Semester – III

16MBU313 INDUSTRIAL MICROBIOLOGY – PRACTICAL (3H – 2C)

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

End Semester Exam: 9 Hours

SCOPE

The paper provides knowledge in the large scale production of industrial product, and teaches the modern trends to cater the needs of industry.

OBJECTIVES

- To encompasses the use of microorganisms in the manufacture of food or industrial products.

EXPERIMENTS

1. Study of different parts of fermenter
2. Microbial fermentation – Production and estimation (qualitative and quantitative) of
    a) Enzymes : Amylase and Protease
    b) Amino acid : Glutamic acid
    c) Organic acid : Citric acid
    d) Alcohol : Ethanol
3. A visit to any educational institute/industry to see an industrial fermenter, and other downstream processing operations.

SUGGESTED READINGS

1. Stanbury PF, Whitaker A and Hall SJ. (2006). Principles of Fermentation Technology. 2nd edition, Elsevier Science Ltd.
2. Crueger W and Crueger A. (2000). Biotechnology: A textbook of Industrial Microbiology. 2nd edition. Panima Publishing Co. New Delhi.
3. Okafor N. (2007). Modern Industrial Microbiology and Biotechnology. 1st edition. Bios Scientific Publishers Limited. USA.
4. Glaze A.N. and Nikaido H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. 1st edition. W.H. Freeman and Company.
5. Casida LE. (1991). Industrial Microbiology. 1st edition. Wiley Eastern Limited.
6. Patel A.H. (1996). Industrial Microbiology. 1st edition, Macmillan India Limited.
7. Waites M.J., Morgan N.L., Rockey J.S. and Higton G. (2001). Industrial Microbiology: An Introduction. 1st edition. Wiley – Blackwell.

DEPARTMENT OF MICROBIOLOGY
KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act, 1956)

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II-B.Sc., Microbiology (Batch 2016-2019)
Industrial Microbiology Practicals (Semester-III) 16MBU313

List of Practicals

S.No	Name of the Experiment
01	Study of different part of fermentor
02	Production and estimation (qualitative and quantitative) of amylase
03	Production and estimation (qualitative and quantative) of protease
04	Production and estimation (qualitative and quantitative) of Glutamic acid
05	Production and estimation (qualitative and quantitative) of citric acid
06	Production and estimation (qualitative and quantitative) of ethanol

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EXPERIMENT NO:1
DESIGN OF A FERMENTOR

FERMENTATION:

Fermentation can be defined as a metabolic process in which cheap raw material such as a sugar or carbohydrates are converted into economically important products like acids, gases and alcoholic by microorganism. This process is cauised out in a equipment called as fermentation

FERMENATOR:

- A fermanator can be defined as a vessel in which sterile numient media and fure curness pf microorganism are mined and fermentation process caused our under aseptic and optimum condition.
- Fermentor provides a sterile environment and optimum condition that are important for growth of microorganism and synthesis of desired products.
- A fermentor should be constructed in suitia way that it can make frrouisims of the below activities:
    1. Sterilization
    2. Temperature control
    3. Ph control
    4. Foam control
    5. Auction and agitation
    6. Samping point
    7. Frioutation point for microorganism media
    8. Harvesting of produces
    9. Drainage point for damage of fermented media
    10. Cleaning
    11. Facility of providing hot, cold water and sterile compound air

MATERIALS USED FOR FERMENTATION:-

The material used for derigning of a fermentor should have some important functions

- It should not be overcome
- It should tolerate steam sterilization process

- It should be able to tolerate high pressure and resist PH change

The fermentor material used in also aided on type of fermentation process of eg: in case of bees, mine are made up of wooden material. Whereas material such as iron, copper, glass and stainless steel can be used in some case . Most of the time 30 A and 31B stainless steel is used for designing of fermentor and these fermentor are mostly coated with epoxy a glass lining.

A fermentor should provide the facility of control and monitor various parameters for a successful fermentation process.

