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P-XXII

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

Production and estimation of Ethanol by using *Saccharomyces cerevisiae* var. ellipsoids

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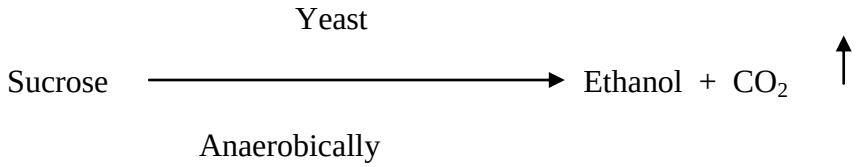
Aim: - To produce and estimate ethanol by using *Saccharomyces cerevisiae* var. ellipsoids

Theory / Approach: -

Saccharomyces cerevisiae var. ellipsoides is yeast, which produces ethanol by converting sugars into ethanol and carbon dioxide anaerobically, whereas in the presence of air it operates glycolysis and Kreb's cycle yielding increased biomass.



For the fermentation of sugar substrate used is sugarcane molasses, which contains about 50 % sugar. At this high concentration of sugar yeast will not grow hence it is diluted to obtain 10 to 15 % sugar.

After the fermentation, ethanol produced is recovered and estimated by using standard curve.

Production of ethanol

Requirements: -

1. Yeast Peptone Dextrose (YPD) broth
2. Sugar cane molasses
3. *Saccharomyces cerevisiae* var. ellipsoids
4. Urea
5. Condenser

Procedure: -

1. Inoculate the YPD broth with yeast culture and incubate at 30 ^{0} C for 3 days.
2. Dilute the molasses (1.1 kg in 8 litre of distilled water to get 12 brix) and adjust the pH to 5.5.
3. Transfer 250 ml of this solution into 500 ml flask and autoclave.
4. Inoculate the sterile sample with yeast and incubate at 30 ^{0} C for 12 hours. This will act as inoculum for fermentation medium.
5. Again prepare fermentation medium by mixing 2.1 kg molasses in 8 litre of distilled water to get 22 brix. Adjust the pH to 5.5 and add 100-ppm urea.
6. Transfer the medium in fermentation jar of suitable capacity and pasteurise it. (250 ml in 500 ml flask).
7. Inoculate the flask with inoculum obtained at step 4 and incubate it at 30 ^{0} C for 2-3 days.
8. Withdraw sample (10 ml) at every 6 hours and estimate ethanol production by using standard curve.

Estimation of ethanol by specific gravity method

The term 'specific gravity' refers to the density of any liquid. By convention, pure water is assigned an SG or specific gravity of 1.00 at 15.5C (60F) and is used as a standard. The 1.00 refers to the fact that the density of H_2O, in metric units, is 1 kg per liter. Since ethanol is less dense than water the Terminal Gravity is less than the Original Gravity. Pure ethanol has an SG of 0.79 kg/L. Specific gravity is a measure of the density of a substance relative to the density of a reference substance (normally water).

A specific gravity bottle measures the weight of a specific volume of an alcohol and water at a known temperature. The alcohol weight is then divided by the water weight to give the apparent specific gravity of the sample. Reference charts (AOAC table) convert apparent specific gravity to percent alcohol.

In the US, distilled spirits alcohol content is reported in degrees proof at 60oF (15oC), with proof representing twice the percent alcohol by volume. A whisky at 40% alcohol by volume in the US is reported as 80^0 proof. In Canada and the UK alcohol content is reported in percent alcohol by volume at 20^0 C.

Requirements: -

1. Specific gravity bottle
2. Weigh balance
3. Alcohol sample
4. Reference chart by AOAC (Association of Official Agricultural Chemists OR Association of Official Analytical Chemists OR ASSOCIATION OF ANALYTICAL COMMUNITIES)

Procedure: -

1. Weight of empty specific gravity bottle is taken and called as A
2. Weight of specific gravity bottle with water is taken and called as B
3. Weight of specific gravity bottle with alcohol is taken and called as C
4. Calculate the apparent specific gravity of the alcohol by the formula.
5. From the reference chart of AOAC determine the alcohol percentage at existing temperature range.

