ADARSH COLLEGE

OMERGA, DIST. OSMANABAD

DEPARTMENT OF MICROBIOLOGY

CERTIFICATE

Practical paper – XVII

Certified that Shri / Miss_____

has satisfactorily completed the course of practical work in B. Sc. III YEAR (SEMESTER V & VI) prescribed by Dr. B. A. M. University, Aurangabad under my supervision in the Microbiology Laboratory during the academic year 20 – 20

Staff member incharge

Date:

Head of the department

Date:

Signature of the Examiner

Date:

Exam Seat No. _____

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B.Sc. III

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Expt. No. 01 **Date: -**

Isolation of total RNA from Yeast

Aim: - To isolate RNA from Yeast (*Saccharomyces cerevisiae*)

Approach / Theory: -

Yeasts contain excessive amount of Nucleic acid. Total RNA is obtained by whole cell homogenate with phenol. Here two phases are obtained, mainly the lower phenolic phase containing DNA and upper aqueous phase containing RNA. Denatured proteins present in both phases are recovered by centrifugation. RNA precipitated with alcohol (cold) is free from DNA.

Requirements: -

1. Active culture of *Saccharomyces cerevisiae*
2. Sabouraud's dextrose broth

Water	100 ml
Peptone	1 gm
Dextrose	2 gm
pH	5.5

   Sterilize at 10 lbs for 40 minutes

3. Potassium acetate (20 % w / v)
4. Phenol (aqueous) 90 % w / v
5. Cold Ethanol
6. Centrifuge tubes
7. Boiling water bath
8. Dry test tubes

Procedure: -

1. 100 ml 0f Sterile Sabouraud's dextrose broth is inoculated with pure culture of yeast *Saccharomyces cerevisiae*
2. The flask is incubated on rotary shaker at room temperature for 48 hours.
3. This broth is centrifuged at 8000 RPM for 15 minutes.
4. Supernatant is discarded and the cell pellet is washed 2 to 3 times with D/W.
5. About 2 gm of washed cells are suspended in 10 ml of D/W and mix thoroughly to get thick suspension.
6. This suspension is slowly added with vigorous stirring into 10 ml of 90 % aqueous phenol at room temperature and resultant mixture is stirred for 1 hour.
7. This mixture is then allowed to stand for 15 hours at room temperature.
8. Then this mixture is centrifuged at 5000 RPM for 15 minutes.
9. The upper aqueous layer is removed carefully and to this Potassium acetate is added to give a final concentration of 2 % w / v (0.2 gm).
10. Then 20 ml of cold ethanol is added.
11. Centrifuge at 5000 RPM for 15 minutes to precipitate RNA.

Result: The RNA from yeast is isolated.

Expt. 2 **Date**

Hyperchromacity study of chromosomal DNA using UV – visible spectrophotometer

Aim: To perform Hyperchromacity study of chromosomal DNA using UV – visible spectrophotometer.

Approach / Theory: -

The absorption spectrum is a unique pattern of absorption maxima and minima at different wavelengths. It is often used in biochemistry to characterize or identify compounds. The absorption peak is generally referred as λ_{max}.

DNA shows maximum absorption in the range of 260 nm of wavelength of light.

Requirements: -

1. Standard DNA sample (100 µg / ml in saline citrate buffer)
2. Saline citrate buffer

Water	100 ml
NaCl	8.77 gm
Tri-sodium citrate	3.675 gm

3. UV visible spectrophotometer

Procedure:

1. Saline citrate buffer solution is added in a cuvette and used as reference.
2. Standard DNA sample is added in a cuvette and absorption is read at different wavelengths as per observation table.

Observation table

Sr. No.	Wavelength in nm	Absorbance
1	200	
2	210	
3	220	
4	230	
5	240	
6	250	
7	260	
8	270	
9	280	
10	290	
11	300	

Result: -

Absorbance is maximum at _____nm .

Conclusion: -

λ_{max} of given DNA sample is _____nm.

Experiment No. 03 **Date: -**

Replica plating for isolation of Lac spontaneous mutants of *E. coli.*

Aim: -

To detect the position of Lac mutants of E. coli on master plate by replica plating technique.

Theory / Approach: -

Mutation is a heritable change in the nucleotide sequence of DNA. Mutations may be characterized according to either the kind of genotypic change that has occurred or their phenotypic consequences. Mutations can alter the phenotype of a microorganism in several different ways. There are two classes of mutations: Spontaneous mutations and induced mutations.

Spontaneous mutations: - A mutation without a known cause is called Spontaneous mutations. This occurs at low frequency leading to the chemical instability of purine and pyrimidine bases and also due to low level of metabolic errors, or mistakes during the DNA replication.