1. **IMPELLER**

    - Impeller is an agitation device. They are maintined on the shaft and introduced in the fermentor through in did
    - They are made up of impeller blades and the passion may vary according to its need.
    - These impeller or blades are attach to the motor on did.
    - The impellent function of an impeller is to mix microorganism. Media and oxygen uniformly.
    - Impellers blades reduce the size of as bubbles and disturbance these air bubbles uniformly into the fermentation media.
    - Impeller also helps in wearing foam bubbles in the cheap space of fermentor. This form formed during fermentation process can cause contamination problems and this problem is avoided by the use of impellirs

2. **BAFFLES:-**

    - Baffles are mainted on the walls of a fermentor
    - The important function of baffles is to break the vortex formed during agitation process by the impellers
    - If this vortex is not broken the fermentation media may split out of fermentor and this may result in contamination as well as can lead to different problems. So it is important to break the vortex formed by using barrier.
    - Baffles acts as a barrier which break the vortex

3. INOCULATION PORT :-

- Inoculation port is a device from which fermentation media, inoculation and substance are added in the fermentation tank.
- Case should be taken that the port provides aseptic transfer.
- The inoculation port should be easy to sterilize.

4. SPARGERS:-

- A sponge is an aeration system through which sterile air is introduced in the fermentation tank.
- Glass wool filter are used in a sponges for stylization of air and other gases.
- The sponges pipes contain holes of about 10mm. through these small holes pressurized air is released in the aqueous fermentation media.
- The air released is in the form of tiny air bubbles. These air bubbles help in mining of media.

5. SAMPLING POINT:-

- Sampling point is used for time to time with drawn of samples to monitor fermentation process and quality control.
- The samples point should provide aseptic withdrawal of samples.

6. PH CONTROL DEVICE:-

- The PH controlling device checks the PH of media at specific interval of time and adjust the PH to its optimum level by addition of acid or alkalis.
- Maintaining PH to the optimum level is very imported for growth of microorganism to obtain a desired product.

7. TEMPERATURE CONTROL:-

- Temperature control device generally contains a thermometer and cooling coils or jacket around fermentor.
- During the fermentation process, various reactions take place in fermentor. That is generated and released in temperature is determined to the growth of microorganism which may slow down the fermentation process.
- So it is necessary to control this rise in temperature. This is done by passing cool water through the coil or jacket present around the fermentor.

8. FORM CONTROLLING DEVICE:-

- A form controlling device is placed on the top of fermentor with a inlet into fermentor. This device contains a small tank containing antifoaming agent.
- Form is generated during fermentation. It is necessary to remove or neutralize this foam with the help of anti foaming agent. Tear the media may spill act of fermentor and lead into contamination and a mess.

9. BOTTOM DRAINAGE SYSTEM:-

- It is an aseptic outlet present as the bottom of fermantor for removal of fermenised media and product formed.

EXPERIMENT NO: 2

Production and Estimation of Amylase

Aims :-

To Check The Qualitative and quantitative production of amylase from microorganism

Introduction:-

Amylase is an enzyme that break down starch glycogen . Amylase is produced by a variety of living organism ranging from bacteria to plant and humans . Bacteria and fungi seoretamylase to the outside of their cells to carry out extra cellular digestion when they break down the in soluble starch the and products such as glucose or maltose are observed into their cells.

Amylase production medium (g/l)

Bacteriological peptone - 6g

Magnesium sulfate - 0.5g

Potassium chloride – 0.5g

Starch – 1g

Sterile by autoclaving at 121°c for 15 minutes

Procedure Qualitative method

Isolation of Amylase producers:-

Suspend 1gram of soil sample in the whichFlask contain 99ml of sterile distilled water

- The stock solution prepared is sterially diluted upto to]
- Spread 0.1ml from all the diluted samples to] on nutrient agar plates containing 1% (w/v) solube starch and insoluble at 30®C for 24 hours
- Starch hydrolyzing colonies will have on area of clearing around them . it is confirmed by flooding plates with grams's iodine

