Semester – III

18MBU313 INDUSTRIAL MICROBIOLOGY – PRACTICAL (4H – 2C)

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

End Semester Exam: 9 Hours

COURSE 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.

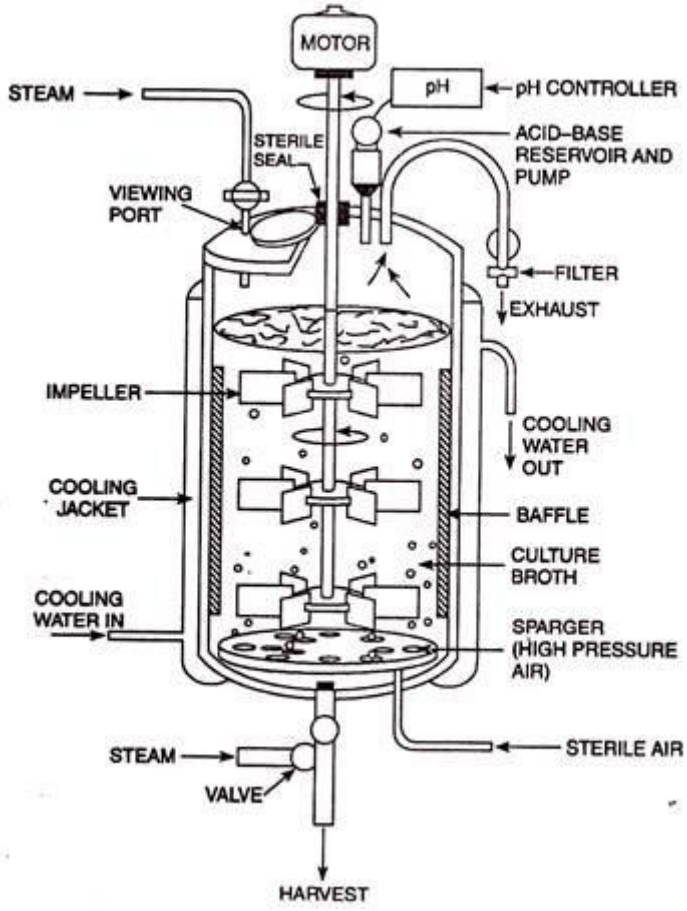
EXPERIMENT NO: 1
Study of different parts of the fermentor

Fermentation:

Fermentation can b defined as a metabolic process where chap a materials such as carbohydrates (sugar) are converted into economically important products like acids, gases and alcohol by microorganisms. This process is carried out in a equipment called as fermentor.

Fermentor:

A fermentor can be defined as a vessel in which sterile nutrient media and pure culture of microorganisms a mixed and process is carried out under aseptic and optimum conditions for the synthesis of desired product.



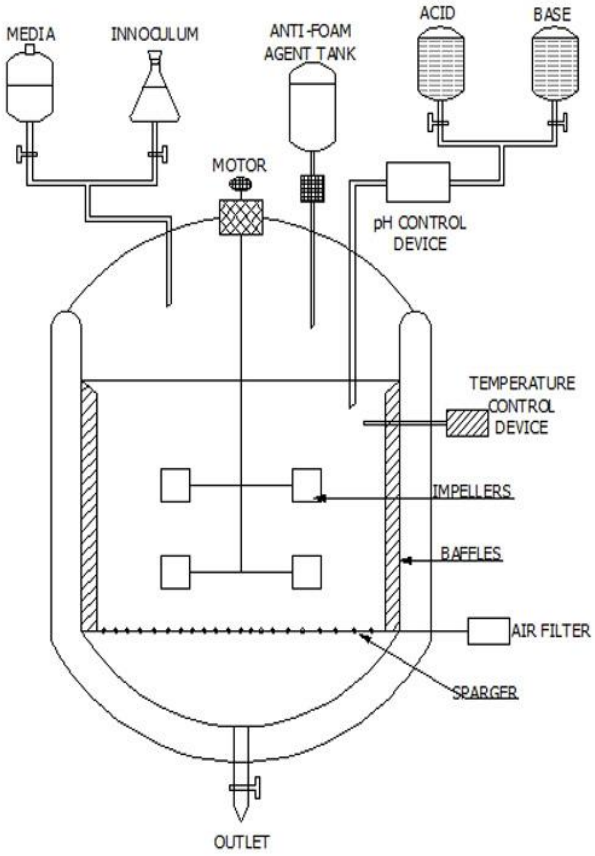


Fig. 1. An industrial fermentor (internal view)

A bioreactor should provide for the following:

- Sterilization
- Temperature
- pH control
- Foam control
- Aeration and agitation
- Sampling point
- Inoculation point
- Drainage point
- Harvesting of product
- Cleaning
- Facility of providing hot and cold water, and sterile compressed air.

