.2) was added to the aqueous layer, the contents were mixed by invert mixing.

13. Then centrifuged at 10000 rpm for 10 min at room temperature.

14. The supernatant was discarded, and 70 % ethanol was added to the DNA pellet.

15. Again centrifuged at 10000 rpm for 10 min.

16. Then the ethanol supernatant was discarded and the pellet was subjected to air drying.

17. After air drying, the pellet was resuspended in 50 µL TE buffer (pH 8.0).

18. After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.

19. 0.8 % agarose gel was prepared using 1X TAE buffer.

20. Isolated DNA samples were loaded into the wells, recording which samples are loaded into which wells as lane 1, 2, etc. The power supply was started after sample loading, with the voltage set to 50 V.

21. Ran the gel until the second dye from the well has reached 3/4 th of the gel.

22. After the run was completed, the gel was observed under UV transilluminater.

24. Then DNA was stored at 4°C short term, –20°C or –80°C long term.

Results and Observations:

EX. NO : 2 Isolation and analysis of plasmid DNA

DATE :

Aim: To isolate plasmid DNA from the bacterial cells by alkaline lysis miniprep method and visualizing the same DNA by gel electrophoresis.

Principle:

Plasmids are extra-chromosomal, double-stranded, closed-circular DNA present in many microorganisms. Plasmid DNA needs to be extracted (from bacterial hosts, mostly *E.coli*) almost routinely in cloning experiments.

Many methods have been described for successful extraction of plasmid DNA; however, the alkaline miniprep method is most useful for quick extraction of plasmids, mostly for analytical use.

In alkaline lysis miniprep method, the bacterial cells are lysed followed by SDS, NaOH treatment. The high pH of NaOH denatures the bacterial DNA but not the covalently closed-circular plasmid DNA. Neutralization of the high pH by sodium or potassium acetate makes the bacterial DNA to precipitate. The plasmid DNA is then purified by organic solvent.

Materials required:

1. Luria Broth - containing Trypton, 1%; Yeast extract, 0.5% NaCl, 1%
2. Antibiotics - Ampicillin, 50 mg/mL
3. Solution I - containing

    50 mM glucose

    25 mM Tris-CI (pH 8.0)

    10 mM EDT A (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, (autoclaved for 15 minutes at 10 lb/sq and stored at 4°C). add RNase A (100mg/ml) to get 100 µg/ml of solution I.

4. Solution II -containing

    0.2 N NaOH (freshly diluted from 10 N stock); 1% SDS

5. Solution III - containing

5 M potassium acetate 60 mL

Glacial acetic acid 11.5 mL

Distilled Water 28.5 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

6. 50 % PEG 6000 solution

7. 5M NaCl

8. TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0

9. 70% and 100% ethanol

Procedure:

Harvesting and Lysis of Bacteria

Harvesting - A single bacterial colony was inoculated into 2 mL of LB medium containing the appropriate antibiotic in a loosely capped 15-mL tube.

1) the culture was incubated for overnight at 37°C with vigorous shaking.
2) 1.5 mL of the culture was transferred into a centrifuge tube.
3) Centrifuged at 12000 g of 5 min using microcentrifuge.
4) the medium was removed by decantation, leaving the bacterial pellet as dry as possible.

Lysis by Alkali

1) The bacterial pellet was resuspended in 300 µL of ice-cold Solution I by vigorous vortexing.
2) 300 µL of freshly prepared Solution II was added.
3) The tube was closed tightly and the contents were mixed by inverting the tube rapidly for 5 minutes. (Make sure that the entire surface of the tube comes in contact with Solution II. Do not vortex). Then 300 µL of ice-cold Solution III was added.
4) the content was invert mixed and incubated on ice for 3–5 minutes.
5) After incubation, centrifuged at 14000 rpm for 10 minutes in a microcentrifuge. Then the supernatant was transferred to a fresh tube.

6) To the supernatnant, 166 µl of 50 % PEG6000 and 118 µl of 5M NaCl were added and mixed thoroughly by vortexing. Then centrifuged at 14000 rpm for 10 minutes in a microcentrifuge,
7) The supernatant was removed by gentle aspiration. The tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. Any drops of fluid adhering to the walls of the tube was also removed.
8) The pellet of DNA was rinsed with 200 µL of 70% ethanol.
9) Centrifuged at 12000 rpm for 10 minutes in a microcentrifuge.
10) The supernatant was removed as described in previous step, and the pellet of DNA was allowed to dry in the air for 1 minute.
11) The DNA pellet was resuspended in 50 µL of TE (pH 8.0). Vortexed briefly.
12) The isolated plasmid DNA was analyzed in 1.2 % agarose gel electrophoresis.

Results and Observations:

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EX. NO.3: Isolation and analysis of total RNA

DATE :

Aim: To isolate the RNA molecules from yeast cells

Principle:

Effective cell lysis is a required first step for extraction of good yields of high quality RNA. Yeast and many bacterial strains can be difficult to disrupt because, unlike eukaryotic cells, they are protected by cell walls. Mechanical disruption or enzymatic pretreatment are typically used to thoroughly disrupt bacterial and yeast cells for RNA isolation.

Materials required:

• Selective medium or YEPD
• Sterile dH_2O
• Buffer A

for 1 L

50 mM Sodium acetate (16.7 ml 3M Sodium acetate)
10 mM EDTA (20 ml 0.5 M EDTA)
Make upto to 1 lit using dH_2O (963.3 ml dH_2O)

• Complete Buffer A (0.5 ml per sample)

for 1 L
900 ml Buffer A
100 ml 10 % SDS
Add 1 % DEPC just before use

• Buffer A saturated phenol (1.2 ml per sample)
• TE saturated phenol: Chloroform (0.6 ml per sample)
• 3 M NaOAc (pH 5.2) (90 µl per sample)
• DEPC-treated dH_2O (1.5 ml per sample)
• Absolute ethanol (2 ml per sample)
• 70 % ethanol (1 ml per sample)

Procedure:

A. Preparation of required materials

1. RNase free tips, microcentrifuge tubes, pipettes.
2. Label the microcentrifuge tubes.
3. Dry ice/ethanol bath.
4. 65°C water bath.
5. Aliquot the solutions required.
6. Add 1% DEPC to Complete Buffer A.
7. Equilibrate the Buffer A saturated phenol to 65°C.
8. Ice.

B. Harvest cells:

1. Grow cells to an OD_{600} of 0.4 - 0.6 in 10 ml of selective medium or 5 ml of rich medium. This can be done in two steps. First, grow an overnight culture to saturation in 3 ml of medium. Second, inoculate 10 ml of selective with 5 µl, 10 µl, 50 µl and 100 µl of the saturated overnight culture the afternoon of the second day. The YEPD can be inoculated with smaller amount of overnight culture. This procedure will generally ensure that at least one of the cultures will be at the correct OD_{600} the following day.

2. Transfer the cultures to sterile 45 ml centrifuge tubes. Pellet the cells by centrifugation at 4,000 g for 5 minutes.

3. Resuspend the pelleted cells in 1.0 mls of sterile dH_2O.

4. Pour the cell slurry into an RNase free 1.5 ml microcentrifuge tube.

NOTE: all subsequent steps should be using RNase free labware and solutions. Follow the general guidelines in Sambrook et al., (1989) for the preparation of RNase free solutions and labware.

5. Pellet the cells in a microcentrifuge at full speed for 20 seconds.

6. Remove the liquid and freeze in a dry ice/ethanol bath. Store the frozen pellets at -70oC.

C. RNA extractions:

1. Remove the tubes from the -70°C and immediately add 500 µl of **Complete** Buffer A (remember to add 1% DEPC before use). Vortex to resuspend the cells.

2. Add 600 µl of Buffer A saturated phenol equilibrated to 65°C. Mix each tube for ~ 10 seconds. Place tubes in a 65°C water bath. Repeat mixing of each tube every 30 seconds for a total of 5-6 minutes.

3. Centrifuge the tubes in a microcentrifuge for 30 sec at full speed.

4. Remove the phenol layer (yellow, bottom layer) using an RNase free blue tip.

NOTE: Leave the interface, and the pellet of unbroken cells and cell debris in the tube or you will loose some of the RNA.

5. Add 600 µl of Buffer A saturated phenol equilibrated to 65°C. Repeat mixing and incubation at 65°C for 5-6 min.

6. Centrifuge the tubes in a microcentrifuge for 2-3 minutes at full speed.

7. Remove aqueous layer (top layer) to a new tube.

8. Add 600 µl of 1:1 phenol buffered with TE:chloroform at room temperature. Mix the samples by vortexing for 20 seconds. Separate the layers by centrifuging the tubes in a microcentrifuge for 2-3 minutes at full speed.

9. Remove the aqueous layer (top layer) to a new tube. Add 50 µl of 3 M NaOAc (pH 5.2) and 1 ml of absolute ethanol. Mix. Incubate the microcentrifuge tube on ice for 15 minutes to precipitate the RNA. Centrifuge at full speed in a microcentrifuge for 10 minutes to pellet the RNA. Aspirate. Remove as much of the supernatant as possible. Incubate the open tube at

37°C for 5 minutes to dry the pellet. Be careful not to over dry the pellet. Dehydrated RNA is very difficult to resuspend.