- Transfer isolated amylase producing bacteria to nutrient agar and allow to grow for 24hours they are store in the refrigerator until needed

Quantitative method
Amylase production :-
Breed culture:-

- Prepare amylase production medium ,sterile properly
- Molecular loopful of amylase producing culture
- Incubate for 24hrs at 37C

Fermentation:-

- Prepare 250ml of amylase production medium in 500ml conical flask
- Sterile at 121®C for 15 minutes
- Inoculate 25ml of used mixture
- Withdraw outside and subject to extraction of enzyme

Extraction of enzyme from bacteria

Pour the bacterial culture into centrifuge tube and spinfor 20 minutes at 5000rpm

Separate the supernatent into a sterile beaker which is the crude enzyme extract.

Enzyme activity

- Pipette 1ml of mixture extract "enzyme " into attest tube
- Add 1ml of 1% soluble starch in citrate phosphate buffer (ph 6.5)
- Incubate in water bath at 40C for 30min
- Setup blank consisting of 2ml of the enzyme extract that has been boiled for 20 minutes (boiling inactivities the enzyme).added to the starch solution and treated with the same reagents as the experimental tubes.
- Stop the reaction by adding 2ml of reagent and estimate reducing sugar
- Enzyme activity may be defined as the amount of glucose produced per ml in the reaction
- Mixture for unit time

Estimation of reducing sugar by DNS method

Reducing agar was analysed by dinitro saricylic acid (DNS) method. It is a simple sensitive and adaptable method. Reagent react colour to light brown colour

Procedure:-

Preparation of standard graph :-

- Preparation an aqueous solution of glucose about a concentration of 1mg 1ml
- To a series of 10 test tube, add a glucose stock solution corresponding to the required sugar concentration [0.1-.1ml]
- Make up the volume to 3ml using double distilled water ,(use distilled water as a blank)
- Add 2mlof DNS and heating in boiling water bath at 80 C for 15 mins and cooled
- Read the absorbency at 580 min
- Plot the values on a graph

Analysis reducing sugar from sample:-

- Trace 5 ml of sample aseptically and centrifuged at 5000rpm for 10 mins
- Add 1 ml of supernatant to attest tube and makeup the volume to 8 ml using double distilled water (use distilled water as a blank)
- Add 2ml of DNS and heated in boiling water bath at 80 degr C for 15mins and cooled
- Read the absorbence at 580nm
- The concentration of reducing sugar 1ml is determined using the standard graph

Observation & result:-

Dark yellow colour is noticed on the test sample which indicates the presence of the reducing sugar.

EXPERIMENT NO: 3

PRODUCTION AND ESTIMATION OF PROTEINS

AIM

To check quantitative and qualitative production of protease from microorganism

INTRODUCTION

Protease and metalloprotease constitute one of the most important group of industrial enzymes accounting for about 60% of the total enzyme market. Among the various protease bacterial protease are the most significant compared with animal and fungal protease and among bacteria, Bacillus sps are specific producers of extracellular protease. These enzymes have wide industrial application, including pharmaceutical of protein hydrolyzate,waste processing industry. Thermostable protease are advantages in some application because higher processing temperature can be employed resulting in faster reaction rates, increases in the solubility of nongaseous reactant and products are reduced incidence of microbial contamination by mesophile organism.

PROCEDURE

Qualitative method

Isolation of protease producers

- Suspend 1g of soil sample in the conical flask containing 99ml of sterile distilled water(stock solution)
- The stock solution prepared is serially diluted upto 10^{-7} dilution.
- Spread 0.1ml of the diluted sample gelatin hydrolyse medium and incubate at 30C for 24 hours.
- Gelatin utilising colonies havearea of clearance around them.
- It is confirmed by flooding plates with mercuric chloride solution.
- Transfer distinguishable protease producing bacteria, streak on nutrient agar plate containing 1% gelatin.