Calculations: -

1. Weight of water = B - A
2. Weight of alcohol = C – A

$$\text{Specific gravity of alcohol} = \frac{\text{Weight of alcohol}}{\text{Weight of Water}}$$

Result: - The ethyl alcohol percentage in the given sample is _____.

Conclusion: - We can determine the percentage of alcohol by measuring Specific gravity of alcohol.

Expt. No. 02 **Date: -**

Production and estimation of Citric acid by using *Aspergillus* spp.

Aim: - To produce citric acid by carrying out fermentation using *Aspergillus niger*.

Theory: -

Commercially using fermentation process, citric acid is one of the most important organic acids produced by microorganisms. Citric acid is used as –

- Acidulant in food, beverages and in pharmaceutical industries
- Iron citrate is used as a source of iron and preservative for stored food, tablets, ointments and cosmetic preparations.
- It is used as antifoaming agent in chemical industries.
- In detergent industries, sodium citrate is used inplace of phosphate.
- It is used as chelating and sequestering agent and plastisizer.

Aspergillus niger is mostly used for the production of citric acid. It is aerobic process and adequate aeration is required. The concentration of sucrose used is about 15 %.

Requirements:-

1. Fermentation medium

Composition

D/W	100 ml
KH_2PO_4	50 mg
Na_2NO_3	50 mg
$MgSO_4$	25 mg
$CuSO_4$	06 mg
$ZnSO_4$	25 mg
$FeSO_4$	01 mg
Sucrose	15 gm
pH	5

2. Potato dextrose agar slants (PDA)

Composition

1. 20 gm chopped pieces of potato are boiled in 20 ml water for 20 minutes and supernatant is filtered through muslin cloth and filtrate is taken in a flask.
2. Dextrose 2 gm
3. Agar agar 2.5 gm
4. Water to make volume 100 ml
5. pH 5

Pour in test tubes and autoclave at 10 lbs for 40 minutes and after keep tubes in inclined position to form slants.

3. *Aspergillus niger* culture.
4. Sterile saline (0.85 % NaCl)

Procedure: -

1. *Aspergillus niger* culture is subcultured on PDA slants and incubated at 30^0 C for 48 hours.
2. Thick spore suspension is prepared in 10 ml saline.
3. This 10 ml suspension is poured in 100 ml sterile fermentation medium.
4. The flask is incubated on shaker at 30^0 or at room temperature for 3-4 days.
5. Filtrate is prepared by filtration and then by centrifugation. The amount of citric acid produced is estimated by titrable acidity method.

Estimation of citric acid by titrable acidity method

Aim: - To estimate citric acid in the fermented medium

Requirements: -

1. Fermented citric acid medium
2. 1 % Phenolphthalein indicator
3. 0.1 N NaoH

Procedure: -

1. 1 ml of fermented medium is taken in a beaker and one drop of 1 % phenolphthalein is added in it.
2. The solution is titrated with 0.1 N NaOH.
3. The end point is colourless to pink.
4. The amount of NaOH required is noted and the amount of citric acid is calculated using relationship –

1 ml of 0.1 N NaOH = 7 mg of citric acid

Observations: -

The amount of NaOH required in titration is _____ ml hence amount of citric acid in the fermented medium is _____ mg / ml.

Result: - The amount of citric acid produced is _____ mg / ml.

Conclusion: - Maximum amount of citric acid is produced after 4 days by submerged fermentation method.

Expt. No. 3 **Date: -**

Paper Chromatography

Aim: - To separate amino acids in a given mixture by paper chromatography.

Theory / Approach: -

Chromatography is an analytical or separatory procedure, which permits the separation of one or more biological compounds from a mixture. This technique was employed by M. Tswet to separate coloured pigments. It is highly efficient laboratory technique. It consists of two phases. One is stationary phase and other is mobile phase. The choice of stationary or mobile phase is made so that the compounds to be separated have different distributory coefficients.