10. Resuspend the pellets in 400 µl of dH2O. Add 40 µl of 3 M NaOAc and 1 ml of ethanol. Mix and precipitate again as described in Step 9.

11. Wash the pellets by adding 1 ml of 70% ethanol and vortexing for 20 seconds. Centrifuge the microcentrifuge tubes at full speed for 5 minutes.

12. Remove the supernatant by aspiration. Incubate the open tube at 37°C for 5 minutes to dry the pellet.

13. Dissolve the RNA in 50 µl dH_2O. Heat to 65°C for 10 minutes to assist resuspension. Vortex. Centrifuge briefly.

14. Dilute 5 µl RNA into 495 µl of dH_2O. Determine the absorbence at A_{260} and A_{280}.

15. Dilute the RNA to 1 µg/µl.

Results and Observation:

EX. NO : 4 Restriction digestion of DNA, Ligation of DNA

DATE :

Exp. No.4.a: Restriction digestion of DNA

Aim: To perform restriction digestion of isolated plant genomic DNA with *EcoR* I and *Hind* III enzymes and to analyze the restriction pattern by agarose gel electrophoresis.

Principle:

- Type II restriction enzymes hydrolyse the backbone of DNA between deoxy ribose and phosphate groups within the recognition sites and generate two different types of ends.
- This leaves a phosphate group on the 5' ends and a hydroxyl group on the 3' ends of both strands.
- The 5' or 3' overhangs generated by enzymes that cut asymmetrically are called sticky or cohesive ends, because they will readily stick or anneal with their complementary sequences by base pairing (Example: *EcoR* I).
- Some enzymes cut at precisely opposite sites in two strands of DNA and generate blunt ends without overhangs called blunt ends (Example – *Hae* III).

Materials required:

1. 10X restriction enzyme buffer (see manufacturer's recommendation)
2. DNA
3. restriction enzymes – *EcoR* I and *Hind* III
4. sterile water
5. phenol:chloroform (1:1)

Procedure

1. The following contents were added to a microfuge tube:

2 µL of appropriate 10X restriction enzyme buffer, 0.1 to 5 mg DNA, and sterile water to a final volume of 19 µL (Note: These volumes are for analytical digests only. Larger volumes may be necessary for preparative digests or for chromosomal DNA digests.)

2. 1 to 2 µL (3 to 20 units) enzyme was added, mixed gently and centrifuged for a few seconds in the microfuge.

3. Incubated at the appropriate temperature (usually 37°C) for 1 to 2 hours.

4. Meanwhile, 1 % agarose gel was prepared for electrophoresis.

5. After an restrict digestion incubation, 3 µL of 6x gel loading buffer was added to the samples.

6. The digested samples, 10 µL of control DNA, 10 µL of DNA size marker were loaded, (note down the order of loading).

7. Electophoresis was performed at 50 – 100 V for 1-2 hours.

8. After electrophoresis, the gel was observed under UV transilluminator.

(Note) If the DNA is to be used for another manipulation, heat-inactivate the enzyme (if it is heat-labile) at 70°C for 15 min, phenol/chloroform extract, and ethanol precipitate, or purify on DNA purification column.

EcoR I restriction site:

Hind III restriction site

Results and Observations:

Exp. No.4.b. Ligation of restriction digested DNA

Aim: To perform ligation of λ/*EcoR* I digest using T4 DNA ligase and to anlayze the ligated sample by agarose gel electrophoresis.

Principle:

Construction of recombinant DNA molecule is dependent on the ability to covalently seal single stranded nicks in DNA. This process is performed both invitro and invivo by the enzyme called DNA ligase. It catalyses the formation of phosphodiester bond between 5' phosphate and 3' hydroxyl terminals of double stranded DNA. Thereby joins the double stranded DNA restriction fragments having either blunt end or homologus cohesive ends.

- *E.coli* ligase and T4 DNA ligase are the two DNA ligases used in recombinant DNA technology.
- T4 DNA ligase has the unique ability to join cohesive and blunt ended fragments.
- T4 DNA ligase produced from bacteriophage T4 and requires ATP as energy source. Cohesive end ligation is carried out at 12 to 16º C to maintain a good balance between annealing of ends and enzyme activity.
- If reaction is set at higher temperatures, annealing of the ends become difficult, while lower temperatures reduces the ligase activity.

Materials required:

1. λ/*EcoR* I digest
2. 2X ligase assay buffer
3. T4 DNA ligase
4. Sterile distilled water
5. Agarose
6. 50X TAE buffer
7. 6X gel loading buffer

Protocol:

1. Ligase assay buffer and λ/*EcoR* I digest were thawed.

2. T4 DNA ligase vial was placed on ice 1

3. To the tube, labeled as ligated sample, 10 µL of λ/*EcoR* I digest was added to it.

4. Then 10 µL of 2X ligase assay buffer and 1 µL of T4 DNA ligase were also added and mixed by tapping.

5. It was then incubated at 16ºC for 2 hrs in waterbath.

6. Meanwhile, 1% agarose gel was prepered for electrophoretic analysis.

7. After incubation, 2 µL of 6X gel loading buffer was added to the ligated sample and the control sample containing the 10 µL of λ/*EcoR* I digest alone.

8. Both the samples were loaded onto the wells

9. Electrophoresis was ran at 50 – 100V for 1-2 h.

10. After electrophoresis, the gel was viewed under UV-transilluminator.

11. The bands of ligated λ DNA was compared with the λ/*EcoR* I digest.

Results and Observations:

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EX. NO :5 Transformation of plasmid DNA using calcium chloride

DATE :

Aim: To prepare chemically competent *E.coli* cells and to perform transformation of plasmid DNA into the competent *E.coli* cells.

Principle:

Bacteiral transformation is a process in which the bacterial cells managed to uptake free or external DNA from the environment or surrounding medium. The transformation ability by most bacteria is limited in nature. However, bacterial cells can be artificially induced to take up DNA by treating them with calcium chloride.

Culture of bacterial cells that are capable of taking up the DNA is said to be competent. During the process of competence, the bacterial cell wall changes and receptors of some kind are either formed or activated on the cell wall which are responsible for initial binding of the DNA. Then the competent cells are briefly exposed to a temperature of 42ºC (Heat shock), wherein pores are created and DNA is taken up. Further the immediate chilling on ice ensures closure of pores. These are then said to be transformed and are then screnned for transformants or recombinants.

Materials required:

1. **E.coli** starin
2. Luria broth
3. Ampicillin – 100 mg/ml stock solution
4. 0.1 M $CaCl_2$
5. Plasmid DNA
6. X – Gal
7. IPTG
8. Luria agra plate

Protocol:

Day 1: Revival of bacterial strain

1. The lyophilized vial was opened, to that 0.1 ml of LB medium was added.
2. A loopful of suspension was streaked onto LB plates and incubated at 37ºC for overnight.

Day 2:

A single colony was inoculated into 5 ml of LB medium and incubated at 37ºC for overnight.

Preparation of competent cells:

1. One ml of overnight grown culture was inoculated to 100 ml of LB medium and incubated at 37 ºC for 2-3 hrs, until OD A_{600} reaches to 0.23 - 0.26.
2. Then the culture flask was chilled on ice for 20 mins (To arrest the bacterial growth).
3. The culture was aseptically transferred into sterile centrifuge tubes and centrifuged at 6000 rpm for 5 min at 4 ºC.
4. The supernatant was discarded and 15 ml of cold 0.1 M $CaCl_2$ solution was added to the cell pellet aseptically. The cell pellet was gently suspended in the solution using a pre-chilled pipette (Care should be taken not to remove the tubes from ice during resuspension).
5. The tube was placed on ice for 30 min. Then centrifuged at 6000 rpm for 5 min at 4 ºC.
6. The supernatant was discarded and resuspended gently in 0.6 ml cold 0.1M $CaCl_2$ solution.
7. 100 µl aliquots of competent cells were aseptically transferred to 6 pre-chilled 1.5 ml sterile microfuge tubes and placed on ice.

Transformation procedure:

8. 5 µl (10 ng) of plasmid DNA was added to 5 aliquots of 100 µl of competent cells. Gently tapped and incubated on ice for 20 mins. One aliquot of competent cell was used without transformation as control.
9. After incubation, the cells were subjected to 42 ºC for 2 min in water bath and then the vials were kept in ice for 5 mins.
10. Then 1 ml of LB medium was added to the tubes aseptically and incubated at 37 ºC for an hour.

11. Meanwhile, LB agar plates with ampicillin (100 µg/ml of medium) and X- Gal and IPTG were prepared.

12. After 1 h growth of transformed cells, 100 µl were spreaded onto the plates using a spreader.

13. The control plate (LB agar alone) was prepared by plating 100 µl of competent cell alone that has not been transformed (to check the cell contamination).