Quantitative method

Protease production

- Prepare protease production medium and sterilize it properly.
- Inoculate loop full of protease producing Bacillus species
- Incubate for 24 hours at 37C

Fermentation

- Prepare 250ml of protease production medium in 500ml conical flask.
- Sterilize at 121C for 15 min.
- Inoculate the above medium with 1ml of an overnight culture and incubated at 37C for 24 hours in a rotatory shaker operated at 15 rpm.
- At time intervals the turbidity of the culture is determined by measuring the increase in optical density at 470nm with spectrophotometer
- Withdraw culture and subject to extraction of enzymes.

Extraction of enzyme from bacteria

- Pour the bacterial culture into centrifuge tubes and spin for 20min at 5000rpm.
- Decant the supernatant into sterile beaker which is the crude enzyme extract

Enzyme activity

- Pipette 1ml of cultural extract enzyme into test tube.
- Add 1ml of 1% soluble casein in citrate phosphate buffer (ph 6.5).
- Incubate in a water bath at 40C for 30min.
- Setup a blank consisting of 2ml of the enzyme extract that has been boiled for 15min, added to the casein and treated with some reagent as the experimental tube.
- Estimate protein content by using folin's method.
- Enzyme activity can be defined as the amount of casein produced per ml in the reaction mixture per unit time.

Estimation of protein by lowry's method

Protein react with the folin–ciocareace reagent to give a colored complex. Tryptophan residue of protein reduce sodium tungstate and molybdate anions in folin reagent which when combine with copper sulphate gives blue colored complex hetro-polymolebdenum and tungsten blue. The copper atom present in copper sulphate complexed with nitrogen atom of the peptide bond of protein

during reaction time that is also a reason for blue or purple color formation. The intensity of color depends on the amount of these aromatic amino acid present and will thus vary for different protein

Materials Required

Solution: alkaline sodium carbonate solution

Take 1gm of sodium hydroxide in 400ml of double distilled water mix well and then add 1gm of anhydrous sodium carbonate shake well and make up to 500ml using distilled water.

Solution 2: copper sulphate solution.

Dissolve 1gm of copper sulphate in 500ml of distilled water

Solution 3: sodium potassium tartarate solution.

Dissolve 1gm of sodium potassium tartarate in 50ml of water.

Solution 4: folin ciocatteaus reagent.

Dilute the commercial reagent with an equal volume of distilled water one the day of use. This is a asolution of sodium tungstate and sodium moleumdate in phosphate and hydrochloric acid.

Solution 5: standard protein bovine serum albumin (BSA).

Dissolve 10mg of BSA in 10ml of double distilled water.

Solution 6: working standard.

Make up 1ml stalk standard to 10ml. It give 100mg per ml concentration.

Procedure.

- Pipet out various concentration of working standard solution into a series of test tube and made up the volume to .2ml with distilled water.
- To each test tube add 1ml of the mixed reagent and mix thoroughly and allow to stand at room temp for 10min or longer.
- Add 0.3ml of dilute folin ciocolcate reagent rapidly and mix properly.
- Incubate all tube for 60min.
- Measure OD of the standard and test solution at 660 nanometer and plot the standard graph.
- Run the blank.

- The test protein sample is performed as like the standard solution and calculate the amount of protein present in the given sample. Blue color was noted and read using spectrophotometer.

- **Estimation of protein lowry's method**

SL.NO	Volume of standard (ul)	Volume of water (ul)	Concentrating working	Mixed reagent (ml)	Follin's reagent (ml)	OD value (660 nm)
1	10	190	10	1	0.3	0.02
2	20	180	20	1	0.3	0.04
3	30	170	30	1	0.3	0.05
4	40	160	40	1	0.3	0.07
5	50	150	50	1	0.3	0.09
6	60	140	60	1	0.3	0.12
7	70	130	70	1	0.3	0.14
8	80	120	80	1	0.3	0.17
9	90	110	90	1	0.3	0.20
10	100	100	100	1	0.3	0.22
Test sample 200ul			Unknown nil	1	0.3	0.22
Blank 200ul water				1	0.3	0.60

RESULTS

Concentration of protein present in the given sample is 95µg/ml.