Induced Mutations: Mutations that results from exposure of organisms to mutagenic agents such as ionizing irradiation, ultraviolet light or various chemicals that react with nucleic acids.

A simple velveteen covered colony transfer device is used to transfer the colonies in nutrient agar medium supplemented with or without a particular antibiotic or nutrient. The fibers of velvet act as fine inoculating needles, picking up the bacterial cells from the surface of this master plate. The velvet with its attached microbes is then touched to the surface of a sterile agar plate, inoculating it. In this manner, microbes can be repeatedly stamped onto media of differing composition. By comparing the presence of colonies following incubation we can indirectly determine the mutant colonies by their absence in the selective environment.

The process involves making of identical copy of master plate by using velvet cloth, which acts as several inoculating needles at a time. The given culture of *E. coli* is grown on nutrient agar plate to form well-isolated colonies. This is called as master plate. This does not contain lactose.

This plate is replicated on MacConkey's agar plate (containing lactose) with the help of sterile velvet stamp. Both the plates are incubated at 37^0 C for 24 hours. Wild type *E. coli* utilize lactose and produce pink coloured colonies on MacConkey's agar plate, while the mutant *E. coli* (Lac^-) produce colourless colonies. The position of Lac mutant on master plate is determined by corresponding position of colourless colony on replica plate. As master plate

was not exposed to any mutagenic agent, the type of mutation is spontaneous mutation.

Requirements: -

1. *E. coli* culture
2. Nutrient agar plate
3. MacConkey's agar plate
4. Velvet stamp

Procedure: -

1. A round piece of velvet cloth is wrapped on the bottom of stamp having a diameter of petri plate. It is sterilized.
2. From the given culture of *E. coli*, 0.1 ml is spread on the Nutrient agar plates and incubated at 37^0 C for 24 hours.
3. The plate showing isolated colonies is selected. This plate serves as master plate. This plate is labelled as A and B at two ends of diameter.
4. Similar labelling is made with MacConkey's agar plate called as Replica plate.
5. Velvet stamp is also labelled as A and B at two ends of diameter.
6. The stamp is gently pressed on the surface of master plate and then on Replica MacConkey's agar plate by coinciding the A and B marks both the plates.
7. The plates are incubated at 37^0 C for 24 hours.
8. Lac^- mutants *E. coli* grow on the replica plate but produce colourless colonies, where as wild type *E. coli* produce pink coloured colonies. The position of mutant colony on master plate corresponds to the position of these colonies on replica plate. Thus we can prove spontaneous mutation, which occur in absence of mutagenic agents.

Observation: -

Colourless colonies appeared / not appeared on replica plate.

Result: -

Spontaneous mutation has taken place / not taken place which is indicated by appearance/ not appearance of colourless colonies on replica plate.

Conclusion: -

By knowing the position of colonies on replica plate, we can detect the position of Lac mutant on master plate.

Expt. No. 04 **Date: -**

Ultraviolet damage and Photoreactivation

Aim: - To study the ultraviolet damage and photoreactivation of *E. coli*

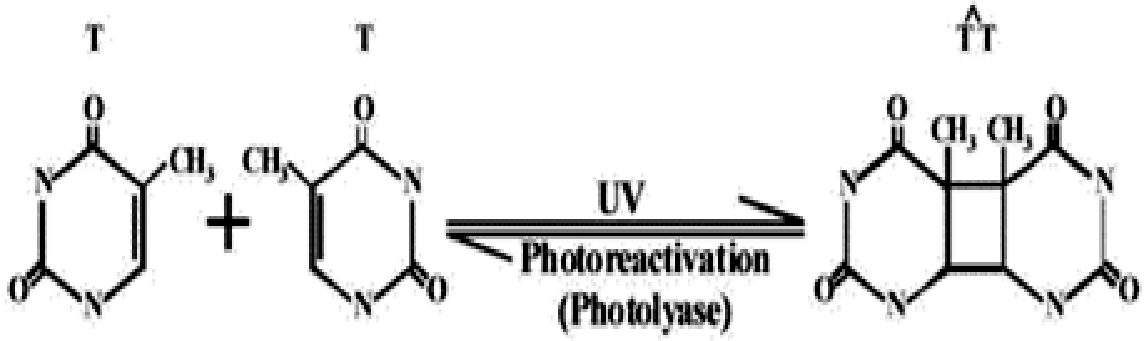
Theory / Approach: -

Ultraviolet rays range from 100 to 400 nm in electromagnetic spectrum. Due to short wavelength they have high energy. Edenberg in 1934 discovered that U.V. light is a mutagenic agent.