14. All plates were incubated for overnight at 37 ºC.

Transformation efficiency is expressed as Number of transformants/ µg of plasmid DNA

Results and Observations:

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EX. NO :6 DNA AMPLIFICATION BY THE POLYMERASE CHAIN REACTION (PCR) METHOD

DATE :

Aim: To amplify the given sample of DNA using PCR.

Principle:

- Polymerase chain reaction (PCR) is a very simple method for in vitro DNA amplification *Taq* DNA polymerase.
- A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the 4 nucleotides, large quantities of the primer sequence, and DNA polymerase. The polymerase is the *Taq* DNA polymerase, isolated from *Thermus aquaticus*.
- The three steps of the polymerase chain reaction are carried out in the same vial, but at different temperatures.

Thus, this technique involves DNA synthesis in 3 simple steps.

Step 1. Denaturation of the template into single strands.

Step 2. Annealing of primers to the template.

Step 3. Extension of new DNA.

- The three steps in the polymerase chain reaction—the separation, of the strands, annealing the primer to the template, and the synthesis of new strands—take less than two minutes. Each is carried out in the same vial.

- The first step of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90°C–95°C for 30 seconds.
- But the primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 55°C. At this temperature, the primers bind or "anneal" to the ends of the DNA strands. This takes about 20 seconds.

- The final step of the reaction is to make a complete copy of the templates. Since the *Taq* DNA polymerase works best at around 75°C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised.
- The *Taq* DNA polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on, to the end of the DNA strand. This completes 1 PCR cycle.
- At the end of a cycle, each piece of DNA in the vial has been duplicated. But the cycle can be repeated 30 or more times.
- Each newly synthesized DNA piece can act as a new template, so after 30 cycles, 1 million copies of a single piece of DNA can be produced.
- Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be ready in about 3 hours.

Materials Required

1. *DNA template:* Between 1 and 5 ng of cloned DNA or between 40 and 100 ng of genomic DNA should be used per reaction. It is convenient to dilute template stocks to an appropriate concentration, e.g., 5 ng/mL in dH_2O for cloned DNA.

2. *Primers:* Primers should be resuspended in dH_2O at 100 ng/mL. Each primer should be used at ~100 ng per reaction.

3. *Buffer:* Buffer should be prepared as a 10X stock.

10X PCR buffer: 100 mM Tris. HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$.This buffer can be prepared containing 0.1% gelatin.

4. *Taq DNA polymerase:* Taq should be used at 2.5 U per reaction.

5. *Magnesium:* Extra magnesium can be added to the PCR reaction.

- If using the buffer above, a final Mg^{2+} concentration of 1.5 mM will be obtained. If necessary, magnesium can be titrated to obtain an optimal concentration.
- Suggested concentrations for this would be 1.5, 3.0, 4.5, 6.0 and 10 mM.
- Magnesium can be prepared as $MgCl_2$ at 25 mM and autoclaved.

- Increasing the magnesium concentration has the same effect as lowering the annealing temperature.

6. *Nucleotides:* dNTPs should be prepared from 100 mM commercial stocks as a 10X stock at 2 mM of each dNTP. This is most easily done by adding 2 µL of each dNTP to 92 µL dH_2O in an eppendorf tube.

7. *Water:* Water should be autoclaved and used solely for PCR.

Milli-Q water is fine for PCR or "water for injection" if the distilled water is in doubt. It can be aliquotted into 1-mL volumes and kept separate from DNA and other sources of contamination. Each aliquot should be discarded following a single use.

8. *Paraffin oil:* In some instruments, paraffin oil must be added to prevent evaporation of the sample.

9. PCR machine.

Procedure

1. Add 38 µL of sterile milliQ water (or autoclaved double distilled water) to a sterile microfuge.
2. Add 5 µL of 10 X Taq polymerase assay buffer with $MgCl_2$ to the microfuge.
3. Add 3 µL of 2.5 mm dNTP mixed solution to the microfuge.
4. Add 1 µL of control template DNA.
5. Add 1 mol each of forward and reverse primers.
6. Add 1–2 units (0.5–0.7 µL) of Taq DNA polymerase.
7. Gently mix.
8. Layer the reaction mixture with 50 µL of mineral oil to avoid evaporation (if required).
9. Carry out the amplification using the following reaction conditions:
10. Initial denaturation at 94°C for 1 min.
11. Denaturation at 94°C for 30 sec.
12. Annealing at 48°C for 30 sec.
13. Extension at 72°C for 1 min.
14. Final extension at 72°C for 2 min.
15. Meanwhile, prepare a 1 % agarose gel for electrophoresis.

After PCR, add 3 µL of 6x gel loading dye to the PCR products.

6. Load the PCR products along with DNA size marker, note down the order of loading.

7. Electrophorese the samples at 50 – 100 V for 1-2 hours.

8. After electrophoresis, observe the gel under UV transilluminator.

Result and observation:

The specific segment of DNA was amplified by PCR and analyzed using agarose gel electrophoresis.

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BLOTTING TECHNIQUES—SOUTHERN, NORTHERN, WESTERN BLOTTING

These are techniques for analyzing cellular macromolecules: DNA, RNA, and protein.

Theory: Complementarity and Hybridization

- Molecular searches use one of several forms of complementarity to identify the macromolecules of interest among a large number of other molecules.
- Complementarity is the sequence-specific or shape-specific molecular recognition that occurs when 2 molecules bind together.

For example: the 2 strands of a DNA double-helix bind because they have complementary sequences; also, an antibody binds to a region of a protein molecule because they have complementary shapes.

- Complementarity between a probe molecule and a target molecule can result in the formation of a probe-target complex. This complex can then be located if the probe molecules are tagged with radioactivity or an enzyme. The location of this complex can then be used to get information about the target molecule.

In solution, hybrid molecular complexes hybrids of the following types can exist:

1. DNA-DNA. A single-stranded DNA (ssDNA) probe molecule can form a double-stranded, base-paired hybrid with a ssDNA target if the probe sequence is the reverse complement of the target sequence.

2. DNA-RNA. A single-stranded DNA (ssDNA) probe molecule can form a double-stranded, base-paired hybrid with an RNA (RNA is usually a single-strand) target if the probe sequence is the reverse complement of the target sequence.

3. Protein-Protein. An antibody probe molecule (antibodies are proteins) can form a complex with a target protein molecule if the antibody's antigen binding site can bind to an epitope (small antigenic region) on the target protein. In this case, the hybrid is called an "antigen-antibody complex" or "complex" for short.

There are 2 important features of hybridization:

1. *Hybridization reactions are specific;* the probes will only bind to targets with complementary sequences.

2. Hybridization reactions will occur in the presence of large quantities of molecules similar but not identical to the target. That is, a probe can find 1 molecule of target in a mixture of many of the related but noncomplementary molecules.

- These properties allow us to use hybridization to perform a molecular search for 1 DNA molecule, or 1 RNA molecule, or 1 protein molecule in a complex mixture containing many similar molecules.
- These techniques are necessary because a cell contains tens of thousands of genes, thousands of different mRNA species, and thousands of different proteins.
- When the cell is broken open to extract DNA, RNA, or protein, the result is a complex mixture of all the cell's DNA, RNA, or protein.
- It is impossible to study a specific gene, RNA, or protein in such a mixture with techniques that cannot discriminate on the basis of sequence or shape.

Hybridization techniques allow us to pick out the molecule of interest from the complex mixture of cellular components and study it on its own.

Basic Definitions

Blots are named for the target molecule.

Southern blot - DNA cut with restriction enzymes-probed with radioactive DNA.

Northern blot - RNA-probed with radioactive DNA or RNA.

Western blot - Protein-probed with radioactive or enzymatically tagged antibodies.

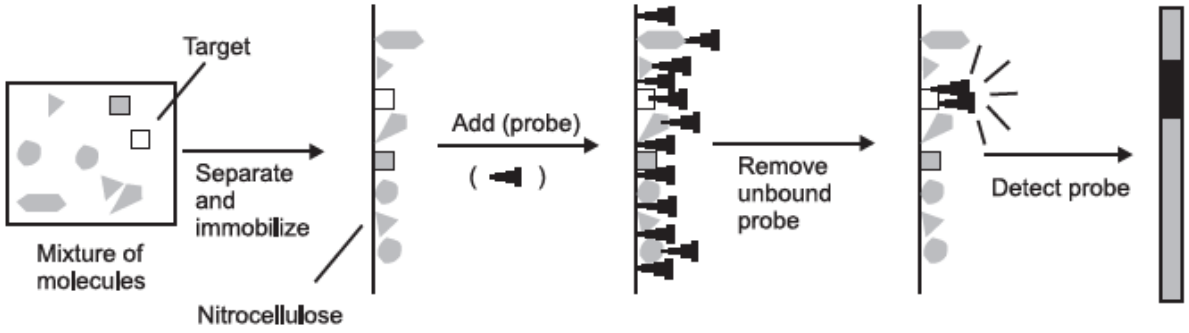
Overview

The formation of hybrids in solution is of little experimental value—if you mix a solution of DNA with a solution of radioactive probe, you end up with just a radioactive solution. You cannot tell the hybrids from the nonhybridized molecules.

