Citric Acid : Structure, Fermentation, Process and Uses in Food | Industries | Biotechnology

Article shared by : Dashmeet

In this article we will discuss about:- 1. Introduction to Citric Acid 2. Chemical Structure of Citric Acid 3. Fermentation Process 4. Uses.

Introduction to Citric Acid:

Citric acid is formed as an intermediate in the Kreb's cycle, but it is accumulated in greater quantities in the fungus Aspergillus niger, may be due to metabolic abnormality. This aspect of the fungus is being exploited for the fermentative production of citric acid. It is also found as a natural product in many fruits specially citrus fruits. Prior to the development of fermentation technology, citric acid was extracted from the juice of these fruits. Mexico contributes 1% of world production of citric acid.

Citric acid can also be produced from glycero, but it is very expensive. Though Aspergillus wentii, A. clavatus, Penicillium divaricatum, P. citrinum, P. luteum, Mucor pyriformis, Citromyces pleffencinus, Candida guilleirmondii, Saccharomyces lipolytica, Trichoderma viride, Arthrobacter paraffinicius and Corynebacterium spp. can produce citric acid, A. niger is employed extensively.

Citric acid production by surface fermentation was started in 1923, while deep fermentation in 1930. Citric acid produced in 1929 was 5000 tons, which has increased to 4.0 lakhs tons by 1992. Sixty percent of citric acid produced is used in food and beverage industry as a flavouring agent and preservative, while 10% in pharmaceutical industry in the form of iron citrate, about 25% of citric acid is used in chemical industry.

Chemical Structure of Citric Acid:

Structurally, citric acid is a hydroxypropane-1, 2, 3 tricarboxylic acid and is shown in Fig. 4.1.

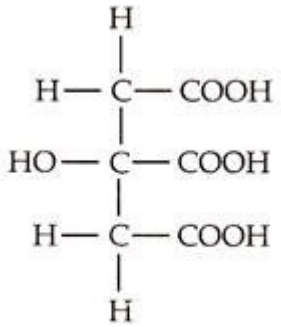

Fig. 4.1: Structure of citric acid

Fermentation Process of Citric Acid:

Various fermentation processes used for the manufacture of citric acid are shown in Fig. 4.2.

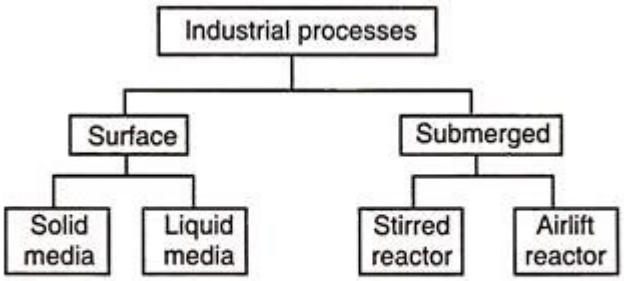

Fig. 4.2: Various fermentation processes for citric acid production

The ratio of sugar consumed to amount of citric acid produced is normally observed in the ratio of 1:1.

Aspergillus niger is employed in most of the processes for the following reasons:

1. Can easily be cultivated.

2. Process uniform biochemical properties.

3. Produces only of small amount of oxalic acid under controlled conditions.

4. Yield large amount of citric acid.

Surface culture process employing solid medium and submerged culture process employing stirred reactor are briefly described here:

1. Surface Culture Process:

Though it is an old process, it is still employed. It is a kind of stationary fermentation process.

This process consists of four phases:

(i) Inoculum production,

(ii) Preparation of medium,

(iii) Fermentation process and

(iv) Harvest and recovery.

(i) Inoculum Production:

Spore suspension is used as inoculum for the production of citric acid. Suitable and high yielding strain of A. niger is selected from a stock culture. The stock culture is inoculated on to the surface of a sporulating medium present in glass bottles. The bottles are incubated for 10-14 days at 25°C.

The composition of trace elements like salts of manganese, zinc or iron in sporulating medium should be suitably maintained, otherwise they will affect the yield of citric acid in the actual fermentation. Suspension of spores is obtained by suspending the grown up spores in a suitable diluents such as

water containing a wetting agent, sodium lauryl sulphate. Besides the total number, the viability of spore crop is critical.

(ii) Preparation of Medium:

The medium, used in the production of citric acid, should have carbohydrate source and inorganic salts. A variety of materials can be used as carbon source. But, generally sucrose and beet molasses are used as carbon source.

Sucrose is the best source of carbon among different organic substances tested. A medium with less than 15% sucrose is reported to give high yield of citric acid. Reduced yield of citric acid is observed when a part of sucrose was substituted by fructose or glucose. Commercially, beet molasses is also extensively used as carbon source in the production of citric acid employing A.niger.

Apart from sugars, beet molasses also contains excessive amounts of inorganic salts. To eliminate these excessive inorganic salts, it is treated with ferrocyanide or ferricyanide before it is employed in the medium preparation. Alternatively, the inorganic salts are removed by passing the beet molasses through a cation exchange resin.

The elements like nitrogen, potassium, phosphorus and magnesium are also needed in the medium, apart from carbon source. They are added in the form of ammonium nitrate, potassium dihydrogen phosphate or potassium monohydrogen phosphate and magnesium sulphate into the medium in minimum quantity as given in table 4.2.

Table 4.2: Composition of citric acid production medium

Chemical	G/l
Sucrose	125 – 150
NH_4NO_3	2.0 – 2.5
KH_2PO_4	075 – 1.0
$MgSO_4 \cdot 7H_2O$	0.20 – 0.25
pH is to be adjusted to 3.4 – 3.5 by adding 4 – 5ml of HCl	

The presence of these elements at higher concentration lowers the yield of citric acid and increases the yield of oxalic acid.

pH of the medium should be adjusted to 3.4-3.5 by using hydrochloric acid. Low pH is reported to be most favourable because it facilitates less contamination, formation of more citric acid, suppression of formation of oxalic acid and easy sterilization of the medium.

Currie (1917) reported a fermentation medium table 4.2 with the following composition for the production of citric acid as the most favourable. Salts and sugars are dissolved in one liter of distilled water. The medium is to be sterilized at $55\text{-}10^3$ to $69\text{-}10^3$ Nm^{-2} steam pressure per square inch for 30 min.

(iii) Fermentation Process:

The production medium is placed in shallow pans in such a way that a thin layer of medium with a depth of 1 to 2.5 cm is formed. The spores of inoculum are added to the medium to keep them floating on its surface. This is achieved by suitable modulating devices. Incubation is done in the incubation chambers at 30-40°C. Figure 4.3 shows the layout of the typical fermentation.

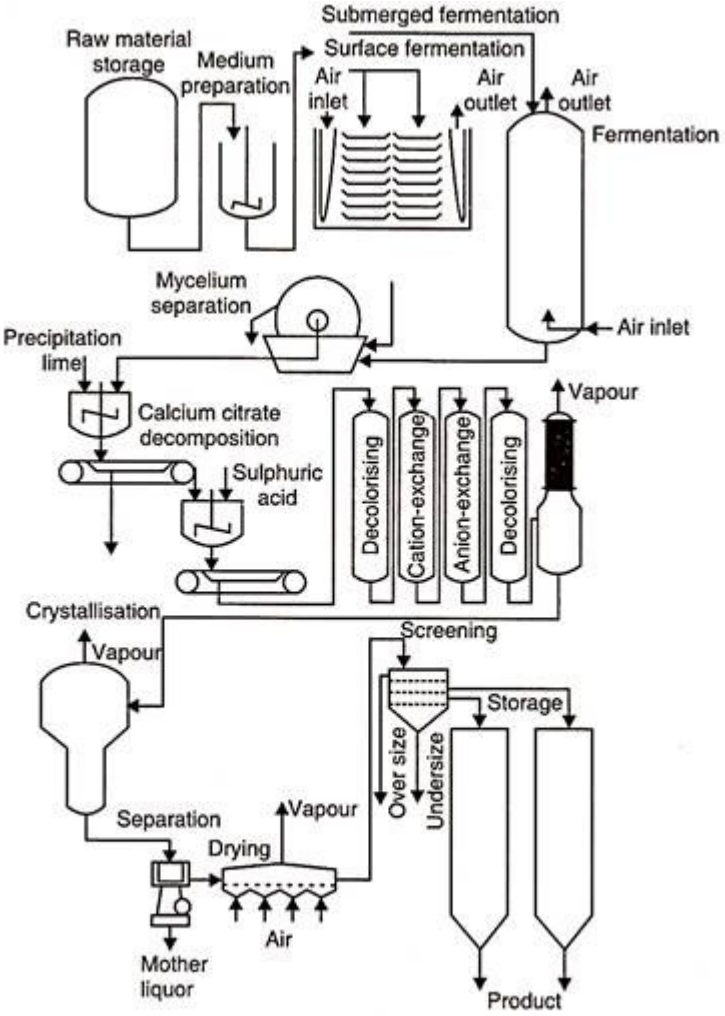

Fig. 4.3: Flow-sheet of citric acid produced by surface or submerged process

The temperature is kept constant at 30°C during the fermentation. Air current ventilation is also important for gas exchange because the rate of citric acid production falls if CO_2 in the atmosphere increases to 10%. Within 24 hrs after inoculation the germinating spore form a thin layer of mycelium on the surface of the nutrient solution. As a result of the uptake of ammonium ions, the pH in the culture liquid fall to 1.5 to 2.0.

After 30 hrs of fermentation, if the iron concentration is more oxalic acid and yellowish pigment is formed which in turn hinders the recovery process. The fully developed mycelium floats as thick convoluted white layer on the liquid medium. The fermentation is stopped after 8-14 days.

The rate of bioconversion of sugar to citric acid depends on the ratio of surface area to the volume of the medium. There will be higher yield of citric acid if the

ratio is lower. In this shallow pan method, the ratio of surface area to the volume of the medium is lower due to which large surface area of the mycelial mat is exposed to shallow layer of the medium.

Under these conditions more and more of sugar is converted into citric acid. That is why this process is considered to be superior to the submerged culture process. Yield per hour from this process amounts to 1.2 – 1.5 kg citric acid monohydrate per square metre of fermentation surface.

(iv) Harvest and Recovery:

The mycelium is separated from the fermentation broth. Any intracellular citric acid present in the mycelium is obtained by pressing the mycelium. The filtered broth is treated with calcium hydroxide. It is filtered and washed. It is then treated with equal volume of sulphuric acid to liberate citric acid. Calcium sulphate is formed as a precipitate in this process.

The precipitate is separated by filtration. An impure solution of citric acid is obtained which is decolorized by treating with activated carbon and also demineralized. Finally pure citric acid crystals are produced by evaporation. It is also recovered alternatively by counter current extraction method.

2. Submerged Culture Process:

In this method A. niger is made to grow uniformly dispersed throughout the liquid production medium. Fermentation is generally carried out in large fermenters having 4klt a capacity of thousands of gallons and are provided with mechanical agitator and sparges. Eighty percent of the world's supply of citric acid is produced by this process. Cost of production decreases by 25% by this method. It involves low labour cost, longer incubation period, more energy consumption and sophisticated techniques.

Three factors are important for production in submerged culture process. They are quality of the metal used for the construction of fermenter, mycelium

structure and oxygen supply. Candida lipolytica, an alkane utilizing fungus can also be employed in citric acid production under continuous fermentation. It yields, 45% higher than normal citric acid production.

(i) Inoculum Production:

Mycelial mats called pellets are used as inoculum for fermentation in this process. Suitable and high yielding strains of A. niger are selected from a stock culture. The spores are induced to germinate in a seed fermenter. A nutrient solution containing 15% sugar from molasses is used in this seed fermenter. To induce the formation of mycelial pellets, cyanide ions are added to the medium.

Pellet formation largely depends upon the concentration of cyanide ions in the medium. Lower yield of citric acid occurs if the cyanide ions are in less concentration. This is because lower concentration of cyanide ions induce formation of normal mycelium instead of pellets. The spores germinate at 32°C and form pellets of 0.2 — 0.5 mm diameter within 24 hrs. During this period the pH falls to 4.3. These pellets are then used as inoculum for production fermenters.

(ii) Preparation of Medium:

The medium, employed for surface culture process is also employed in this process.

(iii) Fermentation Process:

Mostly fermenters used for citric acid production are constructed in the range of 10 — 220 klt. They must be made of stainless steel to prevent leaching of heavy metals. Normal steel, if it is used in the construction of fermenters, at low pH level of 1-2 may inhibit the formation of citric acid. Small fermenters with a capacity upto 1000 lt should have plastic lining even though they are

made of stainless steel because of large surface/volume ratio. However, such plastic lining is not necessary for large stainless steel fermenters.