The various chromatographic techniques based upon the choice of stationary and mobile phases are –

1. Column Adsorption chromatography
2. Thin layer chromatography
3. Paper chromatography
4. Gas liquid chromatography
5. Ion exchange chromatography

Paper chromatography: -

In paper chromatography, paper absorbs water from the atmosphere. This water is held between cellulose fibres of the paper and is regarded as one of the solvents. The mobile phase (a non aqueous solvent) is allowed to travel along the paper by capillary action. The solute molecules on the paper distribute themselves between the two phases in a ratio, which is characteristic of their distribution coefficients. The more soluble a solute is in the mobile phase, the further will this compound travel along the paper in the direction of flow of solvent. The distance moved by the solute in the direction of solvent flow during a chromatographic separation is characterised by the term $\mathbf{R_f}$ value, which is defined as

$$\mathbf{R_f} \text{ value} = \frac{\text{The distance travelled by the solute}}{\text{The distance travelled by the solvent}}$$

Requirements: -

1. Mixture of amino acids
2. Solvent system (n – butanol, Acetic acid and water in a proportion 4 : 1 : 5)
3. Locating reagent Ninhydrin
4. Whatmann filter paper no. 1

5. Chromatographic jar

Procedure: -

1. Cut a piece of Whatmann filter paper no. 1 into strips. Draw a line with pencil across the width of the paper 2.5 cm from the bottom of the strip.
2. At the centre of this line apply the amino acid mixture 2 to 3 times with the help of capillary tube or thread and allow to dry.
3. Put the paper strip into the chromatographic jar containing solvent system so that the bottom of the strip should dip into the solvent 1 cm. A care should be taken that the line of application of mixture should not dip into the solvent system. The strip should not touch to the sides of the chamber.
4. Close the jar by lid and allow the solvent system to flow about 10 to 15 cm along the strip.
5. Remove the strip, air dry and mark solvent front with pencil.
6. Spray ninhydrin reagent and dry in oven at 60^{0} C for 5 to 10 minutes.
7. Observe for colour spots and mark the circle around the spots with pencil and find out the centre of the spots.
8. Note down the distance travelled by the spots from the origin and calculate the $\mathbf{R_f}$ value.
9. Compare the $\mathbf{R_f}$ values with standard $\mathbf{R_f}$ values and identify the amino acids.

Observation: -

The distance travelled by the solvent =

The distance travelled by the spot 1

The distance travelled by the spot 2

Calculations: -

$\mathbf{R_f}$ value of spot 1 = _____

$\mathbf{R_f}$ value of spot 2 = _____

Result : -

In the given mixture the amino acids are –

1.

2.

Conclusion: - By the paper chromatography method amino acids can be separated from the mixture and can be identified by comparing the observed $\mathbf{R_f}$ values with standard $\mathbf{R_f}$ values.

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

Separation of serum proteins using Agarose Gel Electrophoresis

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Aim: - To separate serum proteins by agarose gel electrophoresis

Theory / Approach: -

Electrophoresis is the term used to describe the movement of charged ions in the solutions under the influence of an electric field. The positive charged particles move towards cathode and negatively charged particles towards anode.

As a result of different rates of migration, a complex mixture can be separated into a number of fractions of different ionic mobility. The greatest usefulness of electrophoresis is in clinical diagnosis for serum, urine, spinal fluids, gastric juice and other body fluids.

The electrophoresis is of following types---

1. Paper electrophoresis
2. Cellulose acetate electrophoresis
3. Starch gel electrophoresis
4. Agar gel electrophoresis
5. Polyacrylamide gel electrophoresis (PAGE)

The pH of the solution plays major role in determining the mobilities of ions, since the net charge carried by most ions is pH dependent. This is particularly important where Zwitter ionic species such as amino acids or proteins are concerned.

According to the position of separating media it is either vertical electrophoresis or horizontal electrophoresis. Great varieties of buffer such as Barbiturate, Phosphate, Acetate, Citrate, EDTA etc, which cover the pH range 1 to 11 are used in the electrophoresis.