For this reason, you must first physically separate the mixture of molecules to be probed on the basis of some convenient parameter.

These molecules must then be immobilized on a solid support, so that they will remain in position during probing and washing. The probe is then added, the nonspecifically bound probe is removed, and the probe is detected. The place where the probe is detected corresponds to the location of the immobilized target molecule.

This process is diagrammed below:



In the case of Southern, Northern, and Western blots,

the initial separation of molecules is done on the basis of molecular weight

In general, the process has the following steps:

- Gel electrophoresis
- Transfer to solid support
- Blocking
- Preparing the probe
- Hybridization
- Washing
- Detection of probe-target hybrids

The procedure for these 3 blots is summarized below:

Southern Blot	*Northern Blot*	*Western Blot*
1. Extract DNA from cells	1. Extract RNA from cells	1. Extract protein from cells
2. Cut with restriction enzyme	2. Denature with formaldehyde	2. Denature with SDS
3. Run on gel (usually agarose) Denature DNA with alkali	3. Run on gel (usually agarose)	3. Run on gel (usually polyacrylamide—called "SDS-PAGE")
4. Transfer to nitrocellulose (usually by capillary action)	4. Transfer to nitrocellulose (usually by capillary action)	4. Transfer to nitrocellulose (usually by electrophoresis)
5. Block with excess DNA	5. Block with excess RNA	5. Block with excess protein
6. Hybridize with labeled DNA probe	6. Hybridize with labeled DNA probe	6. Hybridize with labeled antibody probe
7. Wash off unbound probe (use of controlled stringency)	7. Wash off unbound probe (use of controlled stringency)	7. Wash off unbound probe
8. Autoradiograph	8. Autoradiograph	8. Autoradiograph or develop with chromogenic substrate

The important properties of the 3 blots are shown below:

	Southern	*Northern*	*Western*
What is separated by molecular weight (target)?	DNA cut with restriction enzymes	RNA denatured with formaldehyde	Protein denatured with SDS
Probe	Radioactive gene X DNA	Radioactive gene X DNA	Antibody against protein X, labeled with radioactivity or enzyme
What do you learn from it?	Restriction map of gene X in chromosome	How much gene X mRNA is present? How long is gene X mRNA?	How much protein X is present? How big is protein X?

EX. NO. :7 Southern blotting (Demonstration)

DATE :

Introduction

This method to know the presence or absence of a particular fragment in genomic DNA was first developed by E. D. Southern in 1975. The advent of Southern blotting technique was a turning point in the field of molecular biology.

It involves the capillary transfer of DNA fragments from an agarose gel to various types of membranes. Restriction Fragment Length Polymorphisms can be analyzed using the technique, wherein DNA fragments are separated on agarose gels denatured in situ and transferred onto membranes for analysis.

Materials required:

- Denaturation solution: NaCl, 1.5 M and NaOH, 0.5 M
- Neutralization solution: NaCl, 1.5 M; Tris-Cl (pH 7.5), 0.5 M and EDTA (pH 8.0), 1 mM
- 20X SSC: NaCl, 1.5 M and trisodium citrate, 0.1 M
- Depurinization solution: 0.25 N HCl
- Nylon or nitrocellulose membrane

Procedure

1. After agarose gel electrophoresis, photograph the gel and soak it in 0.25 N HCl for 15 minutes at room temperature, with gentle shaking.
2. Decant the acid solution and denature the DNA by soaking the gel in several volumes of denaturation solution for 30 minutes at room temperature, with constant shaking.
3. Neutralize the gel by shaking in several volumes of neutralization solution for 30 minutes at room temperature, with shaking.
4. Wrap a piece of Whatman 3-mm paper around a glass plate. Place the wrapped support on a large plastic tray with the ends of the 3-mm paper dipping into the 20X SSC solution in tray.

5. Invert the gel and place it on a damp 3-mm paper on the support. Make sure that there are no air bubbles between the 3-mm paper and the gel.
6. Cut a piece of nylon membrane slightly bigger than the gel. Use gloves and forceps to handle the membrane.
7. Float the membrane on 20X SSC until it wets completely.
8. Place the wet nylon membrane on top of the gel. Remove all the air bubbles that are trapped between the gel and tile membrane.
9. Wet 2 pieces of Whatman 3-mm paper, cut to exactly the same size as the gel in 10X SSC, and place them on top of the membrane. Again remove the air bubbles.
10. Cut a stack of coarse filter paper just smaller than the gel size. Keep on top of the Whatman filter papers.
11. Put a glass plate on the top and place (about 1 kg) on it to exert pressure.
12. Allow the transfer of DNA to proceed for about 12–24 hours.
13. Remove the stack of coarse filter papers and the 3-mm paper above the gel.
14. Turn over the dehydrated gel and membrane and lay them gel side up on a dry sheet of 3-mm paper. Mark the position of the wells on the membrane with a soft pencil.
15. Peel off the gel. The transfer can be checked by restaining the gel. If the transfer is complete, no DNA should be retained on the gel.
16. Soak the membrane in 6X SSC at room temperature for a few minutes.
17. Allow excess fluid to drain off from the membrane and set it to dry at room temperature on a sheet of 3-mm paper.
18. Place the dried filter between 2 sheets of 3-mm paper.
19. Fix the DNA on the membrane by baking for 2 hours at 80°C under a vacuum or cross linking on a UV transilluminator for a few minutes.
20. Wrap the membrane with saran wrap or keep it in an envelope made up of Whatman No. 1 filter paper and store.

Notes

- Nylon membranes are preferable over nitrocellulose for transfer.

- In case nitrocellulose is used, the DNA has to be fixed by baking at 80°C for 2 hours under a vacuum. Nitrocellulose, being combustible, will become brittle if baked in the presence of oxygen.
- DNA can be fixed on nylon membrane by UV-cross linking using longwave UV rays or by baking at 120°C for 2 hours.
- Care has to be taken so that the buffer passes to the filter paper through the gel only. A layer of parafilm may be put on the glass plate around the gel to avoid the filter papers touching the buffer directly.
- While photographing the gel, keep a fluorescent ruler alongside the gel for proper orientation later on.
- Never touch the membrane with bare hands. Any grease or powder on the membrane will prevent transfer of DNA.

Observations

- Restain the gel in ethidium bromide (5 mg/mL) for 45 minutes and view on a UV transilluminator after proper washings. There should not be any DNA on the gel, as the entire DNA should have been transferred to the membrane.
- There will be only one band in the lane. In the case of genomic DNA, a continuous smear should be visible, as digestion will result in many pieces of varying sizes.

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EX. NO : 8 Northern blotting (Demonstration)

DATE :

Aim: To learn the technique of Northern Blotting for the detection of a specific RNA fragment in a sample.

Introduction: Northern blotting or Northern hybridization is a widely used technique in molecular biology to determine the molecular weight of mRNA and to measure relative amounts of mRNA present in different samples and for identifying alternatively spliced transcripts and multigene family members. Northern Blotting involves separation of RNA samples according to size by agarose gel electrophoresis and detection with hybridization probe complementary to part of or the entire target sequence. Northern Blot refers to capillary transfer of RNA from the electrophoresis gel to the blotting membranes.

Principle:

- Northern blotting is a commonly used method to study gene expression by detection of RNA (or isolated mRNA) in samples. Northern blot technique was developed by James Alwine and George Starck and was named such by analogy to Southern blotting.
- In Northern Blotting the total RNA or mRNA is isolated from an organism of interest, and then electrophoresed on denaturing agarose gel, which separates the fragments on the basis of size.
- The next step is to transfer fragments from the gel onto nitrocellulose filter or nylon membrane. This can be performed by the simple capillary method.
- The transfer or a subsequent treatment results in immobilization of the RNA fragments, so the membrane carries a semi permanent reproduction of the banding pattern of the gel.
- The RNA is bound irreversibly to the membrane by baking at high temperature (80°C) or by UV crosslinking.
- For the detection of a specific RNA sequence, a hybridization probe is used. A hybridization probe is a short (100-500bp), single stranded nucleic acid either DNA or RNA probe that will bind to a complementary piece of RNA.

- Hybridization probes are labeled with a marker (radioactive or non-radioactive) so that they can be detected after hybridization.
- In non-radioactive detection the probe is labeled with biotin or dioxigenin.
- The membrane is washed to remove non-specifically bound probe and the hybridized probe is detected by treating the membrane with a conjugated enzyme, followed by incubation with the chromogenic substrate solution.