The mycelial pellets developed in the seed tank are transferred aseptically to the fermenters and incubated at a constant temperature, 30°C. The structure of the mycelium that forms in the fermenter is vital to a successful production process. Little citric acid is produced if the mycelium is loose and filamentous with limited branches and no chlamydospores.

Optimal citric acid is formed if the mycelium is in the form of pellets. The ratio of iron to copper in the medium determines the nature of mycelium. In some cases, production fermenters are inoculated directly with spores.

Although A. niger requires relatively little oxygen, it is sensitive to oxygen deficiency. There must be minimum oxygen concentration of 20 to 25% of the saturation value throughout the fermentation process. Short interruptions in the oxygen supply ceases the production irreversibly. The aeration rate should be 0.2-1.0 volume per min. during the acid production phase. Due to low viscosity, stirring is not necessary. Thus, although some plants use stirred fermenters, airlift reactors can also be used.

Foaming is a problem in the submerged culture process. However, it can be controlled by adding antifoam agents such as lard oil at frequent intervals. A foam chamber, $1/3^{rd}$ the size of the fermenter volume, is needed in both airlift and stirred bioreacters. Mechanical antifoam devices can also be used. Progress of the fermentation process is monitored regularly by calculating the content of sugar and citric acid in the fermentation.

Uses of Citric Acid in Industries:

Industrially, citric acid is used in the following ways:

1. It is used in the production of carbonated beverages.

2. As a chelating and sequestering agent in the tanning and textile industry.

3. Citrate esters are used as plasticizer.

4. It is abundantly used in food industry as an acidulent in the preparation of food items like jams, preserved fruits and fruit juices etc.

5. It is used in frozen foods to prevent its change in colour and flavour.

6. Metal painting industry

7. In pharmaceutical industry

8. In the manufacture of astringent, hair rinsers and hair setting fluids.

9. In beverage industry as a preservative to prevent oxidation of alcohol, emulsifier of dairy products like cheese and ice creams.

10. It is used as preservative and to prevent change in colour, flavour and in the oxidation of alcohol.

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Fermentation of Penicillin | Antibiotics | Biotechnology

Article shared by : Geetha H

In this article we will discuss about the fermentation of penicillin.

Requirements:

1. Starter cultures on M_2 Agar.

2. **M_2 agar (pH 7.0):**

Glycerine	10.0	g
Glucose	10.0	g
NaCl	10.0	g
Yeast extract	5.0	g
KH_2PO_4	0.5	g
$MgSO_4.7H_2O$ trace	0.062	g
Agar	20.0	g
Distilled water	1000.0	ml

3. **Seed medium:**

Sucrose	20.0	g
Corn steep liquor	10.0	g
$NaNO_3$	3.0	g
KH_2PO_4	1.2	g
$MgSO_4.7H_2O$ trace	0.062	g
$CaCO_3$	5.0	g
Distilled water	1000.0	ml

4. **Fermentation medium:**

Peanut meal	25.0	g
Lactose	50.0	g
Sodium thiosulphate	1.0	g
Corn steep liquor	100.0	g
K_2HPO_4	1.2	g
$MgSO_4.7H_2O$ trace	0.062	g
Phenyl acetic acid	1.5	g
$CaCO_3$	8.0	g
Distilled water	1000.0	ml

Dissolve 1.5 g of phenyl acetic acid in warm distilled water. Adjust pH to 7 and make up the volume to 100 ml. Add 1 ml of this to the medium.

5. Flasks.

6. Shaker.

Procedure:

1. Grow P. chrysogenum on M_2 agar for 120—144 hours at room temperature (28°-30°C).

2. Make a suspension of spores and inoculate 10 ml of it on seed medium in Erlenmeyer flasks with 500 ml capacity.

3. Incubate at room temperature (28°— 30°C) on a shaker (240 rpm) for 48-72 hours seed.

4. Inoculate this seed at the rate of 10% (V/V) to 45 ml of fermentation medium in 500 ml Erlenmeyer flasks (45 and medium + 5 ml seed).

5. Incubate at room temperature (28°- 30°C) for 120 hours on a rotary shaker (240 rpm).

6. Check the pH of the medium first before inoculation (pH will be around 7.0-7.5). Adjust it to 5.2 using 20% H_3PO_4.

7. After fermentation filter the broth and use the filtrate for chemical and biological assays.

Chemical Assay— Iodimetric Method:

Principle:

Active penicillin molecule does not absorb iodine. Alkali inactivation product, Na penicillate does absorb I_2. Penicillin G inactivated with alkali absorbs nine atoms of I_2 per penicillin molecule. Residual I_2 is estimated with sodium thiosulphate.

Requirements:

1. Standard curve.

2. 1(N) NaOH (40 g NaOH in 1000 ml distilled water.).

3. I_2 solution 0.1 (N1).

12-12-7 g

KI-18.0 g

Grind I_2 and KI in a mortar and pestle. Add little water in it and remove the solution. Add, little more water and grind. Repeat the process till all the I_2 and KI are brought into solution. Make up the volume to 1000 ml and keep in brown bottle. Take 10 ml of this and make it upto 100 ml = N/58 I_2.

4. Sodium thiosulphate 0.1N-24.3g. $Na_2S_2O_3$ in 1000 ml distilled water + 1 ml chloroform as preservative.

5. Starch solution: lg starch powder—boil in 100 ml water and keep it at low temperature.

6. Methyl orange 0.04% in 20% ethanol.

7. Buffers for penicillin.

$P0_4$ buffer pH 6.0

KH_2P0_4-5.81g + lN NaOH-6.7ml made upto 1000 ml. Check pH on pH meter.

Acetate buffer pH 4.5

Na acetate-5.44 g + Glacial acetic acid – 2.4 ml made upto 1000 ml.

8. 1.2N HCl-100 ml conc. HCl 12(N) made upto 1000 ml.

9. Titration apparatus.

10. Test tubes.

11. Flasks.

Procedure:

1. For standard curve, dissolve the contents of Penicillin G from a vial (5,00,000 units) in 50 ml phosphate buffer (pH 6.0). One ml of this solution has 100,00 units of penicillin.

2. Prepare dilutions of penicillin G. as follows:

1) Total units in the vial=500,000 units

2) Solution of this in 50 ml PO_4 buffer = 500,000/50 = 10,000 units/ml

3) 1 ml of dilution (2) + 9ml PO_4 buffer 10000/10 = 1000 units/ml (stock)

4) 1 ml of dilution (3) + 99 ml PO_4 buffer = 1000/100 = 10 units/ml (working stock)

5) 0.5 ml of dilution (4) + 9.5 ml PO_4 buffer = 5/10 = 0.5 units/ml (working stock)

6) 0.7 ml of dilution (4) + 9.3 ml PO_4 buffer = 7/10 = 0.7 units/ml

7) 1.0 ml of dilution (4) + 9.0 ml PO_4 buffer = 10/10 = 1.0 units/ml

8) 1.2 ml of dilution (4) + 8.8 ml PO_4 buffer = 12/10= 1.2 units/ml

9) 1.5 ml of dilution (4) + 8.5 ml PO_4 buffer = 15/10 =1-5 units/ml

10) 2.0 ml of dilution (4) + 8.0 ml PO_4 buffer = 20/10 = 2.0 units/ml

Add reagents to known and unknown samples as shown below.

	Penici. soln ml	Acet buff pH 4.5 ml	IN NaOH ml		1.2N HCl ml	1N NaOH ml	Acet buff pH4.5 ml	N/50 ±2 ml		N/100 $Na_2S_2O_3$ (in bure.) ml	Diff. B-E
B_S	1	5	–	incu-	2	2	–	5	stopper		
E_S	1	–	2	bate	2	–	5	5	tubes		
B_T	1	5	–	for 15	2	2	–	5	incubate		
E_T	1	–	2	minutes	2	–	5	5	for 15 min at R. temp		

B_S = Blank standard.

E_S = Experimental standard.

B_T = Blank test (fermented broth).

E_T = Experimental test (fermented broth).

$$\text{Penicillin factor: } F=\frac{\text{wt. of penicillin powder in mg} \times \text{potency}}{B_S - E_S \times \text{volume of standard}} = \frac{65 \times 1592}{B_S - E_S \times 50} = X$$

Penicillin in the fermented broth = $B_T - E_T \times F(=x) = Y$ units/ml.

Label these dilutions and use for chemical and biological assay.

Bioassay-Diffusion Method:

Requirements:

1. Test organism-Bacillus subtilis.

2. Fermented broth.

3. Standard graph of penicillin with diameter of zone of inhibition against units of penicillin.

4. Petri plates.

5. Bioassay medium butts.

6. Cork borer.

7. Glass marking pencil.

8. Incubator.

9. Base agar (any fungal medium).

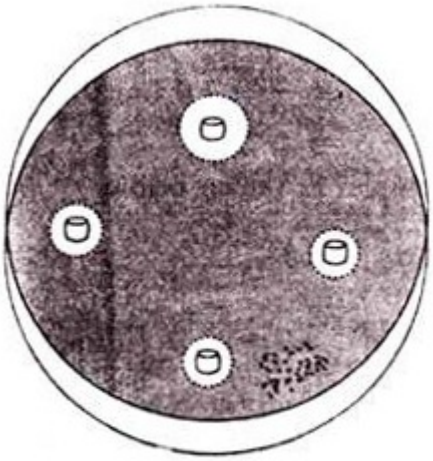

Fig. 2.6: Bioassay

Procedure:

1. Pour base agar into sterile Petri plates and allow it to solidify.

2. Inoculate B. subtilis into sterilised cooled (to 45°C), 10 ml medium in tubes, mix well by rolling it gently between the palms.

3. Pour it to the surface of the base agar, allow it to solidify.

4. With a sterile cork borer punch holes in the agar medium (3—4) in such a way that the zones of inhibition should not overlap.

5. Add different dilutions of standard penicillin along with fermented broth into the wells using a micropipette and label respective concentrations.

6. Keep the plates in a refrigerator (4°C) for an hour to allow even diffusion of the test solutions into the surrounding agar medium.

7. Incubate at 37°C for 24-48 hours and measure the diameter of the zones of inhibition.

8. Prepare a graph with concentration of penicillin against the diameter of the zone of inhibition. From this find out the concentration of penicillin in the fermented broth.

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College of Science

Determination of Ethanol Concentration in Aqueous Solutions

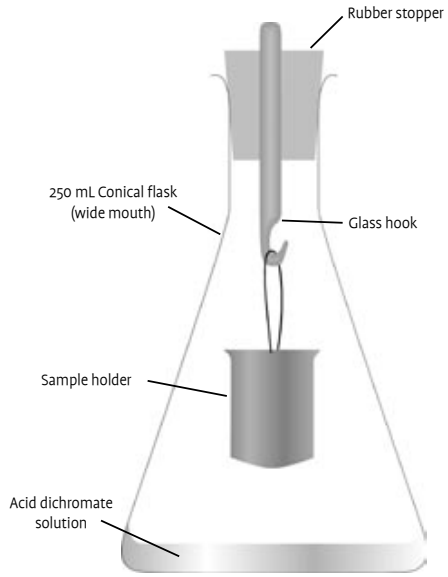
Safety

Lab coats, safety glasses and enclosed footwear must be worn at all times in the laboratory.
The acid dichromate solution needs to be prepared with care. Any concentrated acid spills must be cleaned up by **very** carefully diluting with water before wiping up. Take care to put the **water in the flask first** before adding the acid, and add the acid slowly with constant swirling. The flask will get quite hot.

Introduction

This method uses a redox titration to find the concentration of ethanol in an aqueous solution. The ethanol is oxidised to ethanoic acid by reacting it with an excess of potassium dichromate in acid.

$$2\ Cr_2O_7^{2-} + 16\ H^+ + 3\ C_2H_5OH \rightarrow 4\ Cr^{3+} + 11\ H_2O + 3\ CH_3COOH$$



The amount of unreacted dichromate is then determined by adding potassium iodide solution which is also oxidised by the potassium dichromate forming iodine.

$$Cr_2O_7^{2-} + 14\ H^+ + 6\ I^- \rightarrow 2\ Cr^{3+} + 3\ I_2 + 7\ H_2O$$

The iodine is then titrated with a standard solution of sodium thiosulfate and the titration results are used to calculate the ethanol content of the original solution.

$$2\ S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2\ I^-$$

Because alcoholic beverages such as wine or beer contain other oxidisable substances that could interfere with the titration, the dichromate solution is placed in a flask and the alcoholic beverage sample is suspended in a small container above it (see diagram). The water and ethanol slowly evaporate and as the ethanol comes in contact with the dichromate it first dissolves, and is then oxidised. More ethanol evaporates until eventually all the ethanol from the beverage has left the sample and reacted with the dichromate. Since this transfer takes time, it is necessary to leave the flask with the suspended sample in a warm place overnight.