EXPERIMENT NO:4

MICROBIAL PRODUCTION OF GLUTAMIC ACID

INTRODUCTION

The amino acid business is a multi-billion dollar enterprise. All twenty amino acid are fold, each in greatly different quantities. Amino acids are used as animal feed additives(lysine, methionine, threonine), flavor enhancers, (serine, aspartic acid) and as special nutrients in the medical life (glutamic acid, lysine and methionine accounts for the majority by weight of amino acid. Glutamic acid and lysine are

made by fermentation. Methionine is made by chemical synthesis. The major producers of amino acids are based in Japan, US, South Korea, China and Europe.

All amino acids may be produced by fermentation. Bacterial strains that produce amino acids with some exceptions mainly derived from Corneybacterium sps, Bacterium sps or E.coli strains used in production are wild type natural over production autotrophs or regulatory mutant that have altered feedback inhibition pathways or de-repressed enzyme synthesis and or genetically engineered organism that have multiple copies of genes encoding rare limiting enzymes.

Members of the Actinobacteria were the the most effective producers. Over the years various glutamate producing bacteria have been isolated and classified as Arthobacter, Brevibacterium or as member of other genera but recent works has shown that almost all of these strains belong to the genus corneybacteria wild type cultures produced upto 10g/l glutamic acid.

Small seed cultures are grown on glucose ,potassium phosphate, magnesium sulphate, yeast extract and urea a source of nitrogen. Larger culture use cheaper source of sugar, beet molasses and starch hydrolyses parallel the geographical location of the process. This is corn source used in the united states, tapioca in south asia and cane and beet molasses in Europe.

Sulphate are used as nitrogen source ammonia can be used to control pH during fermentation, cheap source of steep liquor a byproduct of corn. Starch manufacture that is amino acids, nutrients minerals and vitamins.

MATERIALS

Bacillus subtilis

PRODUCTION MEDIUM

Glucose – 10mg

K_2HPO_4- 50mg

KH_2PO_4 – 50mg

$MgSO_4$ – 25mg

$FeSO_4$ – 1mg

$MnSO_4$ – 1mg

Urea – 500mg

Water – 100ml

The pH should between 7 and 8, $CaCl_2$ – 0.5%

PROCEDURE

Mix the ingredient of the medium accordingly to the order and autoclave.

Autoclave 0.5% calcium chloride separately and add to the medium.

Inoculate 0.1ml of exponential growth phase culture of Bacillus subtilis in 50ml of glutamic acid medium.

Place this in a beaker for 10 days.

Filter the B.subtilis cultures using whatmann no:1 filter paper under aseptic condition.

Centrifuge the filtrate at 1000rpm for 10 min. use the cell free supernatant for the estimation of glutamic follow the ninhydrin method of amino acid estimation

ESTIMATION OF GLUTAMIC ACID

INTRODUCTION

The reaction between alpha amino acid and ninhydrin method of amino acid estimation take place as follows

Alpha amino acid + ninhydrin = reduced ninhydrin + H_2O ------(1)

Amino acid + H_2O = Alpha keto acid + NH_3 ----------(2)

Alpha ketoacid +NH_3 = Aldehyde +CO_2 ----------(3)

STEP 1 is an oxidative deamination reaction that removes two hydrogen from the alpha amino acid to yield an alpha amino acid. Simultaneously the original ninhydrin is reduced and acts on oxygen atom with the formation of water molecule.

In STEP2 the NH group in the alpha aminoacid is rapidly hydolysed to form an alpha keto acid with the production of an ammonia molecules. This alpha acod further undergoes decarboxylation reaction in STEP3. Under a heated condition to form an aldehyde that has one less carbon atom than the original amino acid. A carbondioxide molecule is produced here.

The first three step produce the reduced ninhydrin and ammonia that are required for the production of color. The overall reaction for the above is simple.