As a result a visible band can be seen on the membrane where the probe is bound to the RNA sample. The entire procedure can be divided into following steps:

A] Denaturing Agarose Gel Electrophoresis:

- Denaturing Agarose gel electrophoresis is a technique used for separation of RNA molecules according to their molecular size.
- In this gel electrophoresis, formaldehyde (a denaturant) is used along with MOPS electrophoresis buffer.
- RNA has high degree of secondary structures, making it necessary to use denaturing gels.
- Formaldehyde in the gel disrupts the secondary RNA structures so that RNA molecules can be separated by their charge migration.
- For analysis of RNA molecules, 1 to 1.2% agarose gels are used depending on the size of RNA to be separated.
- The position of RNA in the agarose gel is visualized by staining with low concentration of fluorescent intercalating dyes, such as Ethidium bromide.
- This can be added either in the gels or in the RNA sample before loading for better resolution. The integrity and size distribution of total RNA can be checked by observing the stained RNA.

B] Northern Blotting:

- Northern blotting is the capillary transfer of resolved RNA fragments from the denaturing agarose gel to the nitrocellulose/nylon membrane.
- In this upward capillary transfer procedure, a support is used which is placed in a reservoir of transfer buffer to elevate the entire assembly.

- The wet wicks are placed on the support with both ends completely dipped in transfer buffer.
- A gel is placed on the wicks with RNA transferred side facing down.
- Over the gel, a wet positively charged nylon membrane is placed.
- A stack of paper towel is kept on filter papers as shown in figure 1.
- A small weight is placed over this entire assembly and is kept overnight.

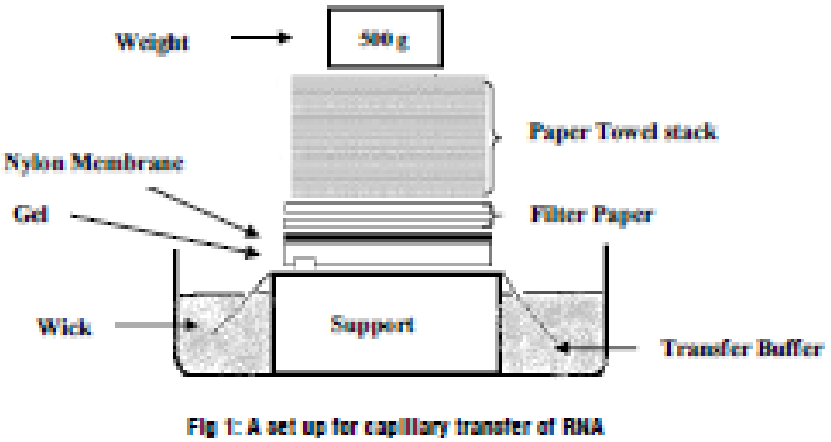


Fig 1: A set up for capillary transfer of RNA

- During capillary transfer the RNA bands are transferred to positively charged nylon membrane in presence of a specific buffer.
- The resolved RNA fragments are transferred to the corresponding positions on the nylon membrane after the capillary transfer.
- The RNA is then immobilized on the membrane either by baking at high temperature or UV crosslinking.
- This results in the covalent linkage of RNA to the membrane, which prevents the nucleic acid from being washed away during the subsequent processing.
- This is followed by hybridization with labelled DNA or RNA probe and then the RNA of interest is detected on the membrane.

C] Detection:

- After capillary transfer, RNA bands bound to the membrane are detected using a chromogen. The RNA of interest is hybridized with a biotinylated probe specific to it.

- The membrane is washed to remove excess unbound probe.
- It is then treated with Horseradish peroxidase (HRP)-conjugated streptavidin which attaches to the hybridized RNA.
- Finally, the membrane is incubated in a substrate solution containing TMB/ H2O2 (Tetramethyl benzidine H2O2 substrate) that reacts with HRP and as a result corresponding blue coloured RNA band develops on the nylon membrane as shown in Figure 2:

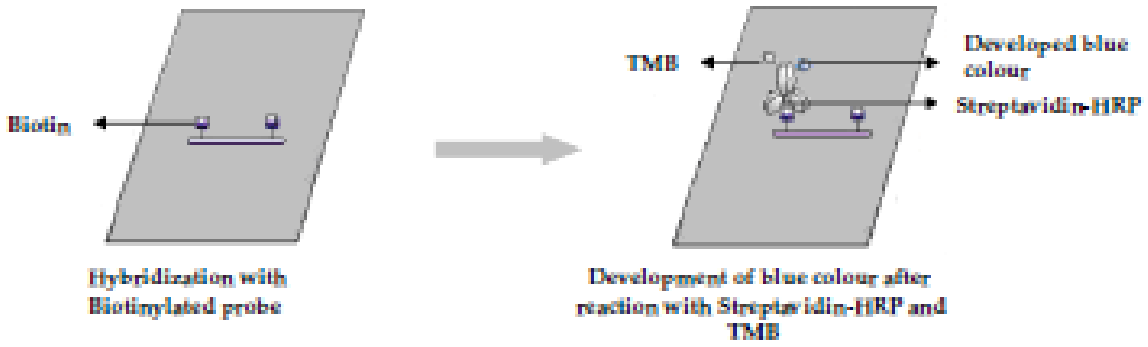


Fig 2: The hybridized RNA is detected after treatment with Streptavidin-HRP, followed by TMB substrate

Materials required:

- Total RNA
- Hybridization Buffer
- Biotinylated Probe 5 x
- RNA Sample Buffer
- 5X RNA Loading Buffer
- Wash Buffer I
- Wash Buffer II
- Blocking Powder
- TMB/H_2O_2
- Streptavidin HRP Conjugate
- Conjugate Dilution Buffer
- 10X MOPS Electrophoresis Buffer
- 10X Transfer Buffer
- Tween 20
- Agarose
- Formaldehyde 37%

- Filter Paper
- Nylon Membrane 1
- Blotting Sheet
- Sterile Disposable Petriplates

Procedure:
Day 1:

1. Denaturing Agarose Gel Electrophoresis Preparation of 1X MOPS Electrophoresis Buffer: To prepare 500 ml of 1X MOPS Electrophoresis Buffer, add 50 ml of 10X MOPS Buffer to 440 ml of RNase free water and add 10 ml of Formaldehyde (37%). Mix well before use.

Precautions to be taken while handling RNA:
1. Prior to starting the experiment, the electrophoresis tank should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol and allowed to dry.
2. Tips, pipettes, electrophoresis unit etc to be used for the experiment must be UV treated for 15-20 minutes.
3. Use sterile, disposable plasticwares and micropipettes reserved for RNA work to prevent cross-contamination \ with RNases from shared equipments.
4. Use RNase-free water for diluting the solutions.

Preparation of Denaturing Agarose gel:

- To prepare 50 ml of 1.2 % agarose solution, mix 5 ml of 10X MOPS Electrophoresis Buffer with 45 ml of autoclaved milliQ in a glass beaker or flask.
- To this add 0.6 g of agarose..
- Heat the mixture in a microwave, burner or hot plate, swirling the glass beaker/ flask occasionally, until agarose dissolves completely (Ensure that the lid of the flask is loose to avoid buildup of pressure).
- Allow solution to cool to about 55- 60°C. Add 0.9 ml of 37% Formaldehyde and mix well and pour the gel solution into the gel tray sealed on both sides with adhesive tape.
- Allow the gel to solidify for about 30 minutes at room temperature (15-25°C).

NOTE: Before running the gel, equilibrate it in 1X MOPS Buffer for atleast 30 minutes.

Loading of the RNA samples:

- To prepare sample for electrophoresis, take 20 µl of total RNA sample and add 40 µl of RNA Sample Buffer to it.
- Heat at 65°C for 5 minutes and immediately chill on ice. Dilute ethidium bromide to 50 ng/µl
- (i.e. dilute the stock 10 mg/ml EtBr to 1:200).
- Add 10 µl of EtBr to denatured RNA sample.
- Load 10 µl of denatured RNA sample mixed with EtBr onto 1.2% agarose gel.

- Load 3 µl of 5X RNA gel loading buffer in one of the wells as a reference to indicate the distance traveled.
- NOTE: EtBr is added to the RNA sample before loading and not to the gel.
- Wear gloves while handling EtBr.

Electrophoresis:

- Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode.
- Electrophorese at 100 volts and 70 mA until dye markers have migrated an appropriate distance, depending on the size of RNA to be visualized.

NOTE: Toxic fumes are released on electrophoresis, hence it is necessary to run the gel in a fume hood.

2. Capillary Transfer of RNA:

1. After electrophoresis, soak the gel in RNase-free water for 5 minutes to remove formaldehyde.
2. Repeat the above step twice and then discard water.
3. Observe the gel under UV transilluminator to excise the gel piece using a gel cutter.

NOTE: Excise the entire piece of gel i.e. 5 lanes.

4. Take care not to excise the individual lanes. Do not expose yourself to UV.
5.Set up capillary blot with transfer buffer as follows.