Equipment Needed

250 mL conical flasks with rubber stoppers

burette

5 mL beakers or small glass vials

beer or wine sample

10 mL and 1 mL pipettes

incubator (optional)

Solutions Needed

Acid dichromate solution: (0.01 $molL^{-1}$ in 5.0 $molL^{-1}$ sulfuric acid) *(see safety notes)*. Add 125 mL of water to a 500 mL conical flask. Carefully add 70 mL of concentrated sulfuric acid with constant swirling. Cool flask under cold water tap and add 0.75 g of potassium dichromate. Dilute to 250 mL with distilled water.

Starch indicator solution: (1.0% solution) Dissolve 1.0 g of soluble starch in 100 mL of recently boiled water. Stir until dissolved.

Sodium thiosulfate solution: (0.03$molL^{-1}$). Add 7.44g of $Na_2S_2O_3.5H_2O$ to a 1L volumetric flask, dissolve in distilled water and dilute up to the mark.

Potassium iodide solution: (1.2$molL^{-1}$) Dissolve 5 g of KI in 25 mL of water.

Method

Sample Preparation

1. Dilute beer samples 1:20 (10 mL in 200 mL) with distilled water.
2. Dilute wine samples 1:50 (20 mL in 1000 mL) with distilled water.

Titration (described for one beverage)

1. Transfer 10 mL of the acid dichromate solution *(see safety notes)* to a 250 mL conical flask with matching rubber stopper.
2. Pipette 1mL of the diluted beverage sample into the sample holder. This can be a 5 mL beaker or glass vial. Prepare three samples of the beverage as the entire contents of the flask are used in the titration.
3. Suspend the sample holder over the dichromate solution and hold in place with the rubber stopper (see figure 1).
4. Store the flask overnight at 25–30°C (an incubator is ideal).
5. Next morning allow the flask to come to room temperature, then loosen the stopper carefully and remove and discard the sample holder.
6. Rinse the walls of the flask with distilled water, then add about 100 mL of distilled water and 1 mL of potassium iodide solution. Swirl to mix.
7. Prepare 3 blank titrations by adding 10 mL of acid dichromate solution to a conical flask, adding 100 mL of water and 1 mL of potassium iodide solution and swirling to mix.
8. Fill a burette with sodium thiosulfate solution and titrate each flask with sodium thiosulfate. When the

Figure 1 Experimental setup for oxidation of ethanol. Conical flask contains yellow acid dichromate solution and is sealed with rubber stopper. Small beaker containing beverage sample is suspended above from hook in rubber stopper.

Figure 2 Titration of the iodine formed. The left flask shows the brown-coloured solution resulting from the formation of iodine. The right flask shows how the brown colour fades to pale yellow as the iodine is titrated with thiosulfate (this is the stage at which starch solution should be added).

Figure 3 Upon addition of starch the solution takes on a blue-black colour due to the formation of a starch-iodine complex.

Figure 4 As more thiosulfate is added and we near the titration endpoint, the blue-black colour from the starch-iodine complex fades.

Figure 5 The endpoint of the titration is reached when just enough thiosulfate is added to react with all the iodine present and the solution becomes colourless.

brown iodine colour fades to yellow (figure 2), add 1mL of starch solution and keep titrating until the blue colour disappears
(figures 3–5). Titrate the blank flasks first, and repeat until concordant results are obtained (titres agreeing to within 0.1 mL). Then titrate each of the alcohol samples. If the three samples of the beverage do not give concordant results, further samples will need to be prepared.

Result Calculations

The blank titration tells you how much acid dichromate was present at the start. As no alcohol was added the full amount of the dichromate is still present. The blank titrations are carried out so the result can be compared with those of the sample titrations.

1. Determine the average volume of sodium thiosulfate used for your sample from your concordant sample results.
2. Determine the average volume of sodium thiosulfate used for the blank titration from your concordant blank results.
3. Subtract the volume of the sodium thiosulfate solution used for the sample titration from the volume used for the blank titration. This volume of the sodium thiosulfate solution is now used to determine the alcohol concentration.
4. Calculate the number of moles of sodium thiosulfate in this volume.
5. Using the equations, determine the relationship between the moles of sodium thiosulfate and the moles of ethanol.
   – as 6 mol of $S_2O_3^{2-}$ is equivalent to 1 mol of $Cr_2O_7^{2-}$
   – and 2 mol of $Cr_2O_7^{2-}$ is equivalent to 3 mol of C_2H_5OH
   – then 1 mol of $S_2O_3^{2-}$ is equivalent to 0.25 mol of C_2H_5OH
6. Use this ratio to calculate the moles of alcohol in the sample solution.
7. Remember to allow for the dilution factor
   eg. if the dilution was 1:20 the result needs to be multiplied by 20.
8. Convert the answer in moles per litre to percentage (grams per 100mL) to compare with the figure given on the bottle of the alcoholic beverage tested.

Contact Us

If you have any questions or comments relating to this experiment, please contact us. Please note that this service is for senior school chemistry students in New Zealand only. We regret we are unable to respond to queries from overseas.

Outreach
College of Science
University of Canterbury
Private Bag 4800
Christchurch
New Zealand
Phone: +64 3 364 2178
Fax: +64 3 364 2490
Email: outreach@canterbury.ac.nz
www.outreach.canterbury.ac.nz

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Estimation of Alcohol by Different Evaluative Methods and Comparisons in Estimated Results of Various Methods

Madhuri Tupe[1], Amol Pawar[2], Nikhil Pawar[3]

[1,2]*Assistant Professor, Dept. of Chemistry, S. M. Joshi College, Maharashtra, India*
[3]*Post Graduate Student of Organic Chemistry, Dept. of Chemistry, A. M. College, Maharashtra, India*

Abstract - *Different methods of preparation are used but the ethanol obtained is initially obtained in admixture with water. From this solution ethanol is obtained by fractional distillation. Ethanol cannot be separated completely by distillation as its boiling point is significantly lower than water. Instead, an azeotropic mixture (i.e. mixture of 95% ethanol and 5% water) is obtained, and the boiling point of the azeotrope is 78.15°C. In a distillation, the most volatile material (i.e. the material that has the lowest boiling point) is the first material to distill out from the distillation flask, and this material is the azeotrope of 95% ethanol, which has the lowest boiling point. If an efficient fractionating column is used, first 95 % alcohol is obtained, then a small intermediate fraction of lower Concentration, and finally water- No matter how efficient the fractionating column used, 95° cannot be further concentrated by distillation. So we have used four different methods which are easily available and cost effective. The four methods used for estimations are Potassium dichromate Method, Specific Gravity Method, Sikes Hydrometer Method and Gas Chromatography Method and evaluated the results.*

Key Words: **Azeotropic Mixture**, Dichromate Method, Pycnometer, Gas Chromatography, Alcohol, Sikes Hydrometer.

1. INTRODUCTION

As we know that a azeotropic mixture is a mixture that cannot be separated from a simple distillation as when the mixture the boiled the vapour contains the same amount of the vapour of both the component present in the mixture so we have researched different cost effective methods by which the percentage of alcohol present in the azeotropic mixture. As this research is an important part of the beverage industry and can be used to find the percentage of alcohol in various beverages such as wines beers etc. In alcohol industry it has a wide scope for determination of percentage in this azeotropic mixture. The low cost methods which we have included are Potassium Dichromate Method, Gas Chromatography Method, Pyconometer and Sike's Hydrometer. We have further compared the results using different parameters to find out the better method for evaluation of the amount of alcohol present in the azeotropic mixture.

2. Materials and methods

All chemicals were used of A.R. grade. The methods used for analysing the amount of alcohol are potassium dichromate method, gas chromatography method, pyconometer and sike's hydrometer.

Experimental Methods:

A) Potassium dichromate Method: About 34 grams of potassium dichromate is dissolved in 500 ml distilled water in a one liter volumetric flask. The volumetric flask is placed in an ice container and 325 ml of conc. H_2SO_4 is added drop wise so that minimum heat is generated. The solution is thoroughly mixed, cooled and the made volume to1 liter with distilled water. Pipette out 1 ml sample in volumetric flask followed by 10ml dichromate reagent. The flask 15 incubated at 60˚C for 20 min. in a water bath and the mixture is cooled. Volume is made up to 50 ml using distilled water. The linearity curve plot by taking concentration from 1 to 10 % ethanol (v/v) the blank solution was prepared with distilled water. The amount of ethanol in the test sample is determined by UV from the linearity Curve plotted at 620 nm.

B) The Specific Gravity Method: The liquid was taken in the specific gravity bottle and the temperature of the liquid was measured then the liquid was completely filled in the bottle and the weight was measured for further calculations.

C) Sike's Hydrometer Method: The azeotropic mixture was taken in the cylinder and exact temperature of the liquid was measured. Spindle was selected likely to be the jar if released carelessly. Impress the measuring cylinder, depress it to the top mark on the scale, shaking free any adherent bubble from it and released gently. A proper spindle was floating at a point with divisions on its steam. Surface of the spirit was brought to the eye level and noted down the division that is cut by surface on seen from below. This was indication of the surface of liquid between any two steam divisions; the division nearest below the surface (seen from below) was recorded as the indication. To find out the strength of spirit was referred to spirit table for use with Sike's hydrometer. Opposite the indication in the table for the recorded temperature will refound this spirit strength.

D) Gas Chromatographic: Instrument Column: Hewlett Packard 6890 plus Gas Chromatograph Porapaq-Q (6 ft. X I/8"-ss packed column), Injector Temperature: 150°C, Injector: Splitless mode, Carrier gas: Hydrogen 20 ml/min

Detector: TCD, Injection volume: 0.4 µL. Samples of different concentration viz. 2%, 4% 6% 8% 10% of alcohol was taken to determine the exact concentration on different experimental methods to get the results.

3. Observations and Results

RESULT TABLE NO.1 – Distilled std. sample of various concentration ranges.

2% standard ethanol sample (distilled)

ALCOHOL %	SP. GRAVITY	G.C. METHOD	SPECTRO (DICHROMATE)		SIKES HYDROMETER	
			587nm	620nm	TEMP	FAHRENHEIT
1	2.14	1.84	2	2.03	0.74	1.14
2	2.12	1.88	1.98	2.07	0.7	1.08
3	2.12	1.85	1.95	2.03	0.74	1.14
MEAN	2.12	1.86	1.98	2.04	0.73	1.12
STDEV	0.012	0.021	0.025	0.023	0.023	0.035
CV	0.543	1.121	1.273	1.130	3.178	3.093

4% standard ethanol sample (distilled)

ALCOHOL %	SP. GRAVITY	G.C. METHOD	SPECTRO (DICHROMATE)		SIKES HYDROMETER	
			587nm	620nm	TEMP	FAHRENHEIT
1	3.6	3.94	4.02	4.07	2.57	2.91
2	3.58	3.91	4.15	4.2	2.68	2.99
3	3.6	3.88	4.12	4.16	2.58	2.88
MEAN	3.59	3.91	4.10	4.14	2.61	2.93
STDEV	0.012	0.030	0.068	0.067	0.061	0.057
CV	0.321	0.767	1.662	1.607	2.331	1.943

6% standard ethanol sample (distilled)

ALCOHOL %	SP. GRAVITY	G.C. METHOD	SPECTRO (DICHROMATE)		SIKES HYDROMETER	
			587nm	620nm	TEMP	FAHRENHEIT
1	5.4	5.89	6.02	6.08	4.51	4.98
2	5.32	5.82	5.85	5.95	4.38	4.82
3	5.4	5.77	5.95	6.02	4.58	4.9
MEAN	5.37	5.83	5.94	6.02	4.49	4.90
STDEV	0.046	0.060	0.085	0.065	0.101	0.080
CV	0.860	1.035	1.438	1.081	2.260	1.633

8% standard ethanol sample (distilled)

ALCOHOL %	SP. GRAVITY	G.C. METHOD	SPECTRO (DICHROMATE)		SIKES HYDROMETER	
			587nm	620nm	TEMP	FAHRENHEIT
1	7.79	7.84	7.6	7.61	6.3	6.7
2	7.89	7.89	7.96	7.8	6.57	6.968
3	7.85	7.77	7.77	7.52	6.4	6.84
MEAN	7.84	7.83	7.78	7.64	6.42	6.84
STDEV	0.050	0.060	0.180	0.143	0.137	0.140
CV	0.642	0.769	2.316	1.870	2.125	2.047

10% standard ethanol sample (distilled)

ALCOHOL %	SP. GRAVITY	G.C. METHOD	SPECTRO (DICHROMATE)		SIKES HYDROMETER	
			587nm	620nm	TEMP	FAHRENHEIT
1	9.7	9.98	9.34	9.55	8.4	8.96
2	9.6	10.05	9.69	9.89	8.55	9.13
3	9.7	9.89	9.69	9.89	8.38	8.96
MEAN	9.67	9.97	9.57	9.78	8.44	9.02
STDEV	0.058	0.080	0.202	0.196	0.093	0.098
CV	0.597	0.804	2.111	2.008	1.100	1.089