The isoelectric point can be defined, as a pH at which there is minimum concentration of an electrically focused ampholite. In alkaline buffer, serum protein migrates towards positive pole. Albumin migrates in advance of other fractions with α_1, α_2, β, and γ globulin in succession.

Requirements: -

1. Normal serum
2. Borate buffer of pH 8.6

Composition: -

A) 0.2 M solution of boric acid (12.4 gms of Boric acid was dissolved in 1 litre of D/W)

B) 0.05 M solution of Borate (19.05 gm of Borate was dissolved in 1 litre of D/W)

C) Complete solution: - 250 ml of A + 87.5 ml of B was added and diluted it to 1000 ml with D/W. The pH of this buffer was adjusted to 8.6

3. Agarose gel 1 % W/V

Preparation: - 1 gm of agarose was dissolved in 100 ml of Borate buffer (pH 8.6). The mixture was boiled to get uniform suspension. Place the combs of electrophoresis set such that it is approximately 2 cm away from the cathode.

Pour the agarose solution in the central part of the tank when the temperature reaches approximately 60^0 C. Do not generate air bubbles. The thickness of the gel should be around 0.5 to 0.9 cm. Keep the gel undisturbed at room temperature for the agarose to solidify.

Pour Borate buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
Gently lift the combs, ensure that wells remain intact.

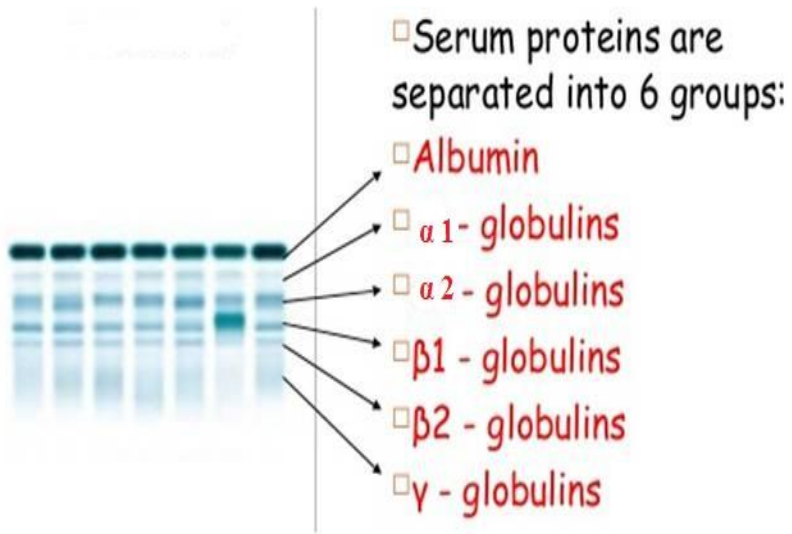
4. Staining reagent (Ponceau S stain 0.2 gm in 100 ml of 5% Trichloro Acetic acid)
5. Loading dye (Glycerol 0.5 ml, Bromophenol Blue 0.5 ml, Serum sample 1 ml)
6. Equipment: - The horizontal electrophoretic chamber

Procedure: - Electrophoresis

1. Connect the power cord to the electrophoretic power supply according to the convention.
2. Red : Anode. Black : Cathode
3. Load the loading dye in one well and samples in the remaining wells.
4. Set the voltage to 100 volts and the switch on the power supply.
5. Switch off the power when the loading dye from the well reaches 3 / 4 th of the gel. This takes approximately 1 to 2 hours.
6. Staining procedure to visualize protein: -

A) Carefully transfer the gel (from gel tank) into a tray containing Ponceau S stain. Make sure that gel is completely immersed.
B) For uniform staining, keep stain approximately 10 minutes. Shake intermittently every 2 to 3 minutes.
C) Pour out the staining dye into a container. Destain the gel by washing with tap water several times till the protein is visible as a dark band against a light background.

Observation: - Dark bands of proteins are observed.



Result: - Proteins from the serum sample are separated.