Nucleic acids absorb the U.V. light; particularly 254 nm UV light is lethal to microorganisms. Target molecule is DNA. The exposure of UV results in the formation of intrastrand dimers by two adjacent pyrimidines. The most important are **Thymine-Thymine dimers,** though others like **Thymine-Cytosine and Cytosine-Cytosine** may even be formed but in less amount.

The significant effects of these dimers are—

→ The DNA helix becomes distorted because the thymines in the same strands come close to each other for the bond formation. As a result of distortion, inhibition of the advancement of replication fork takes place and thus replication is inhibited. This in turn results in the death of organisms.



Thymine-Thymine dimer

The damage caused to DNA by UV rays can be reversed in two ways—

→Dark repair
→Photoreactivation

Dark repair: - The dark repair mechanisms are independent of light and can be accomplished by three distinct mechanisms—

a) Excision repair
b) Recombination repair
c) SOS repair

Photoreactivation: - It is light dependent or light inducible process. In the presence of visible light only the dimers are repaired. It is an enzymatic process involving photolyase, which is only active when it absorbs light. The enzyme cleaves bonds between pyrimidines and the DNA structure is restored.

Requirements: -

1. Sterile Nutrient agar plates
2. *E. coli* culture
3. U.V. light chamber
4. Black Cloth
5. Spreader
6. Absolute ethanol

Procedure: -

1. 0.1 ml of *E. coli* suspension is spread on two sterile nutrient agar plates each.
2. The plates are kept under UV lamp and the lids are removed.
3. The half of the plates are covered with some cardboard and accordingly labelled.
4. Plates are exposed to UV light for 20 seconds.
5. The plates are covered with lids.
6. One plate is wrapped with black cloth and other plate is not wrapped and is kept for photoreactivation by visible light for 10 minutes.
7. Both the plates are incubated for 24 hours at 37^{0} C.
8. Plates are observed for development of colonies.

Observation: -

1. The number of colonies in wrapped plate
    a) On UV exposed area_____
    b) On unexposed area_____
2. The number of colonies in unwrapped plate
    a) On UV exposed area _____
    b) On unexposed area _____

Result: -

1. The number of colonies on UV exposed area is less than the number of colonies on unexposed area in wrapped plate.

2. The number of colonies on UV exposed area of unwrapped plate is more than the number of colonies on UV exposed area of the wrapped plate.

Conclusion: -

From the observations and results it is concluded that there is effect of UV light on *E. coli* and this effect can be reversed by photoreactivation by visible light.

Experiment No. 05 **Date: -**

Transformation of competent cells of *E. coli* using plasmid DNA as a vector

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Aim: - To transform competent cells of *E. coli* using plasmid DNA as a vector.

Theory / Approach: -

This procedure is used to prepare batches of competent bacteria of 5 X 10^6 to 2 X 10^7 transformed colonies per microgram of plasmid DNA. This procedure is based on E. coli research. This procedure allows to preserve most strains at -70^0 C.

Requirements: -

1. *E. coli* (single colony)
2. LB (Luria Bertani Broth) medium
3. Ice cold $CaCl_2$
4. LB plates supplemented with Ampicillin
5. Plasmid DNA
6. Flasks
7. Polypropylene tubes (50 ml)
8. Centrifuge
9. Round bottom test tubes (15 ml)
10. Water bath (42^0 C)

Procedure: -

1. Inoculate 50 ml of LB medium with a single colony of E. coli and incubate it under shaking condition at 250 rpm to get $OD_{590} = 0.375$.
2. Transfer the culture into 8 pre-chilled polypropylene tubes and keep it on ice for 5 to 10 minutes.
3. Centrifuge it for 7 minutes at 3000 rpm in a refrigerated centrifuge, discard the supernatant and suspend the pellets in 10 ml ice-cold 0.1 M $CaCl_2$ solution.
4. Again centrifuge the tubes for 5 minutes at 2500 rpm to form pellets.
5. Resuspend the pellet in 10 ml cold 0.1 M $CaCl_2$ solution for 30 minutes on ice.
6. Centrifuge as earlier, collect pellets, and mix pellets with 2 ml ice-cold 0.1 M $CaCl_2$ solution.
7. Dispense the cells into pre-chilled sterile polypropylene tubes and freeze at -70^0 C.

8. Transfer aliquots of 10 ng of DNA (pBR 322) to round bottom sterile tubes containing 10 to 25 ml cells.
9. Keep on ice for ligation reaction and rapidly thaw between palms.
10. Dispense 100 ml into sterile tubes containing DNA and keep on ice for 10 minutes.
11. Place the tubes into 42 ^{0} C water bath for 2 minutes to give heat shock, add 2 ml LB medium to each tube and spread 1 ml of each on petri plates containing LB medium supplemented with Ampicillin.
12. Incubate the plates overnight at 37 ^{0} C.