Size of Fermentors:

The size of fermentors ranges from 1-2 litre laboratory fementors to 5,00,000 litre or, occasionally, even more, fermentors of up to 1.2 million litres have been used. The size of the fermentor used depends on the process and how it is operated.

Materials used for fermentor:

A fermentor is a large cylinder closed at the top and the bottom and various pipes and valves are fitted into it. The material used for designing a fermentor should have some important features:

- It should not ne corrosive
- It should not add any toxic substance to the fermentation media
- It should have the capacity to tolerate steam sterilization process, high pressure and resist pH change

The material used for fermentor depends on the type of fermentation process. For example, in case of beer, wine and lactic acid fermentation, the fermentor tanks are made up of wooden materials whereas, materials such as iron, copper, glass and stainless steel can be used in most of the fermentation process. Large-scale industrial fermentors are almost always constructed of stainless steel of 304 and 316 and these fermentors are mostly coated with epoxy or glass lining. A fermentor should provide the facility to control and monitor various parameters for successful fermentation process.

Impellers:

The impeller (also called agitator) is an agitating device necessary for stirring of the fermenter. They are mounted on the shaft and introduced in the fermentor through the lid.

The stirring accomplishes two things:

(i) It mixes the gas bubbles through the liquid culture medium and

(ii) It mixes the microbial cells through the liquid culture medium. In this way, the stirring ensures uniform access of microbial cells to the nutrients.

The size and position of the impeller in the fermentor depends upon the size of the fermentor. In tall fermentors, more than one impeller is needed if adequate aeration and agitation is to be obtained. Ideally, the impeller should be 1/3 of the fermentors diameter and the number of impeller may vary from size to size to the fermentor.

Temperature controlling system:

Temperature control device usually contains a thermometer and the fermentor is fitted externally with a cooling jacket through which steam (for sterilization) or cooling water (for cooling) is run. Cooling jacket is necessary because sterilization of the nutrient medium and removal of the heat generated are obligatory for successful completion of the fermentation in the fermentor. For very large fermentors, insufficient heat transfer takes place through the jacket and therefore, internal coils are provided through which either steam or cooling water is run.

Aeration System:

Aeration system is one of the most critical part of a fermentor. In a fermentor with a high microbial population density, there is a tremendous oxygen demand by the culture, but oxygen being poorly soluble in water hardly transfers rapidly throughout the growth medium.

It is necessary, therefore, that elaborate precautions are taken using a good aeration system to ensure proper aeration an oxygen availability throughout the culture. However, two separate aeration devices are used to ensure proper aeration in fermentor. These devices are sparger and impeller.

The sparger is typically just a series of holes in a metal ring or a nozzle through which filter-sterilized air (or oxygen-enriched air) passes into the fermentor under high pressure. The air enters the fermentor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium.

Inoculation port:

Inoculation port is a device from which fermentation media, inoculums and substrate a added in the fermentation tank. Inoculums port should b easy to sterilize.

Sampling point:

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

Baffles:

The baffles are normally incorporated into fermentors of all sizes to prevent a vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the fermentors diameter and attached radially to the walls.

pH control:

pH control sensors are used in fermentor for periodically checking of pH.

Foam controlling system:

If the excessive foaming is not prevented, it results in the leakage of the medium from the lid of the fermentation vessel and hence leads to the contamination of the fermentation medium. A foam sensing device is usually placd on the top of the fermenter. When the foam rises and touches the probe tip, a signal is generated and passes through the circuit of the probe where

antifoam agent (vegetable oil, silicon oil, mineral oil, polyethylene glycol, polypropylene glycol copolymers, etc.,) is released within seconds mechanically or manually.

Bottom Drainage system:

It is an aseptic outlet present at the bottom of fermentor for removal of fermented media and products formed.

Use of Computer in Fermentation:

Computer technology has produced a remarkable impact in fermentation work in recent years and the computers are used to model fermentation processes in industrial fermentors. Integration of computers into fermentation systems is based on the computers capacity for process monitoring, data acquisition, data storage, and error-detection.

Some typical, on-line data analysis functions include the acquisition measurements, verification of data, filtering, unit conversion, calculations of indirect measurements, differential integration calculations of estimated variables, data reduction, tabulation of results, graphical presentation of results, process stimulation and storage of data

EXPERIMENT NO: 2A
Microbial fermentation - Production and estimation (qualitative and quantitative) of amylase

Aim:

To check the qualitative and quantitative production of amylase from microorganisms.