In summary ninhydrin which is originally yellow reacts with ammonia acid turns

Volume of standard amino	Volume of distilled water	Conc.of amino acid	Volume of ninhydrin		Volume solved		Absorbance (970)

deep purple. It is this purple colour that is detected in this method.

Ninhydrin will react with a free alpha amino acid group, NH2-CO-COOH. This group is contained in amino acid, peptides or proteins whereas the decarboxylation reaction will proceed for a free aminoacid will lead to the color development. However one should always check out the possible interference from peptide and proteins by performing blank especially when such solution are readily available. For example one can simply add the ninhydrin reagent to a solution of only protein and see if there is any color development

This test can be used routinely for the detection of glycine in the absence of other species, although this is a fast and semi test for the presence of alpha amino acids. Because of the selectivity it cannot be used to analyse the relative individual content of a mixture of different amino acid. Further most the color intensity developed is depend on the type of amino acid finally it doesnot react with teritiary or aromatic amino acid.

Since ninhydrin is a strong oxidizing agent proper care should be taken in handling this compound. It is especially potent as the elevated temperature under which the reaction is carried out. The ninhydin reagent will stain the skin blue and cannot be immediately washed off completely.

Reagents

Ninhydin reagent: dissolve 350mg of ninhydrin in 100ml of ethanol. Propanol or a mixture of acetone and butenol maybe used in place of ethanol for preparing the reagent.

acid (ml)	(ml)						
0.0	1.0	00	1		5		0.00
0.2	0.8	20	1		5		0.25
0.4	0.6	40	1	Incubation in boiling water bath for 15minutes	5	Incubation at room temperature for 10 minutes	0.53
0.6	0.4	60	1		5		0.80
0.8	0.2	80	1		5		1.03
1.0	0.0	100	1		5		1.39
1.0 (umknown)	0.0		1		5		0.65

PROCEDURE

- Add 1ml of ninhydin to 5ml of sample. Cover the test tube with cotton plug to avoid the loss of solvent due to evaporation.
- With gentle mixing heat at 80-100C for 4-7min
- Cool at room temperature reagent blank in a cold water bath. Record the absorbance with a spectrophotometer at 570 nanometer against reagent balnk. Simultaneously run standards using amino acid and prepare standard graphs.

RESULT

The production of violet color indicate the presence of amino acid .

The concentration of glutamic acid is found to be 50 µg/ml.

EXPERIMENT NO:5

PRODUCTION AND ESTIMATION OF CITRIC ACID

INTRODUCTION

Citric acid is an important organic acid and initially exatracted from citra fruits. Now a days it is largely produced by microbial fermentation, citric acid is commercially used in foods, soft drinks, pharmaceuticals, leather tanning, electroplating etc. Aspergillus niger which are mutant cannot oxidise citric acid and hence accumulate in cuture medium. The composition of the culthure medium is critical for obtaining high yield of the citric acid accumulate in the medium. Acid is added to achieve low pH of 3.5. sucrose serves as a carbon source for the production of citric acid.

Ammonium nitrate is used to prevent the fermentation of oxalic acid to glutamic acid fermentation is aerobic and can be carried out by submerged culture method.

Culturing *Aspergillus niger*

- Preparing the citric acid medium and dispersed above 50ml in 250ml conical flask.
- Autoclave and allow it to cool.
- Inoculate the medium wuth sores of aspergillus niger and incubate it on a shaker water bath at 25C with gentle shaking for 3-5 days.
- After incubation, filter the mycelium using double layered muslin cloth and measure the amount of citric acid in the filtrate by titrimetric method.

Estimation of citric acid by titrimetric method

The filterate obtained is titrated against an alkali of known strength using phenolphthalein as the indicator. The end point is the formation of pale pink colour. The volume of alkali used for neutralization is used to find the normality and the percentage of acid is the sample.

100ml of the filterate is pipetted into a conical flask and 2-3 drops of phenolphthalein indicator is added to it.

This is titrated against 0.1N NaOH taken in the burette till a pale pink color is formed.

The titration is repeated still the concordant values are obtained.