I. Rinse the materials required for transfer with isopropanol followed with RNase-free water thoroughly.

II. Fill the buffer reservoir with 200 ml 10X transfer buffer.

III. Keep a support in the buffer reservoir.

IV. Wet the wicks with transfer buffer. Ensure both the ends of the wicks are completely dipped in the transfer buffer.

V. Place the gel piece upside down i.e. RNA transferred side should face the wick.

VI. Wet the nylon membrane for few minutes in the transfer buffer. Place the membrane supplied on the gel. Ensure that no air bubbles are trapped between the gel and the membrane, as this will affect efficient transfer. V. Wet the filter paper and place over the membrane, ensure no air bubbles are trapped between the membrane and filter paper

VI. Cut blotting sheets of the same size and stack over filter paper.

VII. Carefully place a weight over this stack. Ensure that the book is placed on the centre of this stack, such that even pressure is applied.

VIII. Carry out transfer overnight at room temperature.

Day 2:

1. Immobilization of RNA on membrane:

1. Carefully remove the stack of blotting paper and filter paper after overnight transfer.

NOTE: Mark the side of the membrane that faced the gel as this is the right side i.e. RNA transferred side.

2. Observe the membrane and the gel piece under UV transilluminator to check whether complete transfer has taken place.

3. Expose the membrane to UV light for 5 minutes (placed between the inner layers of tissue paper). This helps in fixing the RNA to the membrane.

4. Switch off the UV, turn over the membrane and expose it to UV light for another 5 minutes.

5. Bake the membrane at 70-80°C for 30 minutes (place the membrane between the inner layers of tissue paper

6. Place the membrane in a ziplock or autoclaved petri plate at 4°C.

7. Mark the lanes on the membrane with a pencil, cut along the length of the membrane.

8. Use one strip for hybridisation and developing and remaining strips can be stored at 4 oC until further use.

2. Hybridization:

1. Set an incubator shaker at 55°C, prior to placing the membrane for Prehybridization.

2. Place the membrane (RNA transferred side facing down) in a petriplate containing 10 ml of hybridization buffer.

3. Carry out Prehybridization at 55°C for 1 hour, with constant shaking (70-80 rpm).

4. Keep 1 vial of biotinylated probe for 10 minutes in boiling water bath and immediately chill by placing it on ice for 5-10 minutes.

5. Remove petriplate and discard the buffer.

6. Add 5 µl of this probe to the 10 ml of Hybridization buffer in the petriplate. Mix thoroughly and add drop wise to the petriplate. Make sure that you don't add probe directly on the membrane.

7. Seal the petriplate and incubate the petriplate at 55°C incubator shaker overnight with mild shaking at about 70-80 rpm.

Day 3:

1. Washes and Detection:

1. Transfer the membrane into a fresh petriplate containing 10 ml of Wash Buffer I.

2. Gently swirl the petriplate for 15 minutes at room temperature. Repeat the wash one more time. Discard the buffer after each wash.°C

3. Add 10 ml of prewarmed Wash Buffer II (65°C) and gently swirl the petriplate. Incubate at 65 °C for 15 minutes in an incubator shaker and gently swirl. Repeat this step. Discard the buffer after each wash. NOTE: Do not let the membrane go dry at any step.

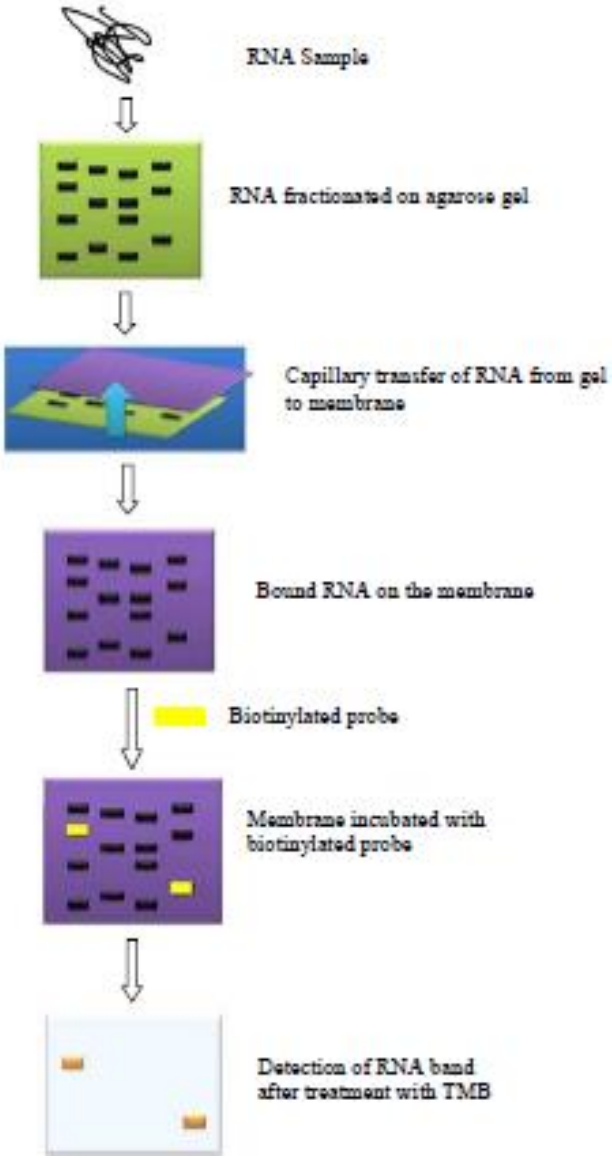
4. Take 30 ml of conjugate dilution buffer in autoclaved test tube and add 30 µl of Tween 20 to it and mix it thoroughly. This is to be used for further washes.

5. Add 9 ml of Streptavidin-HRP conjugate buffer (Refer Important instructions) to the petriplate and incubate at room temperature for 30 minutes with gentle rocking. Discard the conjugate buffer.

5. Carry out washes in a fresh and dried petriplate. Do not carry out washes in the petriplate used for conjugation. Use 10 ml of conjugate dilution buffer (See step 4) to carry out washes of 5 minutes at room temperature.

6. Repeat the above step two more times.
7. Add 5 ml of TMB/H_2O_2 and gently swirl at room temperature until a blue colour band develops.
8. After blue colour band is seen stop the reaction by placing the membrane in distilled water.

Flow chart:



Observation and Result:

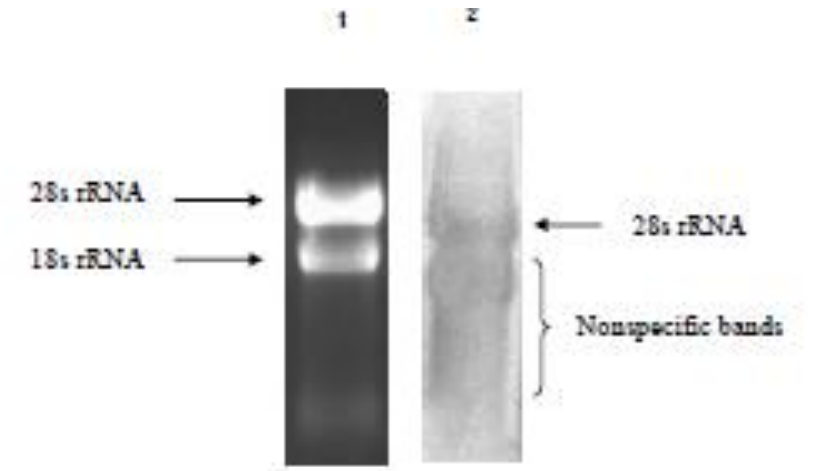


Fig 3: Gel Picture and Immunoblot of the RNA sample after Agarose Gel Electrophoresis and Northern blotting

Lane 1 : RNA sample on 1.2 % denaturing agarose gel
Lane 2 : After Northern hybridization a blue band develops on the nylon membrane

Interpretation:

- In non-radioactive Northern Hybridization a biotinylated probe is hybridized to the complementary target RNA (28s rRNA).
- The biotin of resulting hybridized complex binds to Streptavidin-HRP conjugate.
- In presence of TMB substrate HRP reacts with it and forms a blue colour which appears as a blue band on the nylon membrane.
- A few blue coloured bands can be seen below major 28s rRNA band due to non-specific binding of the probe to the total RNA bound on the membrane.

EX. NO : 9 **Western blotting (Demonstration)**

DATE :

Aim

To separate the proteins through SDS-PAGE and detection followed by characterization of proteins through Western blotting.

Introduction

- Western blotting (also known as protein- or immunoblotting) is a rapid and sensitive assay for detection and characterization of proteins.
- It works by exploiting the specificity inherent in Ag-Ab recognition.
- It is used to identify specific antigens recognized by polyclonal or monoclonal antibodies.
- Western blotting is carried out along with protein (antigen) separation in gel by electrophoresis and the blot development.
- It is essentially a combination of 3 techniques:

  Electrophoresis (PAGE)

  Western blotting

  Immunochemical detection.

Principle

- Identification of protein separated by gel electrophoresis is limited by the small pore size of the gel, as the macromolecule probe for protein analysis cannot permeate the gel.
- This limitation is overcome by blotting the protein into an adsorbent porous membrane. The apparatus consists of a tank containing buffer, in which is located a cassette. Clamping the gel and the membrane tightly together, a current is applied from electrodes, and repeated on either side of the cassette to avoid heating effects.