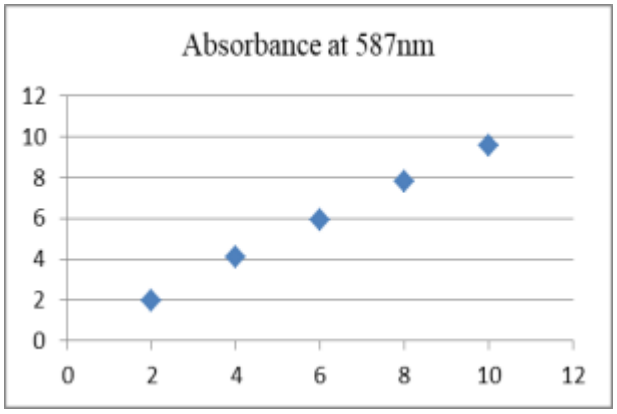

Chart -1: Graph of Absorbance vs. % alcohol at 587 nm

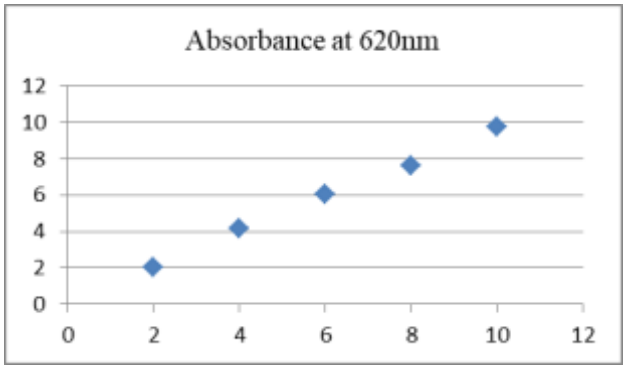

Chart -1: Graph of Absorbance vs. % alcohol at 620nm.

The t-TABLE:-

Results Table:

Comparative t-TEST TABLE of specific gravity, G.C., Sikes spectrophotometric methods for samples for understanding the relation between two tests taken for analysing the % of alcohol.

A

	SP. GRAVITY	G.C. METHOD
ALOCOHOL%	X1	X2
1	96.06	100.29
2	96.06	100.44
3	96.00	99.74
4	95.74	96.51
5	95.69	100.00
6	95.74	98.20
MEAN	95.88	99.20
STD	1.0973	
t-test	5.2343	
t-table	2.228	

B

	SP. GRAVITY	SPECTROPHOTOMTER
ALOCOHOL%	X1	X2
1	96.06	97.81
2	96.06	97.54
3	96.00	97.81
4	95.74	99.00
5	95.69	98.50
6	95.74	99.00
MEAN	95.88	98.28
STD	0.471	
t-test	80823	
t-table	2.228	

C

	SP. GRAVITY	SIKES HYDROMETER
ALOCOHOL%	X1	X2
1	96.06	96.56
2	96.06	96.50
3	96.00	96.50
4	95.74	96.38
5	95.69	96.30
6	95.74	96.38
MEAN	95.88	96.44
STD	0.141	
t-test	6.859	
t-table	2.228	

3. CONCLUSIONS and DISCUSSION

The table T (a), T(b) and T-(c) gives the comparative data between Sp. Gravity, method by Spectrophotometer (potassium dichromate) method, G.C. method, Sike's hydrometer method by the t-Test method . We found that the calculated value is more than the table value, therefore hypothesis are, hence it likely to have the variables in the alcohol determination methods. Then the T-a) is much better than T-b) & T-c) by the t-Test method.

Using t-test, it is observed that for higher concentration, alcohol estimation by specific gravity & G.C.method shows significant difference at 5 % level, because calculated t-value equal to 5.23 is greater than table-t value i.e. 2.228 at 5 % level of significant for 10 ° freedom & alcohol estimation determined by Specific Gravity Method gives average value of 95.88 % which is closed to 96%. Hence Specific Gravity Method is the good method than G.C.Method for alcohol estimation for higher concentration.

CONCLUSION

1) In the given dichromate method the reported ƛ-max is 620nm and Department of Viticulture and Enology, University of California, Davis, California 95616 reference wavelength reported 620nm but actually, We found at it gives 587nm.we took reading at both wavelength i.e. 587nm & 620nm. The plotted linearity graph gives correct or accurate result at 587 run rather than 620nm.

2) Conclusion: Sike's Hydrometer gives the less % of alcohol in Low concentration. As compare to G.C. as well as Specific gravity method.

3) From result table no II: - The Sp. Gravity Method is good for alcohol estimation compared to G.C. Dichromate & Sike's

hydrometer method as well as Sp. Gravity Method & G.C. Method gives nearly same result. It means for lower concentration of alcohol estimation Sp. Gravity method & G.C. Method are more useful than Dichromate & Sike's Hydrometer Method.

4) From result Table no III: - The Sp. Gravity Methods are more usefull for alcohol estimation than Dichromate method & G.C. Method. The G.C method gives much different result as compare to Result Table no I, II & III.

5) Using t-test. It is observed that for higher concentration Alcohol estimation by Sp. Gravity & G.C.method. Indicate significant different At 5% level because calculated t-value equal to 5.24 is greater than table-t value-2.228 at 5% level of significant for 10° of freedom & Alcohol estimation determine by Specific gravity method gives average value of 95.88% which is closed to 96% hence specific gravity method is better Method than G.C.Method for alcohol estimation for higher concentration.

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[5] AOAC Method , or use standard reference material 1590, Stablized ratio (RR)

[6] Indian Standard — Table for alcoholometery-by Pyknometer method. II

[7] AOAC Method , or use standard reference material 1590, Stablized ratio (RR)

Chemical and Biochemical Means to Detect Alcohol

Determination of Ethanol Concentration in Fermented Beer Samples and Distilled Products

Paul Held, Laboratory Manager, Applications Dept, BioTek Instruments, Inc., Winooski, VT

Key Words:

Ethanol

Biofuel

Beer

Distillation

Fermentation

The fermentation of sugars to alcohol is used for a number of different applications, most notably the production of alcoholic beverages. The beer and wine industry ferment extracts of barley and grapes respectively to produce numerous alcoholic beverages, while distilleries further concentrate ethanol using the evaporation and condensation differences of ethanol and water. Ethanol can be biochemically quantitated from a diverse matrix, such as fermenting beer, using alcohol oxidase enzyme, while relatively pure ethanol can be determined chemically using the oxidizer pyridinium chlorochromate (PCC). Here we describe the use of ethanol oxidase and PCC to monitor ethanol production during the fermentation of beer and from distilled products.

Introduction

The production of alcoholic beverages has been associated with mankind from the beginning of civilization. As a result they are a key element of many parts of our culture. One such alcoholic beverage, beer, is produced by the fermentation of starchy grains through a process known as brewing. This has existed since the 6th millennium BC, with recipes being found in some of the earliest Sumerian writings [1]. Brewing is the production of beer through steeping a starch source (usually cereal grains) in water and then fermenting the resultant mixture with yeast (Figure 1). The basic ingredients of beer are water, a starch source, brewer's yeast and a flavoring agent such as hops. All of these ingredients work in concert to provide the individual flavor of different beers. A number of secondary sources of starch, such as corn, rice, millet, sorghum, and cassava root, are also used because of availability, to impart specific flavors or for economic reasons. Water is the primary ingredient in beer and it suffices to say that the dissolved minerals in water have a great deal to do with the style of beer from different regions of the world. Malted or germinated barley is used because the process of germination induces the grain seed to express several key amylase enzymes, such as α-(1→4) endo glycosylase and α-(1→6) endoglycosylase, necessary for the digestion of starch [2]. Hops or more specifically the flower or cone of the hop vine are used a flavoring agent (taste and aroma).

Figure 1. Glass fermentation carboys. Small batch brewing methods often employ the use of glass containers with liquid air-lock systems.

Fermentation of released glucose is carried out by brewer's yeast (Saccharomyces cerevisiea, Saccharomyces uvarum, and Brettanomyces), which convert glucose to ethanol (Figure 2).

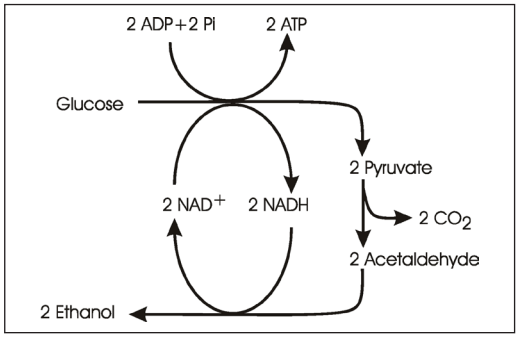

Figure 2. Schematic representation of the fermentation process.

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: **customercare@biotek.com**
www.biotek.com

In order to monitor fermentation, as well as comply with federal and state labeling statues regarding alcohol, brewers need to quantitate ethanol concentration in fermented beer batches. In addition numerous vintners and distillers have similar requirements.

Distillation is a physical process (rather than a chemical process) that separates mixtures based on differences in the volatility of different components. Because ethanol has a lower vapor pressure than water, distilleries can concentrate ethanol produced by fermentation by heating the mixture. At a given temperature ethanol vapor will concentrate in the vapor relative to water. When the vapor is condensed back to liquid form, the effective ethanol concentration will have been increased (Figure 3). Using either a continuous distillation or several batch distillation processes, the ethanol concentration can be increased from the initial fermentation concentration of 5-10% to 95%. Distilled beverages are then aged as necessary to produce desired color and flavors, while bioethanol for industrial purposes can be used immediately.

Figure 3. Copper Distillation Kettles.

There are number of different methods to quantitate ethanol in samples. One of the most commonly used methods is densitometry [3]. First described by Joseph Gay-Lussac in 1824, this method takes advantage of the differences in density between water and ethanol. Using a calibrated hydrometer that has a % vol. scale, also known as an alcoholmeter, one can determine the percent alcohol in a given fluid. Since the 19th century a number of improvements have been developed in order to improve or automate the process. Ebulliometry or boiling point depression has been used to estimate the percentage of alcohol in beer or wine [4]. With increasing amounts of ethanol present the boiling point of the mixture will decrease. There is a linear relationship between ethanol concentration and the change is boiling temperature. Note that residual sugars greater than 4% will influence the value and need to be accounted for. HPLC has been utilized to monitor the fermentation process. This method has the advantage of being able to monitor not only the production of ethanol, but also the reaction substrates and byproducts [5]. FTIR [6], gas chromatography [7], and IR [8] technologies have also been used to detect and quantitate ethanol in samples. While FTIR requires a large investment in instrumentation, the use or less expensive IR technology has been demonstrated to be just as accurate [8]. The chemical methods employed have focused on oxidation of the lone hydroxyl group present on the ethanol molecule. This redox reaction forms an aldehyde or ketone, as well as a reduction of the oxidizing agent. The oxidizing agent pyridinium chlorochromate (PCC) oxidizes ethanol to form a ketone, while the reduction of PCC results in the formation of chromate, which has high molar absorptivity at 570 nm (Eq. 1). Biochemical or enzymatic methods utilize enzymes that specifically react with ethanol. By linking two or more reactions a measureable product can monitored.

Eq. 1. $2C_2H_5NHCrO_3Cl + 3\ CH_3CH_2OH \rightarrow 2C_2H_5NHCl + Cr_2O_3 + 3CH_3CHC{=}O + 3\ H_2O$

Materials and Methods

Pyridinium chlorochromate (cat # 190144), ethanol (cat# A962), methanol (cat# 34860), glycerol (cat# G5516) and 1-butanol (cat# B7906) were purchased from Sigma Aldrich (St. Louis, MO). Amplite™ fluorometric ethanol quantitation kits (cat# 40001) were obtained from AAT Bioquest (Sunnyvale, CA). Clear (3598) 96-well and solid black ½-area (3694) 96-well microplates were procured from Corning (Corning, NY). Barley grain, hops and yeast were purchased from a local homebrew beer supply store. Commercially available beverages were obtained at a local beverage distributor.

Fermentation:

Vienna style lager beer was produced by standard production procedures. Briefly, crushed malted barley was wetted and incubated at 77° C for approximately 60 minutes to convert grain starch to glucose by action of the endogenous α-(1→4) and α-(1→6) glycosylase enzymes present in the germinated barley. The sugar rich aqueous extract was isolated by flow through filtration and boiled for 60 minutes. Hop buds were added at intervals during the boil for flavor. After cooling, the unfermented wort was inoculated with Bavarian lager yeast (Wyeast strain 2206). The culture was sealed with an air lock and allowed to ferment at approximately 16° C. Aliquots (15 mL) were removed daily, centrifuged at 800x g and the supernatant stored at -20° C until assayed for ethanol and glucose content.