Introduction:

An amylase is an enzyme that catalyses the hydrolysis of starch into sugars. Amylases are one of the enzymes which can be derived from several sources such as plants, animals, bacteria and fungi. Microbial production of amylase is more fruitful than that of other sources like plant or animals, because of the short growth period, biochemical diversity and the ease with which enzyme concentrations might be increased by environmental and genetic manipulation.

The bacterial amylases were derived from *Bacillus subtilis, B.licheniformis, B.amyloliquifaciens, B.stearothermophilus, B.gavealeus, B.mesentericus, B.myocodes, B.polymyxa, B.vulgates, B.aterrimus, B.coagulance, B.cereus, Lactobacillus* sp., *Escherichia coli, Proteus* sp., *Pseudomonas* sp. etc. The thermophilic bacterium *B.stearothermophilus* offers an alternative for commercial production of thermostable α-amylases. Alkaline and thermotolerant amylases were produced by *B.licheniformis* and *B.halodurans*. Efficient amylase-producing fungal species include those of genus *Aspergillus* (*A. oryzae, A. niger, A. awamori, A. fumigatus, A. kawachii,* and *A. flavus*), *Penicillium* species *(P. brunneum, P. fellutanum, P. expansum, P. chrysogenum, P. roqueforti, P. janthinellum, P. camemberti,* and*P. olsonii*), *Streptomyces rimosus, Thermomyces lanuginosus, Pycnoporus sanguineus, Cryptococcus flavus, Thermomonospora curvata*, and *Mucor* sp.

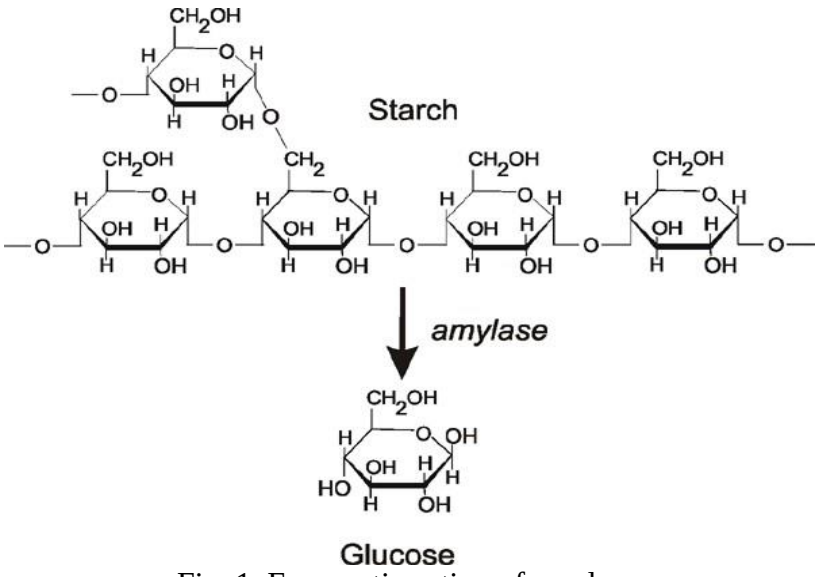


Fig. 1. Enzymatic action of amylase

Gram's Iodine:
Dissolve 1 g of iodine and 2 g of potassium iodide in 300 ml of water.

3,5-Dinitosalicylic acid (DNSA) reagent composition and preparation
Dissolve the following ingredients in 100 mL of distilled water:
Sodium hydroxide (1 g), phenol (2 mL), sodium potassium tatarate (20 g), sodium carbonate (0.05 g) and 3,5-Dinitosalicylic acid (1 g)

Nutrient agar (NA)

Peptone	5.0 g/L
Sodium chloride	5.0 g/L
Yeast extract	1.5 g/L
Beef extract	1.5 g/L
Agar	15 g/L

Amylase production media (APM)

Bacteriological peptone	6.0 g/L
Magnesium sulphate	0.5 g/L
Potassium chloride	0.5 g/L
Soluble Starch	1.0 g/L

Procedure - qualitative method:

- 1 g of soil sample was suspended into conical flask containing 99 mL of sterile distilled water and the sample was serially diluted upto 10^{-7} (10^{-1} to 10^{-7})
- 0.1 mL from 10^{-4} to 10^{-7} dilutions were added to nutrient agar containing 1 % (w/v) soluble starch using spread plate technique
- The plates were incubated at 30 °C for 24 h
- After incubation, individual plates were flooded with Gram's iodine to produce a deep blue coloured starch-iodine complex
- Amylase producing isolates were identified, subcultured and preserved for further experiments

Procedure - quantitative method:
Amylase production:
(I) Seed culture:

- APM was prepared for the production of amylase and sterilized
- A loop full of amylase producing culture was inoculated and incubated for 24 h at 37 °C

(II) Fermentation process:

- APM of 250 mL containing 1 % (w/v) of soluble starch was prepared in 500 mL conical flask and sterilized
- Fermentation media was inoculated with 25 mL of seed culture and incubated in shaker incubator for 24 h at 37 °C
- After incubation, media was subjected for enzyme extraction

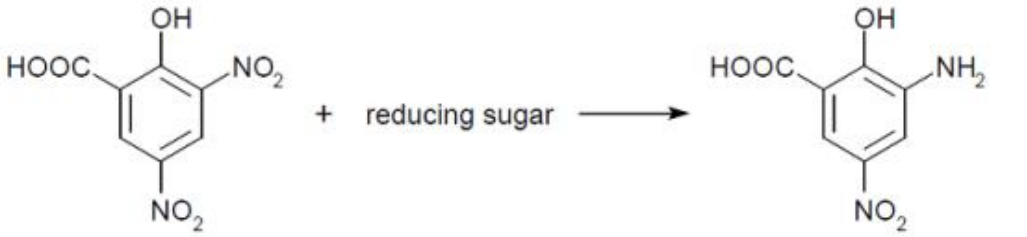
(III) Extraction of enzyme from fermentation broth:
Fermentation broth was centrifuged at 5000 rpm for 20 min and the supernatant

containing crude enzyme was transferred into sterile beaker for further experiments

(IV) Estimation of reducing sugars by dinitrosalicylic acid method:

Principle:

3,5-Dinitosalicylic acid is an aromatic compound that reacts with reducing sugar and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and ketone functional group in fructose. Simultaneously DNSA is reduced to 3-amino,5-Dinitosalicylic acid



3,5-dinitrosalicylic acid(DNS) 3-amino-5-nitro salicylic acid

Procedure:

(a) Preparation of standard graph

- An aqueous solution of glucose at a concentration of 1 mg/mL was prepared.
- Standard glucose solution in the range of 0.2, 0.4, 0.6, 0.8 and 1 mL, were pipette out into 5 separate test tubes.
- A test tube containing a blank solution was also prepared.
- Using distilled water, the volume was raised up to 3 mL in each test tube, including the test tube containing the blank solution.
- 2 mL of DNS reagent was added to each tube and were covered with aluminum foil.
- Contents in the test tubes were heated in a boiling water bath for 15 min.
- Test tubes were cooled to room temperature.
- 1 mL from each test tube was taken and transferred into different cuvettes
- The samples were analysed using colorimeter and the intensity of dark orange red colour as read at 540 nm as the 'absorbance' or OD.

(b) Estimation of reducing sugar from sample

- 5 mL of sample was taken aseptically and centrifuged at 5000 rpm for 10 min.
- 1 mL of supernatant was added to a test tube and the total volume was made up to to 3 mL using double distilled water

- 2 mL of DNSA reagent was added and the sample was heated in boiling water bath for 15 min and cooled
- 1 mL from the sample test tube was taken and transferred into a cuvettes
- The sample were analysed using colorimeter at 540 nm

Observation and result:

Table 1:

Sl. No	Conc. of glucose (mg/L)	Vol of std glucose (mL)	Vol of D/W (mL)	Vol of DNSA (mL)	Vol of D/W (mL)	O.D at 540 nm
1	Blank	Blank				
2		0.2				
3		0.4				
4		0.6				
5		0.8				
6		1.0				
7		Sample (0.5 mL)				
8		Sample (1 mL)				

EXPERIMENT NO: 2B
Microbial fermentation - Production and estimation (qualitative and quantitative) of protease

Aim:

To check the qualitative and quantitative production of Protease from microorganisms.

Introduction:

Proteases are enzymes with highly specialized proteolytic functions. They are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. They not only have several physiological functions and roles in the living beings but are extensively applied enzymes in several sectors of industry and biotechnology.

Protease production is an inherent capacity of all microorganisms and a large number of microbes belonging to bacteria, fungi, yeast and actinomycete are known to produce proteases. Bacteria are the predominant group of alkaline protease producers. Some of the potential alkaline protease producing bacteria are *Bacillus lichiniformis, B. subtilis, B. amyloliquifaciens, Pseudomonas, Flavobacterium, Halobacterium, Vibrio, Serratia, Staphylococcus, Brevibacterium, Alcaligenes*. Among actinomycetes, strains of *Streptomyces, Nocardia* and *Nocardiopsis* are potential ones. In fungi, *Aspergili* is the most exploited group and the strains of *Neurospora, Penicillium, Ophiostoma, Myxococcus, Rhizopus* etc. are common producers of proteases. Only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance.