- The proteins are separated according to their electrophoresis mobility and blotted onto the membrane identified, using suitable immunochemicals to locate the protein of interest. The individual techniques are explained below.

SDS-PAGE

Stage 1. Prepare a PAGE gel slab and fix to a vertical electrophoretic apparatus. Treat the sample with suitable buffer and load onto the gel slots.

Stage 2. Apply electric current. After a few minutes, proteins in the sample migrate according to their electrophoretic mobility in the stacking gel. The stacking gel has a polyacrylamide concentration resulting in higher pore size and a lower pH of 7. This enables the protein to concentrate into sharp bands due to isotachopharesis, or band-sharpening effect. At the end of the stacking gel, it meets the separating gel, which has a higher polyacrylamide concentration and higher pH. In the separating gel, the proteins travel according to their size.

Stage 3. When the dye front reaches the bottom of the separating gel, the proteins in the sample are resolved depending on their size. However the protein cannot be visualized directly. The gel needs to be stained with suitable stainer to visualize all the proteins. The identification of protein of interest can be done using a suitable probe and a developing system.

Western Blotting

Blotting is the transfer of resolved proteins from the gel to surface of suitable membrane. This is done commonly by electrophoresis (known as electroblotting).

In this method, the transfer buffer has a low ionic strength which allows electrotransfer of proteins. Methane in the buffer increases binding of proteins to niotrocellulose and reduces gel smelling during transfer. The use of the membrane as a support for protein enables the case of manipulation efficient washing and faster reactions during the immunodetection, as proteins are more accessible for reaction.

(*a*) The membrane is in close contact with PAGE gel containing proteins. The proteins are electrotransferred to nitrocellulose membrane.

(*b*) At the end of electrotransfer, all proteins would have migrated to the NC membrane.

The protein was transferred to the corresponding position on the membrane as on the gel. A mirror image of the gel was formed. However, the protein location and detection can only be assessed after immunodetection.

Immunodetection

The transferred proteins are bound to the surface of NC membrane and are accessible for reaction with immunochemical reagents. All the unoccupied sites on the membrane are blocked with inert proteins, detergents, or other suitable blocking agents. The membrane is then probed with a primary antibody and a suitable substrate so the enzyme identifies the ag-ab complex form on the membrane.

Applications

To characterize proteins and to identify specific antigens for antibodies.

Preparation of Reagents

1. *Blotting Buffer.* Add 25 mL of blotting buffer component A and 25 mL of component B to 150 mL of distilled water.

2. *Other Buffers.* Dilute the required amount of buffer concentrate to 1X concentration with water.

3. *Antibody.* Dilute primary Ab and label secondary HRP conjugate in an assay buffer.

4. *Substrate.* Dilute TMB/H2O2, 10X concentration 10 times with heat just before use.

Procedure

1. Run SDS:Polyacrylamide gel electrophoresis.

2. Electroblot.

3. Assemble the blotting sandwich within the blotting cassette. Care should be taken to avoid air bubbles between the gel and NC membrane.

4. Insert the cassette into the apparatus filled with blotting buffer so that the gel faces the cathode.

5. Connect the power supply and use a voltage of 50 V for 5 hours for blotting.

Immunodetection

(*a*) Remove the NC membrane gently from the cassette and place it in the blocking buffer for 2 hours at room temperature, or overnight in the cold.

(*b*) Suspend the primary antibody in 10 mL with the assay buffer, using a suitable tube.

(*c*) Immerse the blot in the 10 Ab solute and gently agitate for 30 minutes.

(*d*) Wash the blot by immersing it in wash buffer for 3–5 minutes. Repeat 2 more times.

(*e*) Prepare 1:1000 dilutions of labeled 20 Ab in the assay buffer. Prepare sufficient (10 mL) volume of diluted Ab to cover the blot.

(*f*) Immerse the blot in 20 Ab solute and agitate gently for 30 minutes.

(*g*) Wash the blot in wash buffer for 3–5 minutes and repeat 4 times.

(*h*) Immerse the washed blot in 10 mL of substrate solution with gentle shaking.

Bands will develop sufficient color within 5–10 min.

(*i*) Remove the blot and wash with distilled water. Dry.

(*j*) Although the colored hands fade with time, the rate of color loss can be retarded if the blots are kept in the dark.

Interpretation and Result

The proteins separated through the SDS-PAGE have been successfully transferred onto the nitrocellulose membrane and the transferred proteins detected by immunodetection, which was confirmed by the development of color bands on the nitrocellulose membrane.

Lab manual

Fermentation and Bioprocess Technology Practicals

KAHE

EX. NO.10 Isolation and secondary screening of industrially important microorganisms

DATE:

A. Isolation of bacteria from soil

Aim: To isolate the bacteria from soil sample

Principles

Soil is the principle habitat for a variety of microorganism. The Quality and quantity of microbes depend upon the physico-chemical characteristics of soil. Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilization ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

Materials required

Soil sample, nutrient agar, sterile distilled water, petridish, test tubes, conical flask, colony counter.

Procedure

1. Measure 1-1 g of the garden soil, 1ml of water into the flasks containing different enrichment broths and the preprepared nutrient agar plates directly exposed to air.
2. For better aeration, place the inoculated flasks into a shaker incubator at 28°C for one week.
3. Prepare a five-member, 10-fold dilution series from the enrichment cultures and the original sample.
4. Spread the surface of agar plates having the same composition as the enrichment broths from the individual members of the dilution series.
5. Incubate the cultures at 28°C for one week.
6. Perform germ count estimations and colony morphology examinations after the incubation period.

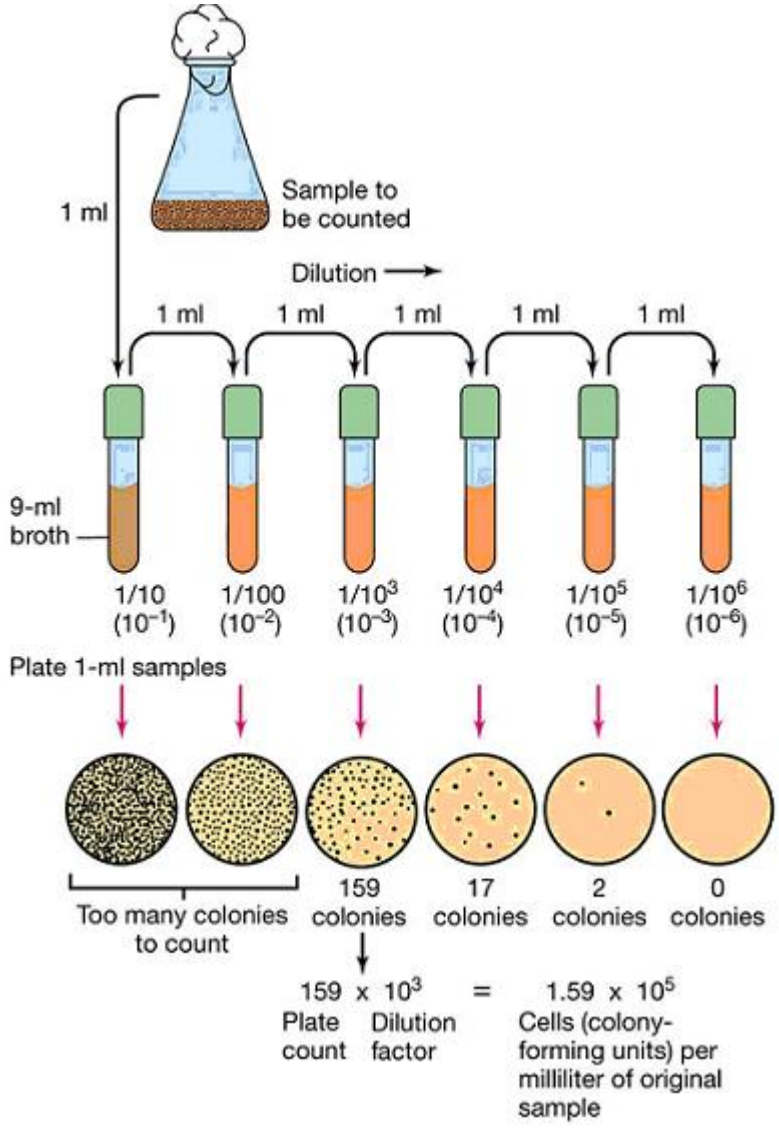
Calculations

Organism per ml of sample = Number of colonies / Amount plated x Dilution factor

Result

The amount of organism per ml of soil sample = ……..colonies.

Serial dilution methods



B. Isolation of bacteria from soil

Aim: To isolate the bacteria from water sample

Principles

Polluted water is the principle habitat for a variety of microorganism. The Quality and quantity of microbes depend upon the physico-chemical characteristics of water. Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilization ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

Materials required

Water sample, nutrient agar, sterile distilled water, petridish, test tubes, conical flask, colony counter.

