علوم الحياة / كورس الأول

2018 / 10/ 21

المادة: جزيئي عملي / 3-4

المرحلة: الرابعة

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السعر (750)

مكتبة كلية مدينة العلم الجامعة Facebook:

Estimation the amount of DNA in *E.coli* by Burton method

1) Grow *E.coli* in nutrient broth and incubate for overnight (18 hrs.) to obtain bacteria in **log phase**.

2) Take (10 ml) from the growth and inoculate it in (50 ml) of new nutrient broth. This is considered as the **zero time** of growth and then incubated for different periods in **shaker incubator** at 37C.

3) Take (7.5 ml) of growth and add (2.5 ml) from the **perichloric acid (P.A) 1N**

4) Put the mixture in **ice bath at 0C for 15 min.** in order to:
   a. Reducing the heat that generated from the addition of P.A (1N)
   b. Freezing the precipitate cell and DNA after opening the cells

5) Centrifuge for (10 min.) at 3000 rpm, discarded the supernatant and keep the pellet that contain the DNA

6) Add (2 ml) of **P.A (0.5 N)** to pellet and mix well, put the mixture in **water bath at 70C**

7) Centrifuge for (15 min.) at 3000 rpm, discarded the pellet and keep the supernatant that contain DNA

8) Add **Burton reagent (1 volume from mixture + 2 volumes from reagent)**

9) Prepare **Blank** from 2 ml of reagent + 1 ml of P.A (0.5 N)

10) Incubate the mixture and the blank for (18 hrs.) at 37C

11) Measure the O.D of mixture and blank at **(600 nm) using spectrophotometer**, the colour will change to **greenish blue** and the colour density depends on DNA concentration.

Preparation standard curve of DNA:

The aim of this curve is to estimate the amount of DNA, which is extracted from *E.coli* for each hour from the above experiment by measuring the O.D of standard gradient concentration of DNA as the following:

1) Prepare stock solution of DNA with (500 µg/ml) by taking (25 mg) from DNA powder and solve it with (50 ml) of P.A (0.5 N)

   25 mg/ml x 1000 = 25000 µg/ml
   25000 / 50 ml = 500 µg/ml

2) Prepare gradient concentration of DNA (25-200 µg/ml) from the orginal solution, P.A is used to complete the volume to 2 ml.

Concentration	C1V1= C2V2	Take from stock	Complete it with
25	500 x V1= 25 x2	0.1	1.9
50	500 x V1= 50 x2	0.2	1.8
75	500 x V1= 75 x2	0.3	1.7
100	500 x V1= 100 x2	0.4	1.6
125	500 x V1= 125 x2	0.5	1.5
150	500 x V1= 150 x2	0.6	1.4
175	500 x V1= 175 x2	0.7	1.3
200	500 x V1= 200 x2	0.8	1.2

3) Add **4 ml of Burton reagent** to each **2 ml of each final concentration.**
4) Prepare **Blank** as it mentioned above
5) Mix the tubes and put them in **water bath in 100 C for 10 min.** then cool the tubes in order to measure O.D at 600 nm.
6) Draw the curve that show the relationship between O.D and gradient concentration of DNA, then point the values of O.D of test sample in the experiment on the curve to obtain the unknown concentration for each O.D after each hour.

The components of Burton reagent:

1. **Diphenylamine:** react with deoxyribose sugar to produce greenish blue colour
2. **Glycial acetic acid:** breaks down hydrogen bonds between base pairs
3. **H_2SO_4:** breaks down the phosphodiester bonds in the nucleotide among the single strand of DNA
4. **Acetaldehyde:** a co-factor that **increase** the reaction between deoxyribose sugar and reagent; and **fix** the colour of mixture.

The principle of Burton reagent work:

Diphenylamine in the reagent will react with deoxyribose sugar release from broken DNA, which lead to the greenish blue colour.

Methodology:

- **Preparation of Burton reagent:**
  1) Take (1.5 gm) from diphenylamine and solve it in (100 ml) of glycial acetic acid, then (1.5 ml) from concentrated H_2SO_4 is added to mixture.
  2) Store the mixture in the dark (to prevent oxidation by the light).
  3) Added (0.5 ml) acetaldehyde to (100 ml) of the mixture.

- **Preparation of acetaldehyde:**
  <u>Ex.:</u> Prepare (10 ml) of (16 mg/ml) of acetaldehyde, if the orginal concentration was (0.78 gm/ml).

  C1V1= C2V2
  0.78 gm/ml x 1000 = 780 mg/ml
  780 mg/ml x V1 = 16 mg/ml x10
  V2 = 0.2 ml from stock and completed to 10 ml with D.W

- **<u>Preparation of perichloric acid</u>**
  The percentage of the orginal concentration is = 70%
  M.wt = 100.64
  Specific gravity = 1.67

$$M= \frac{10\ (\text{percentage con. X S.G}}{\text{M.wt}} \qquad M= \frac{10\ (70 \times 1.67)}{100.46} = 11.6$$

This formula gives the orginal molarity of the solution, and then the normality can be calculated by:

$$N= \frac{M}{\text{Eq. number}}$$

The chemical formula of perichlorite acid is H_2SO_4, so the equivalent number is 1

$$N = \frac{11.6}{1} = 11.6$$ of the orginal solution

<u>Ex.:</u> Prepare (400 ml) of P.A with (0.25 N)?