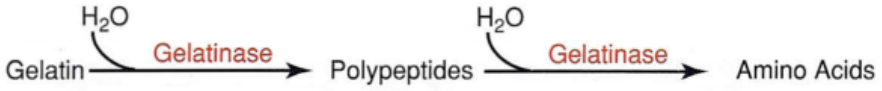


Fig. 1. Enzymatic action of Protease (Gelatinase)

Gelatin agar

Peptone	5.0 g/L
Sodium chloride g/L	5.0 g/L
Agar	15 g/L
Gelatin	1.5 g/L
pH	7.0

Protease production media (PPM)

Gelatin	1.0 g/L
Peptone	10.0
Potassium chloride	0.1 g/L
Magnesium sulphate	0.1 g/L
Dipotassium phosphate	0.5 g/L
Yeast extract	0.2 g/L
pH	7.0

Procedure - qualitative method:

- 1 g of soil sample was suspended into conical flask containing 99 mL of sterile distilled water and the sample was serially diluted upto 10^{-7} (10^{-1} to 10^{-7})

- 0.1 mL from 10^{-4} to 10^{-7} dilutions were added onto gelatin hydrolysis media using spread plate technique
- The plates were incubated at 30 °C for 24 h
- After incubation, individual plates were flooded with Mercuric Chloride ($MgCl_2$)
- Protease producing isolates were identified, subcultured and preserved for further experiments

Procedure - quantitative method:

Protease production:

(I) Seed culture:

- PPM was prepared for the production of protease and sterilized
- A loop full of protease producing culture was inoculated and incubated for 24 h at 37 °C

(II) Fermentation process:

- PPM of 250 mL was prepared in 500 mL conical flask and sterilized at 121 °C for 15 min
- Fermentation media was inoculated with 25 mL of seed culture and incubated in shaker incubator (150 rpm) for 24 h at 37 °C
- After incubation, media was subjected for enzyme extraction

(III) Extraction of enzyme from fermentation broth:

Fermentation broth was centrifuged at 5000 rpm for 20 min and the supernatant containing crude enzyme was transferred into sterile beaker for further experiments

(IV) Estimation of protein by Lowry's method:

Principle: The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex , with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay.

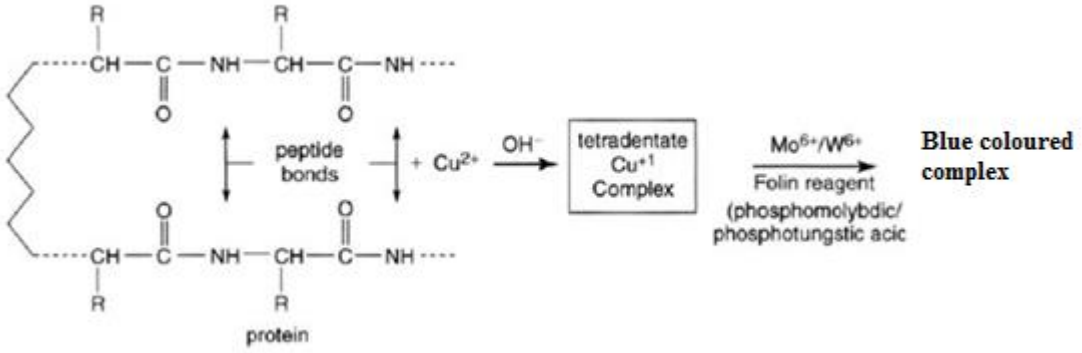


Fig. 2.

Materials required:

1. BSA stock solution (1mg/ml): Dissolve 10 mg in 10 mL of double distilled water.
2. Analytical reagents:

a. 50 mL of 2 % (w/v) sodium carbonate mixed with 50 mL of 0.1 N NaOH solution.
b. 10 mL of 1.5 % (w/v) copper sulphate solution mixed with 10 mL of 2.3 % (w/v) sodium potassium tartarate solution. Prepare analytical reagents by mixing 2 ml of (b) with 98 ml of (a).

3. Folin - Ciocalteau reagent solution (1N): Dilute commercial reagent (2N) with an equal volume of water on the day of use.