Procedure

1. Measure 1-1 ml of the water sample and diluted with fresh water and pored into the flasks containing different enrichment broths and the preprepared nutrient agar plates directly exposed to air.
2. For better aeration, place the inoculated flasks into a shaker incubator at 28°C for one week.
3. Prepare a five-member, 10-fold dilution series from the enrichment cultures and the original sample.
4. Spread the surface of agar plates having the same composition as the enrichment broths from the individual members of the dilution series.
5. Incubate the cultures at 28°C for one week.
6. Perform germ count estimations and colony morphology examinations after the incubation period.

Calculations

Organism per ml of sample = Number of colonies / Amount plated x Dilution factor

Result

The amount of organism per ml of soil sample = ……..colonies.

EX. NO.11: Production of amylase or protease, Enzyme immobilization
DATE:

Exp. No.11 a: Production of amylase or protease

Aim: To produce industrially important enzymes like protease and amylase from the given samples.

Principle: Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes. Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. *Bacillus* produces a wide variety of extra-cellular enzymes, including proteases. The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material

Materials Required: Horikoshi-I medium, mineral medium, skimmed milk, Bacterial culture, Iodine solution, Petri plates,

Procedure:

Protease Screening

1. Polypeptone in Horikoshi-I medium was substituted for skimmed milk. Skimmed milk was autoclaved separately for 5 m at 110 °C (twice).
2. Microorganisms were inoculated on solid media.
3. After 1-3 days of incubation, colonies surrounded by a clear zone were determined as protease producers

Amylase Screening

1. 0.5% soluble starch was added in modified mineral medium
2. After 2 days of incubation at 37 °C, iodine solution (I2 =1 g, KI= 2 g/ 300 ml) was poured onto the plates.
3. Amylase producers were surrounded by a clear halo.

Results and discussion:

Exp. No.11 b: Enzyme immobilization

Aim: To immobilize the enzyme and protect from external factors

Principle: Three different commonly used entrapment media will be introduced in this experiment: polyacrylamide, calcium alginate, and gelatin. All these gels can be formed with a simple set of equipment and share similar procedures. In all the protocols, enzymes are well mixed with monomers/polymers and cross-linking agents in a solution. The solution is then exposed to polymerization promoters to start the process of gel formation. The solution is poured into a mold to achieve the desired shapes. A gel block may be cut into smaller cubes to increase the surface area. Commercially, it is common to force the unpolymerized solution through a set of nozzles to form spherical beads, whose size can be controlled by adjusting the back pressure. The resulting beads may be further hardened to enhance structural integrity.

The various methods used for immobilization of enzymes may be grouped into the following four types:

(i) Adsorption

(ii) Covalent Bonding

(iii)Entrapment and

(iv) Membrane Confinement

Of the three gels, polyacrylamide is the most widely used matrix for entrapping enzymes. It has the advantage that it is non-ionic. The consequence is that the properties of the enzymes are only minimally modified in the presence of the gel matrix. Calcium alginate is just as widely used as polyacrylamide. Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

Materials required:

Beakers, Graduated cylinder, Pipets, Test tubes, Temperature bath, Syringe, Alginic acid, sodium salt, $CaCl_2$ Enzyme, HCl solution 1N, KOH solution 1N.

Procedure:

1. Dissolve 30g of sodium alginate in 1 liter to make a 3% solution.
2. Mix approximately 0.015 g of enzyme with 10 ml of 3% (wt.) sodium alginate solution. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness. See Note 2.
3. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M $CaCl_2$ solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 0.5-2 mm in diameter. Other shapes can be obtained by using a mold whose wall is permeable to calcium ions. Leave the beads in the calcium solution to cure for 0.5-3 hours.

Results and Discussion:

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EX. NO12: Wine Production an alcohol determination by chromic acid method

Aim: To produce wine using *Saccharomyces* fermentation

Principle:

Fermentation is the anaerobic catabolism of a single chemical compound using a series of redox transformations with the goal of generating ATP by substrate-level phosphorylation. *Saccharomyces* is one of the most studied organisms in science and the major producer of commercial ethanol.

Many microorganisms (micro = small), notably yeasts and bacteria, extract energy from their food (glucose) by fermentation. One of the best-known types of fermentation is alcohol fermentation in which the overall chemical reaction is:

$C_6H_{12}O_6$ (glucose) → $2CO_2 + 2CH_3CH_2OH$ (ethyl alcohol)

or, starting from sucrose or maltose,

$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CO_2 + 4CH_3CH_2OH$ (ethyl alcohol)

Various fruits, especially grapes, could also be fermented to produce alcoholic beverages. Thus, alcoholic fermentation is the process which is responsible for the production of wine, beer, and other fermented products. It is the toxic nature of ethanol which acts to preserve these brews, and which leads to intoxication upon consumption. In fact, yeasts cannot generally survive in alcohol concentrations in excess of approximately 12 to 14%.

Materials required: Grapes, yeast culture, sugar etc..

Procedure:

1. Harvesting- This is the most critical stage of the process. The grapes must be harvested when the sugar, acid, phenol and aroma compounds are optimised for the style of wine desired.
2. Crushing and destemming- The grapes are removed from the stems and gently crushed to break the skins. Sulfur dioxide is added to the grapes at this stage to prevent oxidation and inhibit microbial activity. Enzymes may also be added to break down the cell walls and aid the release of juice.

3. Pressing- The juice extraction process depends on the type of wines to be used, but always involves squeezing the berries. After pressing the juice is allowed to stand to separate the solids. If necessary the juice may be clarified by filtration or centrifugation.
4. Fermentation- The juice is innoculated with live yeast, which then carries out the fermentation reaction:

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

This reaction occurs through many intermediary biochemical steps. The process is carried out under a blanket of carbon dioxide as in the presence of oxygen the phenols are oxidised and the sugar and ethanol are converted to carbon dioxide and water.

5. Purification- Unwanted solids, salts and microorganisms are removed through a variety of physical processes, then the wine is bottled and sold.

Results and Discussion:

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EX.NO.13: Down stream processing by Solvent extraction

DATE:

Aim: to extract the microbial metabolites by using solvent extraction

Principles:

Solvent extraction, also called liquid-liquid extraction or partitioning, is a procedure used to separate compounds based on their solubility in two immiscible liquids, usually water and an organic solvent. During solvent extraction of fermented media or biomass, one or more of the solutes in one of the liquid phases migrates to the other liquid phase. The two liquid phases are then physically separated and the desired product is isolated from the phase that contains it. Solvent extraction is one of the most commonly used laboratory purification methods, particularly in organic chemistry labs. Solvent extraction is also widely used in industrial operations like purification of biomolecules from fermented media or biomass. Some industrial applications use batch-mode extraction, albeit usually on a much larger scale than laboratory solvent extractions. Other industrial applications use continuous-mode solvent extractions, often on a gigantic scale, where the two solvents are continuously added to and removed from a large reaction vessel.

Materials required

Soxhlet apparatus

Funnel

Heating mantle

Organic solvents like ethyl acetate, chloroform, methanol and ethanol

Procedure

1. Fill a test tube about halfway with water.
2. Add about 5 mL of organic solvent (petroleum ether) solution to the test tube on fermented media (1ml). Stopper the tube and agitate it until the contents are thoroughly mixed. Record appearance after 10 minutes.
3. After the two layers have separated completely, use the disposable pipette to draw off the top (organic) layer as completely as possible. Add about 5 mL of organic solvent (ethyl acetate) solution to the test tube on fermented media (1ml). Stopper the tube and agitate it until the contents are thoroughly mixed. Record appearance after 10 minutes.

4. Add about 5 mL of organic solvent (chloroform) solution to the test tube on fermented media (1ml). Stopper the tube and agitate it until the contents are thoroughly mixed. Record appearance after 10 minutes.
5. After the two layers have separated completely, use the disposable pipette to draw off the top (organic) layer as completely as possible.
6. Add about 5 mL of organic solvent (methanol) solution to the test tube on fermented media (1ml). Stopper the tube and agitate it until the contents are thoroughly mixed. Record appearance after 10 minutes.
7. After the two layers have separated completely, use the disposable pipette to draw off the top (organic) layer as completely as possible.
8. Add about 5 mL of organic solvent (ethanol) solution to the test tube on fermented media (1ml). Stopper the tube and agitate it until the contents are thoroughly mixed. Record appearance after 10 minutes.
9. After the two layers have separated completely, use the disposable pipette to draw off the top (organic) layer as completely as possible. Transfer the organic layer to the watch glass and set it aside to allow the solvent to evaporate.

Observation and result

Table Solvent extraction of biomolecule – observed data

Layer	**Appearance**
A. aqueous biomolecule solution	
B. Addition of petroleum ether (after settling)	
C. Addition of ethyl acetate (after settling)	
D. Addition of chloroform (after settling)	
E. Addition of methanol (after settling)	
F. Addition of ethanol (after settling)	