Calculation:

No	Initial burette reading	Final burette reading	Volume of NaOH used
1	0	48.8	
2	0	45.2	45.6
3	0	47.8	

Normality of citric acid = N(NaOH) = V(NaOH) / V(citric acid)

% of citric acid = normality X equivalent unit of citric acid X 100

= 0.0456 X 96 X 100 volume of filtrate = 4.37%

(N = Normality, V = Volume, equivalent weight of citric acid = 96)

RESULTS

Thus a consider amount of citric acid was produced and the concentration of was found to be 4.37%

EXPERIMENT NO: 6

FERMENTATION PRODUCTION OF ETHANOL

INTRODUCTION:-

The overall reaction in fermentation hexose by yeast particularly *Saccharomyces cervisiae* can be expressed as under

$$C_6 H_{12} O_6 \rightarrow 2C_2H_6OH + 2CO_2$$

However other than alcohol certain by products like glycerol succinic acid and acetic acid are produced along with ethyl alcohol. Haiden and Young discovered that free inorganic phosphates disappears during fermentation visually though EMP pathway. Ethyl alcohol is mildly applicable as organic solvent. Which is efficiently produced by grouping yeast saccharomyces cervisiae used in the preparation of inoculation media

PREPARATION OF ALCOHOL ESTIMATION:-

Alcohol reacts with oxidising agent ie: potassium deteriomate and get oxidized to acetic acid. Remaining of the potassium deteriomate will react with iodine; this is a light sensitive process. So incubation is carried out in the dark. Liberated iodine is titrated with 0.1N Sodium thiosulphate. In the process starch is used at an indicator.

SODIUM THIOSULPHATE SOLUTION :- (0.03 mol L^{-1})

Add 7.44g of $Na_2 S_2 . O_3 . 5H_2O$ to a 1L Volumetric flask . Dissolve in distilled water and dilute up to the mark.

POTASSIUM IODIDE SOLUTION:- (1.2mol L^{-1})

Dissolve hg of kg in 25ml of water

Day	Gram staining	PH	Measurement of sugar by cole's method					
			Procedure for hydrolysis	Procedure for estimation of sugar	Pilot reading(PR)	Titration reading (TR)	Sugar (gm)	G% Sugar cons
1	Gram positive		20ml of sample 1ml Hcl → hydrolysis for somin neutralization with $NaHCO_3$	20ml of $K_3(FeCN)_6$ + 5ml of NaOH → boiling + 2drops of M.B With hydrolysis sugar	1.6 18.5	10.2 9.8	19.56 19.59	30.44 80.41

PROTOCOL:-

- Transfer aseptically 10% (50ml) of inoculoum in 500ml of production medium and mix it well.
- From production medium aseptically withdraw approx 20.25ml (measure pH and calculate 9% sugar from sample withdrawn)
- Acid hydrolysis for 30 min (20ml of broth 1ml of conc. HCl in boiling water bath for 30 min + neutralize with $NaHCO_3$)
- Estimate the gram percent of sugar by cote's method.
- After 5 day take recover the alcohol as per procedure.

PROCEDURE FOR COLE'S METHOD:-

- Take 20ml of $K_3(FeCN)_6$ in 250ml flask to add 5ml of NaOH
- Keep it for boiling under direct flame over the mix gauze add 2 drops of methylene blue

REACTION:-

$$K_2C_2O + H_2SO_4 + 2C_2H_5OH \rightarrow 3CH_3OOH + 2K_2SO_4 + 2Cl_2(SO_4)_8$$

REQUIREMENTS:-

- 500ml production medium and a 50ml inoculoum medium.
- pH media and water bath
- Methylene blue (1% w/v)
- $NaHCO_3$ (powder)
- Concentrated HCl
- 0.025 molar $K_2C_2O_2$
- 25% (w/v) KI
- 1% (W/V) starch as indicator
- 0.1N $Na_2S_2O_8$
- 25% $ZnSO_4$
- Staining reagent and glassware

ACID DICHROMATE SOLUTION**:-**

Add 125ml of water to a sulphuric acid with constant burning 100l flask under cold water tap and 0.75g of potassium dichromate. Dilute to 250ml with distilled water.