Conclusion: - By this method we can separate and observe the proteins from serum sample.

Expt. No. 05 **Date: -**

Penicillin bioassay

Aim: -

To perform antibiotic assay by using standard penicillin by paper disc method.

Theory: -

Microbiological assays are done for the estimation of potency of the preparation. Effect of desired compound on living susceptible microorganism is recorded. Potency of antibiotic is assayed by comparing the zone growth inhibition of susceptible organisms with those of standard dilutions of antibiotics. The diameter of zone of inhibition is dependent upon the concentration of antibiotics. If the diameters of zone of inhibition of standard penicillin are plotted against the concentration of antibiotic, the resulting graph should be straight line. Using this standard graph, concentration of unknown penicillin sample can be determined.

Penicillin is active against Gram-positive bacteria by inhibiting the process of cell wall synthesis. The test organism used are *Staphylococcus aureus or Bacillus subtilis*.

The Oxford unit of penicillin is the amount of penicillin required to inhibit the growth of *Staphylococcus* aureus in 24 mm diameter zone using cylinder plate method. However, the international unit of penicillin is the amount of penicillin equivalent to 0.6 micrograms of standard crystalline Sodium Penicillin G of about 98 % purity.

Requirements: -

1. Test organism *Staphylococcus aureus*.
2. Basal agar
3. Seed agar
4. Sterile Phosphate buffer solution
5. Penicillin powder
6. Sterile petri plates
7. Sterile pipettes
8. Filter paper discs
9. Forceps

Procedure: -

A) Preparation of Basal agar plate

Pour about 10-to15 ml sterile basal agar in sterile petri plate, allow to solidify.

B) Inoculation of test culture with seed agar

About 0.1 ml of suspension of test culture is spread on sterile agar plate.

C) Preparation of penicillin powder dilutions

The standard penicillin has 1665 units per mg, i.e. 60 mg of penicillin powder has 1,00000 units approximately. Weigh accurately 60 mg of standard penicillin powder and dissolve in 100 ml of buffer. This gives concentration of penicillin 1000 units per ml.

This dilution is referred as stock penicillin solution. From the stock solution, further dilutions are prepared as follows.

1. 0.1 ml of stock solution is taken and 10 paper discs are added to it. 10 units of penicillin will be there on each disc.
2. 0.8 ml of stock solution plus 0.2 ml of buffer solution. This mixture will contain 800 units per ml. Take 0.1 ml of this solution and to it add 10 discs. On each disc 8 units will be there.
3. 0.6 ml of stock solution plus 0.4 ml of buffer solution. This mixture will contain 600 units per ml. Take 0.1 ml of this solution and to it add 10 discs. On each disc 6 units will be there.
4. 0.4 ml of stock solution plus 0.6 ml of buffer solution. This mixture will contain 400 units per ml. Take 0.1 ml of this solution and to it add 10 discs. On each disc 4 units will be there.
5. 0.2 ml of stock solution plus 0.8 ml of buffer solution. This mixture will contain 200 units per ml. Take 0.1 ml of this solution and to it add 10 discs. On each disc 2 units will be there.

D) Application of penicillin dilution

Paper discs of different units are placed on inoculated plates with the help of sterile forceps in sterile condition. The plates are labelled respectively and kept in freeze for 30 minutes for proper diffusion of antibiotic. The plates are then incubated at 37 ^{o} C for 24 hours.

After incubation, diameter of zone of inhibition is measured. A graph is plotted against diameter of inhibition zone and respective dilutions. Using graph the units per ml in the unknown sample is determined.

Observation table

Penicillin units per disc	**Diameter of inhibition zone**
2	
4	
6	
8	
10	
Unknown	

Result: -

The penicillin units in unknown disc is _____ units.

Conclusion: -

From the observation and result, it can be concluded that penicillin is an antibiotic, which inhibits the growth of the test organism *Staphylococcus aureus*. Zone of inhibition is directly proportional to concentration of penicillin. By using standard graph the units of penicillin in a given sample can be determined.