Procedure:

- An aqueous stock solution of BSA at a concentration of 1 mg/mL was prepared
- Working solution in the range of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 1 µL, were pipette out into 10 separate test tubes
- A test tube containing a blank solution was also prepared
- Using distilled water, the volume was raised up to 0.2 mL in each test tube, including the test tube containing the blank solution
- 1 mL of alkaline copper sulphate (Alk. $CuSO_4$) reagent was added to each tube and mixed thoroughly and allowed to stand for 10 min at room temperature
- 0.3 mL of FC reagent was added and mixed thoroughly
- All the test tubes were incubated for 60 min
- Optical density of standards and the test samples were measured using spectrophotometer at 660 nm

Observation and result:

Table 1:

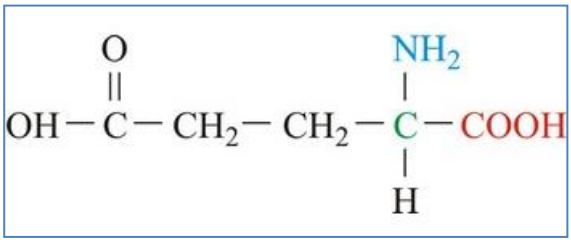
Sl. No	Conc. of BSA (µg/L)	Vol of std BSA (µL)	Vol of D/W (µL)	Vol of Alk. $CuSO_4$ (mL)	Vol of FC reagent (mL)	O.D at 660 nm
B	Blank	Blank	200	1.0	0.3	
1	10	10	190			
2	20	20	180			
3	30	30	170			
4	40	40	160			
5	50	50	150			
6	60	60	140			
7	70	70	130			
8	80	80	120			
9	90	90	110			
10	100	100	100			
S1	-	Sample (100 µL)	100			
S2	-	Sample (200 µL)	-			

EXPERIMENT NO: 3

Microbial fermentation - Production and estimation (qualitative and quantitative) of glutamic acid

Aim: To check the qualitative and quantitative production of glutamic acid from microorganisms.

Introduction: Glutamic acid is a multifunctional amino acid involved in taste perception, excitatory neurotransmission and intermediary metabolism. It plays an important role in gastric phase digestion with multiplicity effects in the gastrointestinal tract when consumed with nutrients by enhancing gastric exocrine secretion. Glutamic acid is also a specific precursor for other amino acids *i.e.*, arginine and proline as well as for bioactive molecules such as γ-amino butyric acid (GABA) and glutathione. GABA possesses several well-known physiological functions (*i.e.*, anti-hypertension and anti-diabetic) and glutathione plays a key role in the protection of the mucosa from peroxide damage and from dietary toxins. At the present time, glutamic acid is largely produced through microbial fermentation because the chemical method produces a racemic mixture of glutamic acid (D- and L-glutamic acid). *Corynebacterium glutamicum* is used for economic production of glutamic acid by submerged fermentation. Other micro organisms found to accumulate significant quantities of glutamic acid are *Corynebacterium lilium, C. callunae, Brevibacterium devaricatum, Br. lactofermentum, Br. saccharolyticum, Br. flayum, Br. immariophilium, Br. roseum* and *Bacillus megaterium.*



Glutamic acid

Screening medium (g/L): Glucose - 5 g, calcium carbonate - 1 g, ammonium sulfate - 1g, KH_2PO_4 - 0.3 g, K_2HPO_4 - 0.7 g, $MgSO_4$ - 0.01 g, FeS_4 - 1.0 mg, Biotin - 10 µg, thymine hydro chloride - 20 µg, pH - 7.0 to 7.2

Production medium (g/L): Glucose - 100 g, calcium carbonate - 20 g, ammonum sulfate - 25 g, KH_2PO_4 - 1 g, $MgSO_4$ - 0.5 g, $FeSO_4$ - 2 mg, $MnCl_2$ - 2 mg, Urea - 500 mg, Biotin - 50 µg, thymine hydro chloride - 200 µg, pH - 7.0 to 7.2