PCC Assay:

A 1 M working stock of pyridinium chlorochromate (PCC) was prepared fresh in deionized water. Samples and standards (100 µL) were pipetted and the reaction was initiated by the addition of 100 µL of working PCC reagent. The reaction was monitored using absorbance measurements at 570 nm.

Amplite™ Ethanol Assay:

Ethanol was measured using the Amplite™ fluorescent ethanol assay kit. The assay was performed according to the assay kit instructions. Briefly, a 250 X Amplite™ reagent stock solution was prepared by dissolving the contents of the pre-weighed vial provided in the assay kit with 40 µL of DMSO. A working enzyme solution (100X) was prepared by diluting the provided lyophilized vial with 1X assay buffer supplied in the kit. Working reaction mixture was prepared by mixing 20 µL Amplite™ reagent, 50 µL enzyme solution, and 4.97 mL of 1X assay buffer. Aliquots (50 µL) of each sample were added to the solid black ½ area microplate and the reactions were initiated by the addition of 50 µL of working reaction mixture. Reaction fluorescence was measured using a Synergy™ H4 Multi-Mode Microplate Reader with an excitation of 540 nm and an emission of 590 nm.

Amplex® Red Glucose Assay:

Glucose production was measured using the Amplex® Red fluorescent glucose assay kit. The assay was performed as previously described [2]. Reaction fluorescence was measured using a Synergy H4 multimode reader with an excitation of 540 nm and an emission of 590 nm.

Results

The reaction with purified ethanol and PCC results in a linear response (Figure 4). Because the reaction does not require any form of stop solution, it can be read continuously. The length of the reaction time can be varied depending on the expected concentrations of the unknown samples.

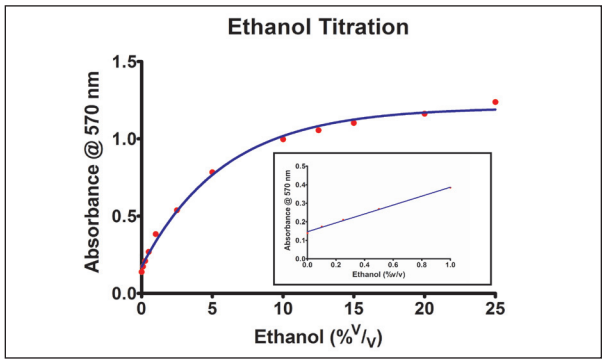

Figure 4. Ethanol titration with PCC reaction.

Pyridinium Chlorochromate is a strong oxidant that changes color as it reacts with ethanol [9, 10]. The formation of chromate results in a change from brilliant orange to darker shades of brown. The color change is demonstrated by the spectral scans of reacted and unreacted wells (Figure 5). Unreacted wells have a marked transition from significant absorbance below 550 nm, to virtually no absorbance above 550 nm. Reacted wells demonstrate absorbance up to 725 nm. A broad peak in absorbance is seen when the absorbance of unreacted well is subtracted from reacted (Figure 5).

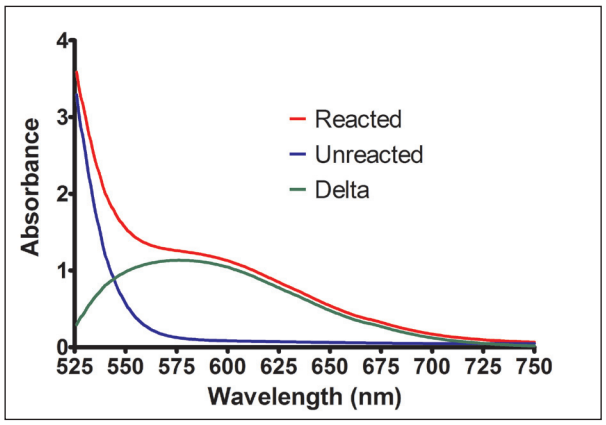

Figure 5. Spectral Scan of Reacted and Unreacted PCC Sample.

The increase in absorbance at 570 nm is not specific to ethanol. When other alcohols are reacted with pyridinium chlorochromate significant increases in the absorbance at 570 nm are also observed (Figure 6). Interestingly, each alcohol had different reactivity with respect to PCC.

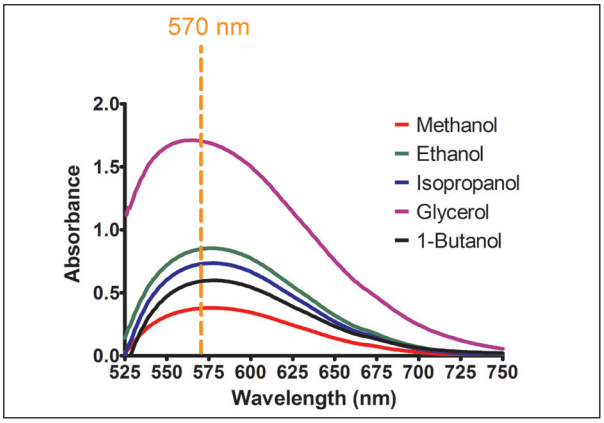

Figure 6. Spectral scan of PCC reaction with different alcohols. Equimolar amounts of different alcohols were reacted with PCC and their absorbance spectra compared.

When equimolar amounts of four different alcohols are reacted with PCC and compared, methanol results in the least increase in absorbance, while ethanol has the greatest change. The three carbon polyol glycerol, which has three hydroxyl groups, demonstrates significant more absorbance than isopropanol, which also has three carbon atoms, but only one hydroxyl group (Figure 7).

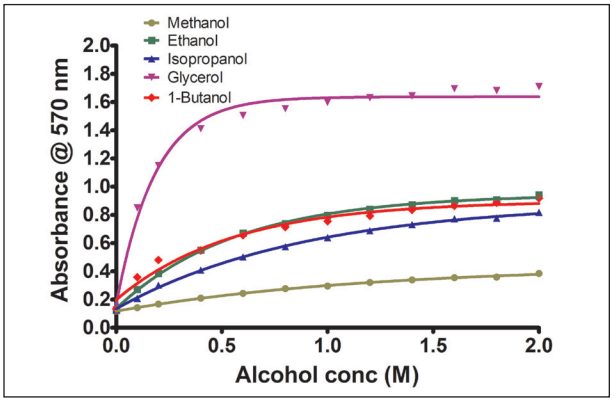

Figure 7. PCC reaction with different alcohols. Equimolar amounts of different alcohols were reacted with PCC and their absorbance compared.

As a result of the reactivity demonstrated with glycerol, the reactivity of the sugars galactose and glucose were investigated. Both sugar moieties, which contain numerous hydroxyl groups, reacted with PCC to a greater extent than ethanol on a molar basis. Despite only differences in isomerization between the two sugars, galactose is more reactive than glucose (Figure 8).

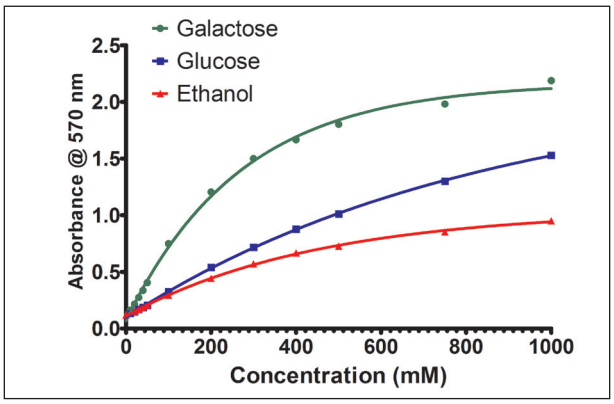

Figure 8. PCC reactivity with sugars.

Using the PCC assay several distilled commercial products were analyzed for ethanol content. As demonstrated in Table 1, there is good agreement between the determined ethanol concentration and what is reported by the manufacturer. The only exception is Southern Comfort, which had a determined content in excess of 50% v/v. The discrepancy can be explained by the sugar content of the spirit, which is reported to be 100 g/L. The other products are simply distilled spirits, which do not contain additives.

Product	% Ethanol Determined	% Ethanol Reported
Finlandia Vodka	44.50	40
Johnnie Walker Scotch Whiskey	41.82	40
Absolut Vodka	42.29	40
Southern Comfort	>50	35
Jameson's Irish Whiskey	41.04	40
Jack Daniels Bourbon	38.56	40
Seagram's 7 American Whiskey	41.58	40
50% Ethanol mixture	47.16	50

Table 1. Determined ethanol concentrations of commercially distilled spirits.

Numerous ethanol containing solutions also possess constituents that have hydroxyl groups that are reactive with PCC. In order to assess the ethanol content of these mixtures, assay methods that are more specific to ethanol are required. The Amplite™ ethanol assay uses the enzyme ethanol oxidase, which is specific for primary alcohols [11]. This assay kit has a linear response to ethanol (Figure 9).

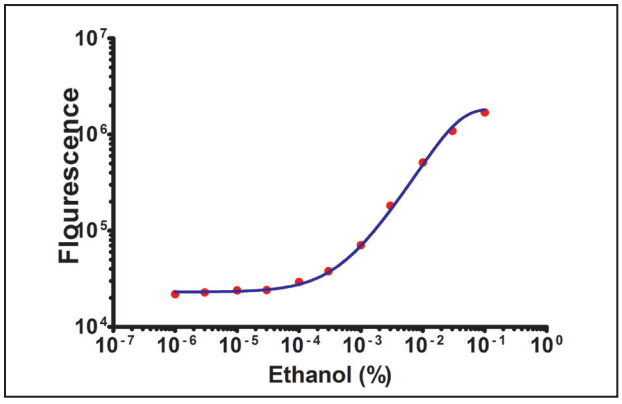

Figure 9. Ethanol titration with Amplite™ Assay.

Using the assay kit production of ethanol in fermenting beer was monitored. As demonstrated in Figure 10 the ethanol concentration of fermenting Vienna lager style beer increased steadily from 0 to approximately 5.5% over a period of 350 hours (14 days). This is in good agreement with ethanol calculations based on the change in specific gravity. The initial specific gravity of the unfermented beer was determined to be 1.055 g/mL, while the final gravity was 1.010 g/mL. Using the change in gravity one can estimate the concentration of ethanol produced to be 5.9% (See Eq. 2), where IG is the initial specific gravity, FG is the final specific gravity, 1.05 represents the mass in grams of ethanol produced per 1 gram of CO_2 generated and 0.79 is the specific gravity of ethanol.

Eq. 2 % ethanol = (((1.05*(IG-FG))/FG)/0.79

During the fermentation process glucose was consumed quite rapidly. Glucose is present in high concentration (approx 80 mM) in the media at the beginning of the fermentation process and is completely consumed in the first 50 hours of fermentation (Figure 10). This rate is much faster than the production of ethanol, suggesting that glucose transporters are quickly sequestering the glucose from the media. Only as the glucose is consumed for energy by the yeast is ethanol produced.

The ethanol concentration of several commercially available beer brands was determined. As demonstrated in Table 2, there is good agreement between the determined concentrations and the ethanol content reported by the manufacturers.

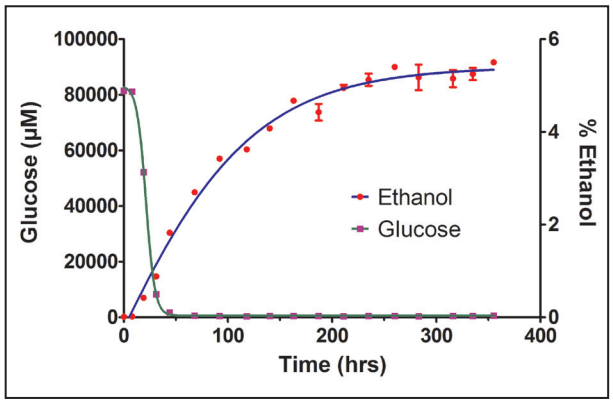

Figure 10. Vienna style lager fermentation.

Product	% Ethanol Determined	% Ethanol Reported
Gaffel Kolsch	6.45	4.8
Sam Smith Oatmeal Stout	3.22	5.0
DeKonnick	4.96	5.0
Scherlenkerla Helles	3.90	5.1
Paulaner Salvator Dopple Bock	5.53	7.9
Theakston Old Peculier	3.53	5.6
Sunner Kolsch	5.14	5.3
Weinhenstephan vitus Werenbock	7.41	7.7
St.Peter's IPA	4.24	4.9
Brooklyn Local #1 (Belgium Tripple)	10.14	9
Spaten Helles lager	5.84	5.2
Spaten Octoberfest	5.77	5.9
Spaten Optimator Dopplebock	7.17	7.6
Zywiec	5.82	5.6
Podge Belgium Imperial Stout	9.10	10.5
Beck's NA Bier Lager	0.34	<0.5

Table 2. Comparison of determined and reported commercial beer ethanol concentrations.