STARCH INDICATOR SOLUTION: - (1.0% solution)

Dissolve 1.0g of soluble starch in 100ml of recent boiled water. Stir until dissolved.

SODIUM THIOSULPHATE SOLUTION

Add 7.44g of Na2S2O3.5H2O to a 1l volumetric flask. Dissolve in distilled ware and dilute upto the mark.

POTASSIUM IODIDE SOLUTION

Dissolve 5g in 25 ml of water

PROTOCOL

Transfer asceptically 10% of inoculum in 500ml of production medium and mix it well.

From the production medium aseptically withdraw approximately 20.25ml.

Acid hydrolysis for 30min(20ml broth, 1ml conc. HCl in boiling water bath for 30 min+ neutralise with NaHCO3.

Estimate the gram % of sugar by Coli's method.

After 5 days take recover the alcohol as per procedure.

PROCEDURE FOR COLE'S METHOD

- Take 20ml of K2(FeCN)6 in 250ml flask to add 5ml of NaOH.
- Keep it for boiling under flame over wire guaze. Add 2 drops of methylene blue
- Titrate with hydrolysed sugar (note the reading as pr)
- From pilot reading the sample for (if pr is 2.0 then . we have to take 2ml of neutralized sample and make the volume up to 10 with (p/ro) (for 8Tr reading total volume should be more than 80ml
- Again repeat the same procedure frome step 1 and note down the titration reading as TR reading should be between 9.11
- Calculate gram % sugar with the help of sucrose factor
- Purity of the culture is checked by grams staining
- Ph is measured on ph meter
- Gram (w/v)% sugar is estimated

Calculation for % sugar by sucrose factor :-

Sucrose % =19.2+co.036×TRmg /TRmin : TR means titration reading

Procedure for recovery of alcohol :-

- Take 10ml of fermented broth , neutralize mixture of 1m NaoH

- Add 25% ZnSO4 allow it to react for 5-10minutes
- Filter the precipitate protein by filter paper
- Collect the filtrated in distillation unit and set the temperature to 70degre c
    - Collect the distillation and dilute the water

    Procedure for estimation of alcohole by potassium dichromate from fermented broth

    - Take different aliquotes of standard alcohol solution 10.1mg/ml ranging from 0.5-2.5ml
    - Take some aliquots of 1ml from diluted sample of distilled broth
    - Make up the final volume to 5ml by distilled water
    - Add 10ml of 0.025ml k2c407 solution in each tube . allow the tubes to stand for 30 min in adark room
    - Transfer them into 250 ml flask and add 100ml f distilled water to each flask and cover it
    - Add 4ml of 25% (w/v) k7
    - Allow them to stand at room temp for 10 minutes
    - After incubation add 1% starch solution on as indicators and titrate against 0.1 Ma2 s2 o3 until it becomes colour less

S_2	**Ethanol (ml)**	**Ethanol (mg/ml)**	**Distilled Water(ml)**	**0.025m $K_2C_2O_{17}$**	**Incubation**	**Distilled water**	**25% KI(w/v) (ml)**	**1% (w/v)axis (ml)**	**0.1ml $Na_1S_2O_2$ (ml)**
Blank	0	0	5		10	100	4		0.5
S_1	0.5	0.05	4.5	10	10	100	4		0.5
S_2	1.0	0.10	4	10	Somin in dark at room	100	4	10 min room	0.5
S_3	1.5	0.15	3.5	10	temperature it to 250ml	100	4	tempe rature	0.5
S_4	2.0	0.20	3	10	flask	100	4		0.5
S_5	2.5	0.25	2.5	10		100	4		0.5
B	3		2	10		100	4		0.5
D	3		2	10		100	4		0.5

Results:-

Thus the concentration of ethanol was found to be 0.30mg/ml