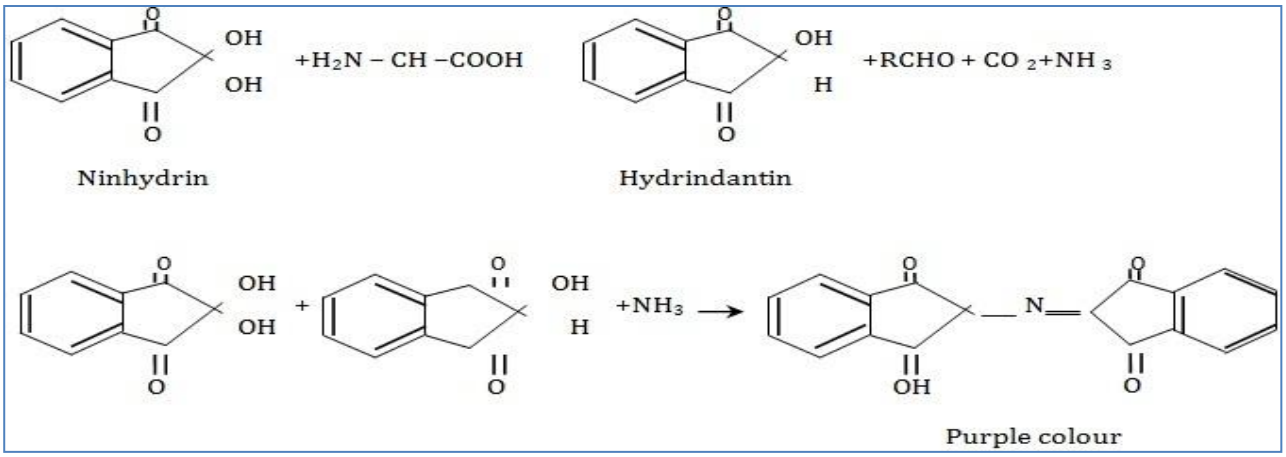
Procedure: **Quantitative estimation**

- All the ingredients were mixed accordingly and calcium carbonate as autoclaved separately and added to the medium

- Selected bacterial colony was inoculated into the production medium and incubated in shaker for 10 days at 37 °C
- After incubation, broth was centrifuged (10,000 rpm for 15 min) and supernatant was collected
- Cell free supernatant was used for the quantitative estimation of glutamic acid

Quantitative estimation of glutamic acid by Ninhydrin method

Principle: Ninhydrin is a powerful oxidizing agent and its presence, amino acid undergo oxidative deamination liberating ammonia (NH_3), carbon dioxide (CO_2) a corresponding aldehyde and reduced form of ninhydrin (hydrindantin). The NH_3 formed from an amino group reacts with another molecule of ninhydrin and hydrindatin to give a deep blue or purple color known as Ruhemann's purple complex. The intensity of the color is directly proportional to the concentration of amino acids in the sample.



Reagents:

Standard amino acid solution: Dissolve 50 mg of amino acid in 50 ml of distilled water in a volumetric flask.

Take 10 ml of standard amino acid solution and make up the volume to 100 ml using distilled water to use as working solution

Ninhydrin reagent: 8.0 g of ninhydrin in 100 ml of acetone.

Solvent: 50 % (v/v) Ethanol

Procedure:

- Desired volumes of standard amino acid solution were pipette into test tubes followed by the distilled water to make up the volume 1 ml

- 1 ml of ninhydrin reagent was added to all the test tubes and covered with aluminum foil to prevent the loss of the solvent due to evaporation
- The contents were mixed and heated for 50 min at 100 °C
- After incubation, the contents were cooled to the room temperature and absorbance at 570 nm were recorded

Table 1:

Sl. No	Vol of std amino acid (mL)	Conc. of aminoacid (µg/L)	Vol of D/W (mL)	Vol of Ninhydrin reagent (mL)	Vol of Solvent (mL)	O.D at 570 nm
B	Blank	Blank	1.0	1.0	5.0	
1	0.1	10	0.9			
2	0.2	20	0.8			
3	0.3	30	0.7			
4	0.4	40	0.6			
5	0.5	50	0.5			
6	0.6	60	0.4			
7	0.7	70	0.3			
8	0.8	80	0.2			
9	0.9	90	0.1			
10	1.0	100	0.0			
T1	Sample-0.1	?	0.9			
T2	Sample-0.5	?	0.5			

Observation and Result:

EXPERIMENT NO: 4

Microbial fermentation - Production and estimation (qualitative and quantitative) of citric acid

Aim: To check the qualitative and quantitative production of citric acid from microorganisms.

Introduction: Citric acid also called as tricarboxylic acid which is found in animal and plants. Citric acid is commercially used in foods, soft drinks, pharmaceuticals, leather tanning, electroplating etc. It is an important organic acid and initially extracted from citrus fruits. Many microorganism including bacteria such as *Bacillus licheniformis, B. subtilis, Corynebacterium spp*., fungi such as *A. niger, A. awamori, A. foetidus* and yeasts such as such as *Candida lipolytica, C. intermedia* and *Saccharomyces cerevisiae* can be used for the production of citric acid. Out of these, *A. niger* which are mutant cannot oxidize citric acid, are used for citric acid production as it grow on wide range of substrate (various carbohydrate rich raw materials such as corn, starch, sugar-beet and molasses are used as some material for sugar), secret citric acid from the mitochondria and the cytosol has led to the massive accumulation of citric acid which was not possible in case of other microorganism. The composition of the culture medium is critical for obtaining high yield of the citric acid. Fermentation is aerobic and can be carried out by submerged culture method.