N1 V1 = N2 V2

11.6 x V1 = 0.25 x 400

V1 = 8.6 ml

Take this volume from the orginal solution and complete it to 400 ml with D.W.

Extraction of DNA molecules

There are many different methods and techniques available for isolation of genomic DNA from prokaryotes (Bacteria), Eukaryotes (animals & plants).

All methods involve disruption and lysis of cell wall and cell membrane, followed by removal of proteins and other contaminants.

Two general types of procedure are used for purification of DNA: **centrifugation** & **chemical extraction.** The DNA yield and purity are highly variable by using these methods.

First: Opening of different cells:

a) Bacterial cells

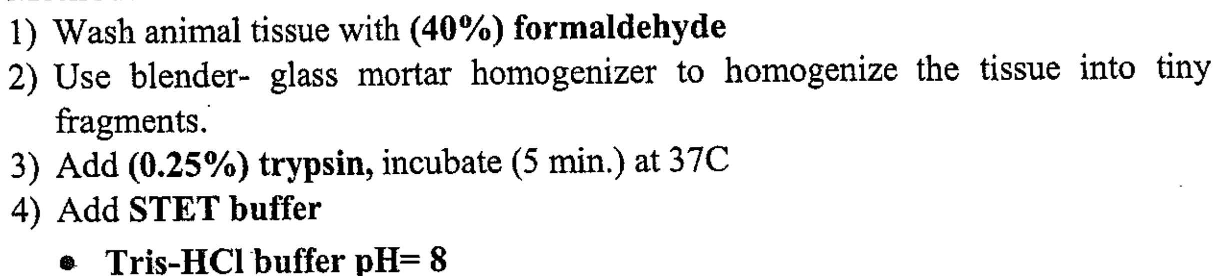
Method:

1) Suspend the overnight bacterial cells in (5 ml) of **TE buffer**, mix well by vortex, spin for (6000 rpm for 10 min. at 4 C).
    - **Tris-HCl:** buffer solution, pH=8
    - **EDTA:** chelating agent squerster divalent cations, such as: Mg^{+2} & Ca^{+2}; this stops DNA as enzyme from degrading the DNA.

2) Add (0.2 ml) of **(2.5% SDS)** to lysis Gram negative bacteria, and (10 mg/ml) of **lysozyme + (2.5% SDS)** to lysis Gram positive bacteria; then incubate (30-60 min.) at 37 C
    - **SDS:** anionic detergent used to soluble the cell membrane to release cell contant.

3) Add (0.6 ml) 0f **(5M Na-perchlorite)** it dissociate DNA from proteins.

b) Cells of animal tissue:



Method:

1) Wash animal tissue with **(40%) formaldehyde**
2) Use blender- glass mortar homogenizer to homogenize the tissue into tiny fragments.
3) Add **(0.25%) trypsin,** incubate (5 min.) at 37C
4) Add **STET buffer**
    - **Tris-HCl buffer pH= 8**

- **Triton x-100:** non ionic detergent (used for lysis cell membrane).

c) Cells of plants tissue:

Method:

1) Plant tissue must be ground into tiny fragments by used **liquid-N_2** with **metal homogenizer.**
2) Add extraction solution **(CTAB)** (cetyl- trimethyl ammonium bromide) as **a cationic detergent makes complete with polysaccharides.**

Seconed: Isolation of DNA from other componants:

Method:

1) Add **[phenol/ chloroform/ isoamylalcohol (25/24/1)]**
   It is an extraction solution that used to remove proteins and other contaminants from nucleic acid sample.
   This method can be carried out in amannae that is very close to quantitative.
   Nucleic acids are remained in **the aqueous phase** and proteins are separated in the **inter phase by phenol,** while most of lipids & polysaccharides are separated in the **lower organic phase by chloroform.**
   isoamylalcohol acts as an anti-foam

2) Centrifuge the mixture in (10000 rpm for 10 min. at 4 C). these layers are separated, the first aqueous layer contains DNA.

3) Transfer the upper layer to another **plastic tube**, then add twice volume of **cold ethanol**.

4) Mix by inversion; after 3 min., DNA will precipitate like spool DNA on a glass rod.

5) Dissolve the DNA in **(50-100 µl)** of **TE buffer.**

NOTE:

Wear plastic gloves to prevent DNAse enzyme on your hands from cutting DNA into small fragments.

References:

1) Gerhardt, philipp. *et al.* Methods for general and molecular bacteriology. Herndon. Vol: ASM press 1997.
2) Synder. Larry & Champness, Wendy molecular genetics of bacteria. Herndon. Vol. ASM press 1997.