In general, the determined ethanol concentrations for dark colored beer styles tended to be slightly below that which was reported, while light colored beers were much closer. For example, the determined ethanol concentration for Theakston Old Peculier was 3.53%, while the concentration reported by the manufacturer was 5.6%. This beer has been described by its manufacturer as having a deep dark ruby color. Alternatively Sünner Kölsch, which has been described as hazy golden color, was measured to have 5.14 % ethanol verses a reported alcohol content of 5.3%.

Discussion

Ethanol is used for a myriad of different applications. Vast quantities of ethanol are produced as an additive for gasoline as a means to increase oxygen content of the fuel. More recently ethanol has been thought of as a means to replace fossil fuel derived energy for transportation. Perhaps more importantly is the presence of ethanol in alcoholic beverages such as beer, wine and distilled products, such as whiskey, and vodka.

These data demonstrate the utility of two different means to determine ethanol concentrations from aqueous mixtures. The oxidation of alcohols by PCC is a fast inexpensive method to determine ethanol concentrations in relatively pure samples such as distilled products. It's reactivity to sugars makes it inappropriate for use in fermenting samples. Its working concentration range (0.5-25%v/v) is amenable to many distilled products with minimal sample dilution. Besides distilled products, this reaction is utilized by many portable breathalyzers for the determination of blood alcohol concentrations. Because the exhaled breath ethanol concentration is proportional to that in the blood, and exhaled breath is relatively free of reactive contaminants, the determination from this non-invasive test can reliably be used by law enforcement agencies to test motorists suspected of driving under the influence.

Quantitation of ethanol in mixed matrices, which is the norm for fermenting products, requires greater specificity than oxidation with PCC. Reactions that are linked to the generation of H_2O_2 by alcohol oxidase are specific to primary alcohols despite the presence of reducing sugars such as glucose. The Amplite™ ethanol assay was very sensitive and capable of making ethanol determinations in fermenting samples. While it was not tested, it stands to reason that this method can be used to test fermenting wine samples as well. The use of hops as a flavoring agent instills beer with antioxidants, which can interfere to some extent with the reaction. For maximal accuracy it is advised to use an equivalent dilution of unfermented wort when making dilutions for the standard curve.

Alcohol oxidase enzyme based assays are very sensitive, capable of detecting concentrations as low as 0.003% v/v ethanol concentrations. As such many potential problems caused by contaminates can be reduced or eliminated through sample dilution. As stated previously there are numerous ways to quantitate ethanol from samples. The methods described here have the advantage of being rapid, inexpensive, and requiring only modest instrumentation. The ability to run these assays in microplate format allows for numerous samples to be run simultaneously.

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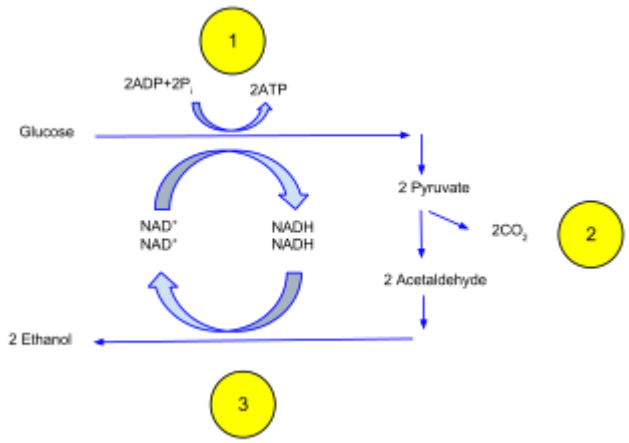
Ethanol fermentation

Ethanol fermentation, also called **alcoholic fermentation**, is a biological process which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as by-products. Because yeasts perform this conversion in the absence of oxygen, alcoholic fermentation is considered an anaerobic process. It also takes place in some species of fish (including goldfish and carp) where (along with lactic acid fermentation) it provides energy when oxygen is scarce.[1]

Ethanol fermentation has many uses, including the production of alcoholic beverages, the production of ethanol fuel, and bread cooking.



In ethanol fermentation, (1) one glucose molecule breaks down into two pyruvates. The energy from this exothermic reaction is used to bind the inorganic phosphates to ADP and convert NAD+ to NADH. (2) The two pyruvates are then broken down into two acetaldehydes and give off two CO2 as a by-product. (3) The two acetaldehydes are then converted to two ethanol by using the H- ions from NADH, converting NADH back into NAD+.

Contents

Biochemical process of fermentation of sucrose

The chemical equations below summarize the fermentation of sucrose ($C_{12}H_{22}O_{11}$) into ethanol (C_2H_5OH). Alcoholic fermentation converts one mole of glucose into two moles of ethanol and two moles of carbon dioxide, producing two moles of ATP in the process.

The overall chemical formula for alcoholic fermentation is:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Sucrose is a dimer of glucose and fructose molecules. In the first step of alcoholic fermentation, the enzyme invertase cleaves the glycosidic linkage between the glucose and fructose molecules.

$$C_{12}H_{22}O_{11} + H_2O + \text{invertase} \rightarrow 2\ C_6H_{12}O_6$$

Next, each glucose molecule is broken down into two pyruvate molecules in a process known as glycolysis.[2] Glycolysis is summarized by the equation:

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ NAD^+ \rightarrow 2\ CH_3COCOO^- + 2\ ATP + 2\ NADH + 2\ H_2O + 2\ H^+$$

The chemical formula of pyruvate is CH_3COCOO^-. P_i stands for the inorganic phosphate.

Finally, pyruvate is converted to ethanol and CO_2 in two steps, regenerating oxidized NAD+ needed for glycolysis:

$$1.\ CH_3COCOO^- + H^+ \rightarrow CH_3CHO + CO_2$$

catalyzed by pyruvate decarboxylase

$$2.\ CH_3CHO + NADH + H^+ \rightarrow C_2H_5OH + NAD^+$$

This reaction is catalyzed by alcohol dehydrogenase (ADH1 in baker's yeast).[3]

As shown by the reaction equation, glycolysis causes the reduction of two molecules of NAD^+ to NADH. Two ADP molecules are also converted to two ATP and two water molecules via substrate-level phosphorylation.

A laboratory vessel being used for the fermentation of straw.

Fermentation of sucrose by yeast.

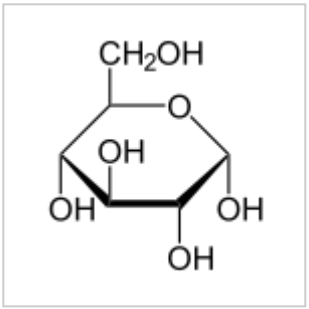
Related processes

Fermentation of sugar to ethanol and CO_2 can also be done by Zymomonas mobilis, however the path is slightly different since formation of pyruvate does not happen by glycolysis but instead by the Entner–Doudoroff pathway. Other microorganisms can produce ethanol from sugars by fermentation but often only as a side product. Examples are[4]

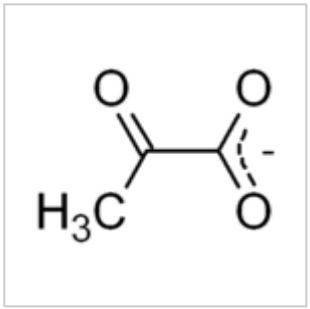
- Heterolactic acid fermentation in which Leuconostoc bacterias produce Lactate + Ethanol + CO_2
- Mixed acid fermentation where Escherichia produce Ethanol mixed with Lactate, Acetate, Succinate, Formate, CO_2 and H_2
- 2,3-butanediol fermentation by Enterobacter producing Ethanol, Butanediol, Lactate, Formate, CO_2 and H_2

Gallery

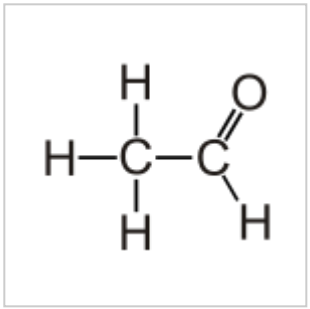
Grapes fermenting during wine production.



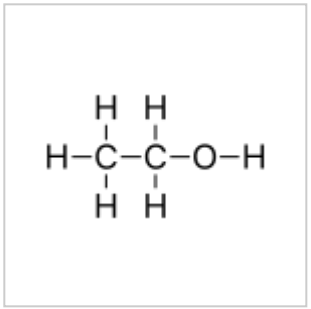
Glucose depicted in Haworth projection



Pyruvate



Acetaldehyde



Ethanol

Effect of oxygen

Fermentation does not require oxygen. If oxygen is present, some species of yeast (e.g., *Kluyveromyces lactis* or *Kluyveromyces lipolytica*) will oxidize pyruvate completely to carbon dioxide and water in a process called cellular respiration, hence these species of yeast will produce ethanol only in an anaerobic environment (not cellular respiration).

However, many yeasts such as the commonly used baker's yeast *Saccharomyces cerevisiae*, or fission yeast *Schizosaccharomyces pombe*, prefer fermentation to respiration. These yeasts will produce ethanol even under aerobic conditions, if they are provided with the right kind of nutrition. During batch fermentation, the rate of ethanol production per milligram of cell protein is maximal for a brief period early in this process and declines progressively as ethanol accumulates in the surrounding broth. Studies demonstrate that the removal of this accumulated ethanol does not immediately restore fermentative activity, and they provide evidence that the decline in metabolic rate is due to physiological changes (including possible ethanol damage) rather than to the presence of ethanol. Several potential causes for the decline in fermentative activity have been investigated. Viability remained at or above 90%, internal pH remained near neutrality, and the specific activities of the glycolytic and alcohologenic enzymes (measured in vitro) remained high throughout batch fermentation. None of these factors appears to be causally related to the fall in fermentative activity during batch fermentation.

Bread baking

Ethanol fermentation causes bread dough to rise. Yeast organisms consume sugars in the dough and produce ethanol and carbon dioxide as waste products. The carbon dioxide forms bubbles in the dough, expanding it a foam. Less than 2% ethanol remains after baking.[5][6]

Alcoholic beverages

All ethanol contained in alcoholic beverages (including ethanol produced by carbonic maceration) is produced by means of fermentation induced by yeast.

- Wine is produced by fermentation of the natural sugars present in grapes; cider and perry are produced by similar fermentation of natural sugar in apples and pears, respectively; and other fruit wines are produced from the fermentation of the sugars in any other kinds of fruit. Brandy and eaux de vie (e.g. slivovitz) are produced by distillation of these fruit-fermented beverages.
- Mead is produced by fermentation of the natural sugars present in honey.
- Beer, whiskey, and vodka are produced by fermentation of grain starches that have been converted to sugar by the enzyme amylase, which is present in grain kernels that have been malted (i.e. germinated). Other sources of starch (e.g. potatoes and unmalted grain) may be added to the mixture, as the amylase will act on those starches as well. Whiskey and vodka are also distilled; gin and related beverages are produced by the addition of flavoring agents to a vodka-like feedstock during distillation.
- Rice wines (including sake) are produced by the fermentation of grain starches converted to sugar by the mold *Aspergillus oryzae*. *Baijiu*, *soju*, and *shōchū* are distilled from the product of such fermentation.
- Rum and some other beverages are produced by fermentation and distillation of sugarcane. Rum is usually produced from the sugarcane product molasses.

The formation of carbon dioxide — a byproduct of ethanol fermentation — causes bread to rise.

Primary fermentation cellar, Budweiser Brewery, Fort Collins, Colorado

In all cases, fermentation must take place in a vessel that allows carbon dioxide to escape but prevents outside air from coming in. This is to reduce risk of contamination of the brew by unwanted bacteria or mold and because a buildup of carbon dioxide creates a risk the vessel will rupture or fail catastrophically, causing injury and property damage.

Feedstocks for fuel production

Yeast fermentation of various carbohydrate products is also used to produce the ethanol that is added to gasoline.

The dominant ethanol feedstock in warmer regions is sugarcane.[7] In temperate regions, corn or sugar beets are used.[7][8]

In the United States, the main feedstock for the production of ethanol is currently corn.[7] Approximately 2.8 gallons of ethanol are produced from one bushel of corn (0.42 liter per kilogram). While much of the corn turns into ethanol, some of the corn also yields by-products such as DDGS (distillers dried grains with solubles) that can be used as feed for livestock. A bushel of corn produces about 18 pounds of DDGS (320 kilograms of DDGS per metric ton of maize).[9] Although most of the fermentation plants have been built in corn-producing regions, sorghum is also an important feedstock for ethanol production in the Plains states. Pearl millet is showing promise as an ethanol feedstock for the southeastern U.S. and the potential of duckweed is being studied.[10]

In some parts of Europe, particularly France and Italy, grapes have become a *de facto* feedstock for fuel ethanol by the distillation of surplus wine.[11] In Japan, it has been proposed to use rice normally made into sake as an ethanol source.[12]

Cassava as ethanol feedstock

Ethanol can be made from mineral oil or from sugars or starches. Starches are cheapest. The starchy crop with highest energy content per acre is cassava, which grows in tropical countries.