Observation: - Colonies are appeared / not appeared on the surface of the medium.

Result: - Transformation of competent cells of *E. coli* occurred / not occurred.

Expt. 06 **Date**

Isolation of coliphages from sewage

Aim: -

To isolate coliphages from sewage sample.

Theory: -

Phages are the viruses that attack microorganisms. They are noncellular, obligate intracellular parasites, always growing in association with its respective host. The phages have host specificity and are named on the basis of their host type. The phages that attack actinomycetes are called actinophages, that attack on algae are called cyanophages, that attack on bacteria are called bacteriophages. The viruses that attack on coliform bacteria are called coliphages. The phages are of great industrial importance because of their ability to destroy desired culture used for product formation. The destruction of the culture or its mutation by transduction results into either decrease or loss of product or decrease the quality of product.

Coliphages can be detected and counted by using phage plaque technique. The sewage sample is inoculated along with a fresh active culture of host (E. coli) and the plate showing inhibition of host are detected. Palques are round clear zones lacking the visible growth.

Requirements: -

1. Bacteria free sewage sample
2. Actively growing E. coli culture in MacConkey's broth
3. MacConkey's agar paltes
4. MacConkey's butts

Procedure: -

The sewage sample is made bacteria free either by filtering through bacteriological filter or by treatment with chloroform. 10 ml sewage sample is mixed with 0.2 ml of chloroform and allowed to act for 15 minutes. The chloroform is evaporated by keeping the sample in oven or water bath adjusted at 45^{0} C.

1 ml of bacteria free sewage sample and 1 ml of fresh culture of E. coli is added to 5 ml of melted and cooled at 45^{0} C MacConkey's agar in a test tube. This mixture is shaken thoroughly and poured on sterile MacConkey's agar plate. The plate is then incubated at 37^{0} C for 24 hours and then observed for plaque formation.

Observation: -

Plaques are observed / not observed.

Result: -

Coliphages are present / absent in given sewage sample.

Conclusion: -

Bacteriophages exist in nature in association with their respective host. The coliforms generally present in sewage sample are from fecal matter of animals. The coliphages are also associated with them. The bacteriophages grow intracellularly causing the death of host bacteria which is observed on the pl ate as a plaque.

Expt. No. 7 Date:

Study of Conjugation in *E.coli*

Aim: To study the process of bacterial conjugation through transfer of genes coding for antibiotic resistance.

Theory:

Bacteria possess several methods for gene transfer for transmission of genes between individual cells. The methods include transformation, transduction and conjugation. Conjugation is a recombination process where two live bacteria come together, and the donor cell transfers genetic material to the recipient cell. This process was first observed in 1946 by Joshua Lederberg and Edward Tatum in a series of experiments with *E. coli*.

Certain "male" types (designated as F^+) donate their DNA and other "female types" (designated as F^-) receive the DNA. F^-cells become F^+ when they acquire a small amount of DNA. Hence the F factor is called as the Fertility factor. The donor's F factors are known to be plasmids which are the extrachromosomal elements. F pili or sex pili, these hairlike fibres contact the recipient bacteria, and then retract so that the surfaces of donor and recipient are very close or touching one another. At the area of contact, a channel or conjugation bridge is formed. Once contact via sex pili has been made, the F factor (plasmid) begins replicating by the rolling circle mechanism. A single strand of the factor then passes over or through the channel to the recipient. When it arrives, enzymes synthesize a complementary strand, and a double helix is formed.

Requirements

1. Streptomycin resistant *E. coli* suspension but sensitive to Tetracycline
2. Tetracycline resistant *E. coli* suspension but sensitive to Streptomycin
3. Sterile LB agar plate with streptomycin (30 µg / ml) and tetracycline (100µg / ml).
4. Luria Bertani broth (2.5 gm in 100 ml water)

Procedure:

1. Add 0.5 ml Streptomycin resistant *E. coli* suspension and 0.5 ml Tetracycline resistant *E. coli* suspension in Luria Bertani broth and incubate at 37 ^{0} C for 2 hours for conjugation process to occur.
2. Spread 0.1 ml from above broth is spread on LB plate containing streptomycin (30 µg / ml) and tetracycline (100µg / ml).
3. Observe for the development of colonies.

Observation: Colonies appeared / Not appeared

Result: Conjugation occured / Not occurred

Conclusion: As colonies appeared on LB plate containing streptomycin and tetracycline plate; it is concluded that the plasmid for resistance is transferred from one type of *E. coli* to another type.