Czapek-Dox broth (g/L)
Sucrose - 30.0
Sodium nitrate - 2.0
Dipotassium hydrogen phosphate - 1.0
Magnesium sulphate - 0.5
Potasssium chloride - 0.5
Ferrous sulphate - 0.01
Bromocresol green dye - 40.0 ml (1.0 % w/v)
pH – 6.0

Procedure - qualitative method:

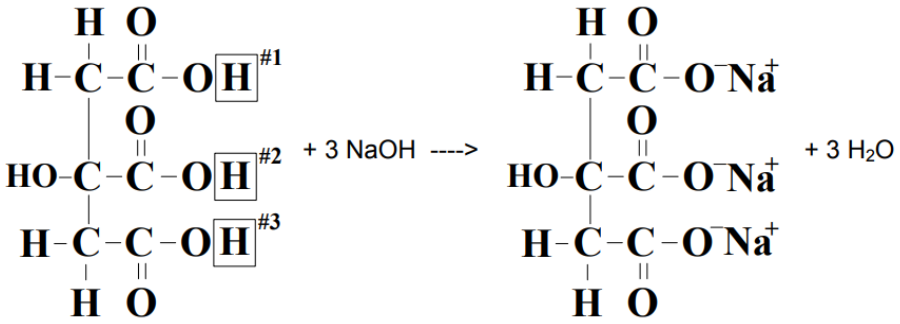
- 1 g of soil sample was suspended into conical flask containing 99 mL of sterile distilled water and the sample was serially diluted upto 10^{-7} (10^{-1} to 10^{-7})
- 0.1 mL from 10^{-4} to 10^{-7} dilutions were added onto Czapek-Dox agar media using spread plate technique
- The plates were incubated at 25 °C for 4-5 days
- After incubation, plates were observed for the formation of yellow zone around the mycelial growth
- Citric acid producing isolates were identified, subcultured and preserved for further experiments

Procedure - quantitative method:

- Czapek-Dox broth was prepared and sterilized
- The medium was inoculated with spores of *A. niger* and incubated on a rotary shaking incubator at 25 °C for 7-10 days
- After incubation, mycelium was filtered using double layered muslin cloth and the amount of citric acid in the filtrate was measured by titrimetric method

Estimation of citric acid by titrimetric method

A titration is a means of quantitative analysis in which the substance to be measured (in a liquid solution) is reacted stoichiometrically with another reagent (called a titrant) until it has completely reacted. The end of the reaction is usually signaled with the appearance of a color from another non-interfering substance called an indicator. In the case of the citric acid titration, when all of the acid has completely been neutralized, the addition of one additional drop of the sodium hydroxide solution causes the solution to become basic. The basic solution will be marked by the appearance of a pinkish color.



Citric acid Sodium citrate

- The filtrate (5.0 mL) obtained was titrated against an alkali (0.1 N NaOH) of known strength using phenolphthalein as the indicator. The end point was the formation of pale pink color. The titration was repeated still the concordant values were obtained
- The volume of alkali used for neutralization was used to find the normality and the percentage of acid is the sample

Observation:

- Solution in the burette: 0.1N NaOH
- Solution in conical flask: 5 mL of filtrate + 2-3 drops of phenolphthalein

Sl. No	Initial burette reading	Final burette reading	Volume of NaOH used
Blank			
T1			
T2			
T3			

Calculations:

Normality of citric acid = [N (NaOH) * V (NaOH)] / V (citric acid)

=

% of citric acid = [Normality * equivalent weight of citric acid * 100] / Volume of filtrate

=

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

Result:

EXPERIMENT NO: 6
MICROBIAL PRODUCTION OF ETHANOL

INTRODUCTION

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

$C_6H_{12}O_6 \rightarrow 2C_2H_6OH + 2CO_2$

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

PRINCIPLE 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 an indicator.

Composition of Media:

Inoculum medium (g/L)

Jaggery 5.0
$(NH_4)_2SO_4$ 0.15
KH2PO4 0.15
Yeast extract 0.50
pH 4.5

Production Media (g/L):

Jaggery 150
KH2PO4 0.15
Yeast extract 0.50
pH 4.5

PROTOCOL:-

- Transfer aseptically 10 % (50 ml) of inoculoum in 500 ml of production medium and mix it well.
- From production medium aseptically withdraw approx 20.25 ml (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 Na2S2O8
- 25% ZnSO4
- 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

• Τρανσφερ ασχεπτιχαλλψ 10% οφ ινοχυλυμ ιν 500μλ οφ προδυχτιον μεδιυμ ανδ μιξ ιτ ωελλ .

• 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 70 °C

 Collect the distillation and dilute the water

Procedure for estimation of alcohol 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 a dark 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 until it becomes color less

Results:-

Thus the concentration of ethanol was found to be