Thailand already had a large cassava industry in the 1990s, for use as cattle feed and as a cheap admixture to wheat flour. Nigeria and Ghana are already establishing cassava-to-ethanol plants. Production of ethanol from cassava is currently economically feasible when crude oil prices are above US$120 per barrel.

New varieties of cassava are being developed, so the future situation remains uncertain. Currently, cassava can yield between 25-40 tonnes per hectare (with irrigation and fertilizer),[13] and from a tonne of cassava roots, circa 200 liters of ethanol can be produced (assuming cassava with 22% starch content). A liter of ethanol contains circa 21.46[14] MJ of energy. The overall energy efficiency of cassava-root to ethanol conversion is circa 32%.

The yeast used for processing cassava is *Endomycopsis fibuligera*, sometimes used together with bacterium *Zymomonas mobilis*.

Byproducts of fermentation

Ethanol fermentation produces unharvested byproducts such as heat, carbon dioxide, food for livestock, water, methanol, fuels, fertilizer and alcohols.[15] The cereal unfermented solid residues from the fermentation process, which can be used as livestock feed or in the production of biogas, are referred to as Distillers grains and sold as WDG, *Wet Distiller's grains*, and DDGS, *Dried Distiller's Grains with Solubles*, respectively.

Microbes used in ethanol fermentation

- Yeast
  - Saccharomyces cerevisiae
  - Schizosaccharomyces
- Zymomonas mobilis (a bacterium)

See also

- Anaerobic respiration
- Cellular respiration
- Cellulose
- Fermentation (wine)
- Yeast in winemaking
- Tryptophol, a chemical compound found in wine[16] or in beer[17] as a secondary product of alcoholic fermentation[18] (a product also known as congener)

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A Method for the Assay of Penicillin

By N. G. HEATLEY, *Sir William Dunn School of Pathology, Oxford*

(*Received* 18 *December* 1943)

In an earlier publication (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941) a very brief account of a method of assay of penicillin was given. A fuller account of this same method (with some minor modifications) is now presented for the following reasons. Experience has shown that the original instructions were not sufficiently detailed to enable other workers to use the method without difficulty. The method has been in regular use in this laboratory since 1940 and is still believed to be as satisfactory, for general use, as other methods. Slight modifications have increased its accuracy. Whatever progress is made in the chemical investigation of penicillin, a biological method of assay is likely to be necessary for some purposes for a considerable time because of its great sensitivity and specificity. This assay method has already been used in the investigation of other antibiotics, and it may have a wide application in this field.

The following account is confined strictly to the procedure used in this laboratory. Modifications have been proposed—sometimes by two or more groups of workers independently—and though some of these are mentioned below the author has had insufficient experience of them to justify a critical appraisal.

PRINCIPLE OF THE METHOD

An agar plate is seeded by pouring on a broth culture of the test organisms, draining and drying. Short open-ended cylinders of glass or vitreous porcelain are then placed on the surface of the agar and the solutions to be assayed are placed in the cylinders. After incubation the surface of the agar becomes covered with a confluent bacterial growth, except for a circular zone around each cylinder where the penicillin has diffused out and inhibited growth. The diameter of this zone of inhibition is related to the concentration of penicillin in the solution in the cylinder, and by setting up a standard containing a known concentration of penicillin at the same time as the unknown, the strength of the latter can be calculated.

PRACTICAL DETAILS OF THE METHOD

Pouring the plates. The Petri dishes (which should be of a uniform size and not too shallow) are poured to a depth of 3–5 mm. with a simple medium such as the following:

Lemco Extract of Meat	1%
Evans's Bacteriological Peptone	1%
Sodium chloride	0·5%
Agar	2%
Made up with tap water	

For the last 2 years it has been customary to add to this 50 ml./l. of M/1·5 phosphate buffer pH 6·8, and phenol red to a final concentration of 0·0025%. The presence of the buffer seems to give sharper zones of inhibition, but opinion is divided as to whether the inclusion of the indicator is an improvement. The medium is sterilized at 15 lb./sq. in. pressure for 20 min. in bottles containing 400 ml., the actual pouring of the plates being simplified and standardized by means of an automatic measuring head. The latter, which is sterilized separately, can be used with at least two successive bottles if ordinary precautions against contamination are taken. 20 ml. is a convenient amount of agar for the ordinary 4 in. Petri dish. Several dozen plates can be poured at one time and stored at room temperature for some days before use. Little infection is encountered nowadays, but at one time plates were all incubated for 24 hr. after pouring, those developing contaminants being discarded.

Seeding the plates. Staphylococcus aureus, no. 6571 of the National Collection of Type Cultures, is used as test organism, though presumably any penicillin-sensitive strain would serve. The plates are seeded by placing on each a small volume of a 16–24 hr. broth culture (or of a 10–100-fold dilution of such a culture). After being tilted and shaken so that the whole surface of the agar is completely covered with the liquid, the plates are left tilted at about 20° to the horizontal for a few moments and the surplus bacterial suspension is drawn off. This edge of the plate is marked (for reasons given below). The surface of the agar is then dried by setting the plates in the 37° incubator for 1–2 hr. with the lid lifted about ½ in. above the bottom of the dish.

Two types of drying-stand have been used; in one the lids rest on two glass rods supported at the correct height, and in the second type a wooden baseboard is fitted with sets of three inward-sloping springy wires which grip the lids and allow them to be set to any desired height over the bottoms of the dishes. The actual time required for

drying the plates depends on conditions of humidity, etc., in the incubator. Usually 1 hr. is sufficient, but in one incubator, although the surface of the agar was quite dry after this period, when the cylinders had been put on and the plate incubated again overnight, a ring of free moisture was found round each cylinder. This interfered with the interpretation of small zones of inhibition. The trouble disappeared when the plates were dried for 2 hr. instead of 1 hr.

The dried seeded plates can be kept in the ice-chest for at least 3 days.

Nearly 2 years ago attempts were made to apply the well-known principle of seeding the agar in bulk prior to pouring the plates, but at the time this modification was thought to offer no advantages and was discontinued. However, Foster & Woodruff (1943*a*) obtain excellent results with a bulk seeding technique, and have described (1943*b*) a further modification in which the agar is pre-seeded with a pasteurized suspension of spores of *B. subtilis*. Independent workers have confirmed that the zones of inhibition are exceptionally sharp, and there is less day-to-day variation in the standard curve, which is somewhat flatter than when *Staph. aureus* is used. Although the accuracy is thereby reduced, the effective range of the method is correspondingly increased.

Preparation of solutions for assay. A considerable practical advantage of the present method is that the solutions to be assayed need not be sterile, since contaminating bacteria are confined to the inside of the cylinder. It is readily conceivable, however, that heavy contamination by bacteria which actively destroy penicillin may reduce the concentration of the latter during the incubation and give falsely low or even negative values. For the same reason it is important that the pH of the solution should be within the range 5·0–8·0. The concentration of penicillin should be preferably between 0·5 and 2·0 units/ml. With other organisms, e.g. *B. subtilis*, a different range may be covered. M/50 phosphate buffer, pH 7·0, is used instead of water in preparing solutions, since traces of acid or alkali in the glassware might otherwise bring the pH to a dangerous level. Very strongly buffered solutions are to be avoided, but the presence of ether or chloroform seems to have no disturbing effect.

Placing and filling cylinders. The cylinders, having been dry-sterilized in a Petri dish, are picked up in forceps, momentarily flamed, and then carefully placed on the surface of the agar. There may be a very brief sizzling sound (though the cylinder should scarcely be hot enough to cause this) and a perfectly fluid-tight seal is made between the agar and the cylinder. The cylinders are *not* pressed into the agar. The solutions to be assayed are then placed in the cylinders, care being taken that there is no air space between the fluid and the surface of the agar. The exact volume of fluid seems to make little difference to the assay value, but in practice the fluid is filled level with the top of the cylinder.

After use the cylinders are placed in strong sulphuric acid containing a few crystals of sodium nitrate, and warmed, which both sterilizes and cleans them. They are well washed under the tap, then in distilled water and finally dry-sterilized in a Petri dish.

Specifications for cylinders. The cylinders may be made of glass or *vitreous* porcelain, glazed or unglazed. Provided they are of uniform size the actual dimensions are unimportant. (The cylinders we have used hitherto are 9·6 ±0·2 mm. high, by 5·1 ±0·2 mm. internal diameter and 7·2 ±0·1 mm. external diameter. One end is bevelled internally and the sharp edge is ground perfectly plane; the other end is coloured for easy identification. Cylinders of vitreous porcelain of this type are obtainable from James Macintyre and Co., Ltd., Burslem, Staffs.) Foster & Woodruff (1943*a*) have stated that the bevel is unnecessary. This is readily understandable as the bevel was introduced originally to give a better seal between the surface of the agar and the cylinder, which was at that time placed on the agar without warming. If the momentary flaming of the cylinders immediately before placing on the agar is adopted, the need for the absolutely plane edge and the desirability of the bevel both disappear.

Dr R. D. Coghill has suggested (private communication) that the medium be made up to contain less than 2% agar. When the cylinders are placed on the softer agar a good seal is obtained without preliminary flaming, thus saving both time and agar. Under these conditions the edge of the cylinder must, of course, be plane, and the bevel is probably an advantage.

Incubation. The plates are placed on a slab of wood or asbestos and transferred to the 37° incubator for 16–24 hr. If the plates are placed directly on the shelf of the incubator, fluid may condense on the lid of the dish, touch the cylinders and cause their non-sterile contents to run down on to the agar. Incubation is usually allowed to proceed overnight (16 hr.) but the zones of inhibition are quite distinct after less than 10 hr. and, if left longer, increase in size only very slightly.

A modification of this procedure which is sometimes used, especially with solutions of low activity, is to place the prepared plate in the refrigerator for 2–8 hr. before incubation. This allows the penicillin partly to diffuse out before the bacteria begin to multiply rapidly, and the zones are somewhat bigger than would otherwise be the case.

By using plates pre-seeded with spores of a quick growing organism of the *B. subtilis* type, Dr C. G. Pope states (private communication) that results can be read after only 4–5 hr. incubation.

Measurement of zones of inhibition. The diameter of the zones of inhibition (which, in millimetres, we have called the 'assay value') can be measured in several ways. The plates may be turned over and the zone measured directly against a millimetre scale laid on the bottom of the dish. Or the plate

may be placed on a transparent scale illuminated from underneath. A more accurate way is to measure the zones with pointed calipers or dividers, the readings being taken off a paper scale which can be burnt, or a metal scale which can be flamed.

Arranging standard and unknown solutions on the plates. In preparing the plates a number of factors such as batch of medium, length of autoclaving, drying time of plates, number of times the incubator is opened during drying, density of bacterial suspension used for seeding, etc., are not all easy to control in practice. It has been found that the shape of the curve relating assay value to concentration of penicillin, as well as the absolute assay value for any given sample, may vary from day to day, probably depending on these variables. Preliminary investigations showed that a quantitative study of the effects involved would be a lengthy and difficult task; the method is still therefore largely empirical. It is, however, a simple matter to determine the shape of the curve afresh each day by setting up with the unknowns three (or four) solutions containing 2 units, 1 unit, and $\frac{1}{2}$ (and sometimes $\frac{1}{4}$ unit)/ml. respectively. The assay values obtained are plotted against the number of units/ml., and a smooth curve drawn through the points. Then, by reference to the curve, the number of units/ml. corresponding to the assay values of the unknowns can be read off. Fig. 1 shows a typical curve, and Table 1 gives the actual values from which the standard curves were constructed in a series of consecutive tests. The day-to-day variation in this particular series is not great, but over longer periods much larger drifts and occasional abrupt breaks may occur (e.g. on changing to a new batch of medium). It is for this reason that it is considered necessary to construct a standard curve each day.

A detail of considerable importance is the way in which the cylinders are arranged on the plate. If the same solution is placed in six or eight cylinders arranged symmetrically near the periphery of a plate, the resulting zones of inhibition will usually be of identical size. It sometimes happens, however, that the zones nearer the edge to which the plate has been drained during the seeding process are smaller than the average, while those on the opposite side are larger than average, the deviation occasionally reaching as much as ± 2 mm. The opposite of this effect has, on rare occasions, been observed. The causes of this occasional asymmetry

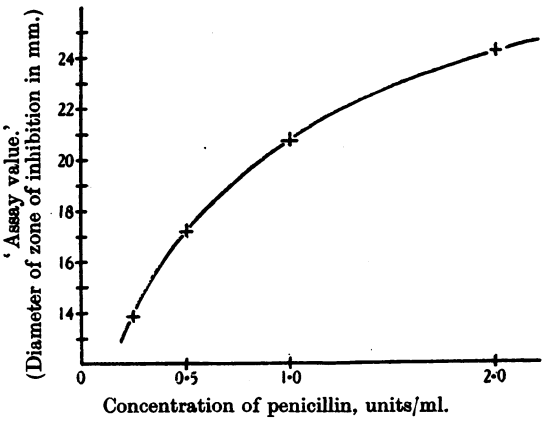

Fig. 1.

are complex and not understood. In the early days replicate assays were usually set up in an identical fashion on successive plates (i.e. each sample would always be in the same position relative to the side to which the plate was drained). Good agreement between replicates was obtained, but it will be realized that if the above-mentioned effect came into play, the mean value for each sample would have a bias depending on the position of that sample on the plates. Errors from this cause can be eliminated or minimized by suitably arranging the replicates on the successive plates. Thus in a quadruplicate assay the samples are arranged in the same order on all four plates, but on each successive plate the series is begun 90° farther round relative to the side to which the seeding fluid was

Table 1. *Assay values produced by standard solutions containing known concentrations of penicillin in nine consecutive tests*

No. of test	Diameter of zone of inhibition in mm. ('assay values') for penicillin solutions containing: 2 units/ml. — Individual data	2 units/ml. — Mean	1 unit/ml. — Individual data	1 unit/ml. — Mean	0·5 unit/ml. — Individual data	0·5 unit/ml. — Mean
1	22·8, 23·6, 22·3, 21·0	22·4	20·2, 21·2, 18·4, 19·1	19·7	16·1, 17·7, 15·1, 13·4	15·6
2	23·8, 23·3, 23·6, 23·5	23·65	18·8, 19·8, 19·6, —	19·4	15·1, 17·8, 15·3, 17·0	16·3
3	22·3, 22·2, 23·3, 23·0	22·7	19·1, 19·2, 19·1, 20·3	19·4	15·1, 15·7, 17·5, 15·9	16·05
4	22·2, 23·2, 22·7, 21·6	22·4	21·0, 17·8, 19·8, 19·7	19·6	17·4, 15·9, 16·6, 15·6	16·4
5	22·9, 23·2, 23·2, 22·8	23·0	20·0, 19·8, 20·2, 17·8	19·45	16·9, 16·2, 17·9, 14·0	16·25
6	23·0, 23·4, 23·7, 24·6	23·7	20·2, 20·6, 20·2, 21·3	20·6	16·4, 17·1, 15·3, 16·9	16·4
7	22·1, 23·3, 22·2, 22·1	22·4	18·8, 18·6, 18·8, 19·4	18·9	17·8, 15·4, 16·1, 17·1	16·6
8	24·3, 24·1, 23 · 22·0	23·6	21·0, 20·2, 19·3, 20·6	20·3	14·8, 16·5, 16·6, 15·6	15·9
9	23·1, 24·7, 23·1, 24·0	23·7	19·5, 20·4, 19·2, 20·1	19·8	14·2, 15·1, 15·3, 15·2	14·95

drained. If this procedure is adopted there will often be a wider scatter in the individual values for one sample, but their *average* values for successive days or runs will agree better.

THE PENICILLIN STANDARD

It is now generally accepted that, for this and most other methods of assay of penicillin, accurate results can be ensured only by comparing the unknowns against a standard of known potency. The first requisite of such a standard is stability, and it has been suggested that mercuric chloride, proflavine or some other stable and easily characterized inhibitor should be used. The arguments against such a course are: (*a*) different strains of test organism will probably respond differently to the proposed standard and to penicillin, which, after all, behaves like no known antiseptic; (*b*) even if a standard test organism is used there is no reason to suppose that if its sensitivity to penicillin changed—and such changes are known to occur spontaneously—its sensitivity to the standard would change in a similar way (Rammelkamp & Maxon, 1942; McKee & Houck, 1943); (*c*) the response to two quite different chemical substances will probably not be identical when slight uncontrolled variations occur in the conditions of the test (see above: *Arranging solutions on the plates*).

For these reasons penicillin itself was chosen as the standard. In 1940 a purely arbitrary unit for internal use in this laboratory was adopted as that amount of penicillin contained in 1 ml. of a certain phosphate buffer solution containing ether. In 1941 a dry sodium salt (containing 42 units/mg.) was standardized against this, and later another primary standard—a barium salt containing 4·4 units/mg.—was prepared. Though substandards in this laboratory and elsewhere have undoubtedly deteriorated, by good fortune the above two primary standards have shown no detectable loss of potency over many months, even when portions were transported to hot climates. The 'Oxford unit' has been adopted by a number of other workers.

Attention may be drawn here to the statement by Florey & Jennings (1942) that 'For those using the dilution method it may be stated that the "Oxford unit" is that amount of penicillin which when dissolved in 50 ml. of meat extract broth just inhibits completely the growth of the test strain of *Staphylococcus aureus*.' It should be pointed out that this method of standardization, though it may give consistent results in the hands of any one group of workers—but see Foster & Woodruff (1943*a*)—is subject to many variables (quality of broth, duration of incubation, etc.) and cannot be taken as necessarily accurate under the conditions prevailing elsewhere.

REPRODUCIBILITY: LIMITS OF ACCURACY

A quantitative idea of the reproducibility of the method is afforded by a series of 27 assays of a given preparation against the same standard. Each single potency value was derived from the mean of four zone measurements, the values being 75, 75, 66, 77, 88·5, 63, 68, 72, 77·5, 77, 77, 85·5, 64·5, 72·5, 76, 66·5, 69, 71·5, 81·5, 65, 71·5, 69, 73·5, 79, 63·5, 81, 73. The coefficient of variation of this series (*Series A*) is 9·0%. Two further series with ten assays in each were run in an exactly similar way with the following results: *Series B*: 56, 56, 62, 61, 51·5, 56·5, 57, 55, 56, 60; coefficient of variation, 5·6%. *Series C*: 70·5, 62, 72, 68·5, 67, 72, 65, 71·5, 65, 68·5; coefficient of variation, 5·0%. In a more detailed statistical analysis of the first series, Dr O. L. Davies, of Imperial Chemical Industries Ltd., found that the error calculated from the variation within single tests was substantially the same as that calculated from variation between tests.

The scatter within tests and between successive tests is illustrated by Table 1, which gives the zone measurements actually recorded for the standard solutions set up in a consecutive series of assays. The unknown solutions would show a similar degree of scatter.

If 9% is taken as the coefficient of variation which may be expected, then for $P = 0{\cdot}01$ the limits of accuracy for a single assay in quadruplicate (i.e. a value derived from measurement of four zones) will be $\pm 23{\cdot}2\%$. For an assay in triplicate the limits will rise to $\pm 26{\cdot}9\%$ and for an assay derived from two zone measurements only they will be $\pm 36{\cdot}7\%$. For $P = 0{\cdot}05$, the limits will be $\pm 19{\cdot}5\%$, $\pm 22{\cdot}7\%$ and $\pm 27{\cdot}7\%$ respectively. In series B and C the degree of reproducibility was considerably greater than this, though the reason is not known.

POSSIBLE MODIFICATIONS AND APPLICATIONS OF THE METHOD

Substitutes for cylinders. Foster & Woodruff (1943*a*) mention that Dowdy, Vincent & Vincent have used, instead of cylinders, circular disks of filter paper which are merely placed on the surface of a seeded plate and impregnated with the solution to be tested. This same method (using disks of thick filter fabric) was proposed in 1940 by Dr C. G. Pope (private communication). In the hole or cup method, which has been used by Fleming (1942) for many years, the solutions to be tested are simply placed in holes punched in a bulk-seeded plate by means of a cork borer and sealed at the bottom with two drops of melted agar. Wilkins & Harris (1943) have used a similar method for studying antibiotics of fungal origin.

Little is known of the upper limits of accuracy of these methods, but a possible disadvantage of all of them is the absence of a barrier to prevent any bacteria contaminating the test samples from spreading over the surface of the plate.

Application to substances other than penicillin. Great caution must be exercised before placing reliance on results obtained by this method when applied to other antibiotics. If the inhibitor is not diffusible, the method is obviously of no use. The composition of the agar may need special attention (e.g. the presence of peptones would presumably interfere with sulphonamides; penicillin B or notatin gives large zones of inhibition in the presence of glucose, but none in its absence). With some substances (e.g. helvolic acid (Chain, Florey, Jennings & Williams, 1943)), the curve relating diameter of inhibition zone to concentration of inhibitor is too flat to be of much use for quantitative work. Results with sparingly soluble inhibitors may need special interpretation. Change in pH of the medium during growth of the bacteria may influence the assay value in a variety of ways. If the potency of the inhibitor depends on the number of bacteria being inhibited, special procedures for ensuring even and reproducible seeding of the plates may be required. Other potential limitations of the method can be imagined, but the method has been successfully applied to the quantitative assay of gigantic acid (Philpot, 1943) and of substances of plant and fungal origin quite different in character from penicillin. The method has been of use in the semi-quantitative investigation of several other substances.

SUMMARY

1. Full practical details are given of a method of assay of penicillin which is applicable also to certain other antibiotics.

2. The method compares favourably as regards speed and accuracy with most other methods and has been in routine use for nearly 4 years.

3. Less than 1 ml. of solution (which need not be sterile) is required for an assay and the presence of ether or chloroform does not interfere.

4. Quantitative information on limits of accuracy is given.

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Technical Notes on the Partition Chromatography of Acetamino-acids with Silica Gel

By A. H. GORDON, A. J. P. MARTIN and R. L. M. SYNGE, *Wool Industries Research Association, Torridon, Headingley, Leeds, 6 and The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1*

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We record here some technical aspects of the experience which we have gained in the use of our partition chromatographic method (Gordon, Martin & Synge, 1943*a*) for the quantitative analysis of amino-acid mixtures.

PREPARATION OF SILICA GEL

The characteristics of different batches of silica gel have been found to vary so much as a result of differences in starting material and minor variations in preparative procedure that we have introduced a standard test to which

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Iodometric Method for Assay of Penicillin Preparations

J.F. Alicino

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between fatty acids and proteins in aqueous media has been known to exist. Thus the free fatty acids in shell eggs which have undergone some deterioration would have an opportunity to combine with protein and the pH should be adjusted to 4 prior to drying the sample.

The lowest moisture content at which interaction of the fatty acids and proteins occurs is not known. However, egg powders containing as much as 8% moisture gave the same free fatty acid values before and after adjustment of the pH. Since few dried egg samples are prepared at higher moisture levels, no adjustment of the pH of dried powders that have been prepared from good shell eggs seems necessary.

INTERFERENCES AND LIMITATIONS

The production of lactic acid in certain types of egg deterioration has been reported (*7*). Lactic acid is, of course, easily soluble in water and is not determined in the method outlined above. Amounts of lactic acid corresponding to 14 to 50 mg. per gram of egg powder were added to samples of the powder and were shown to make no contribution to the acidity of the samples.

The method would not be applicable to samples of materials likely to contain fatty acids that are water-soluble, since they would probably be incompletely partitioned between the aqueous acetone and the petroleum ether in the washing process, thus leading to low results. Since the lower fatty acids have been shown to be absent from egg fat (*10*), such losses do not occur in the determination of egg fatty acids arising from the usual type of glyceride hydrolysis.

Moderate excesses of acids, added to lower the pH of the sample, do not interfere, since they are removed during the washing procedure.

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Iodometric Method for the Assay of Penicillin Preparations

JOSEPH F. ALICINO, Division of Organic Chemistry, Squibb Institute for Medical Research, New Brunswick, N. J.

TWO methods for the chemical estimation of penicillin, recently proposed (*4*, *5*), depend on the liberation of an acidic group upon inactivation of penicillin with penicillinase or with alkali. The simple, rapid inactivation by alkali was being utilized in this laboratory in conjunction with the development of an iodometric assay method which was suggested by observations (*3*) on the behavior towards iodine of penicillin and certain of its inactivation products. Penicillin is inert to iodine in neutral aqueous solution, while penicilloic acid (*1*), the inactivation product formed from penicillin by the action of penicillinase or of alkali, consumes from 6 to 9 equivalents of iodine, depending on the conditions used. The difference in the iodine consumption under standard conditions before and after inactivation by alkali might therefore be expected to be approximately proportional to its penicillin content as ascertained by biological assay. This was found to be the case, with certain limitations.

EXPERIMENTAL

Iodine Equivalent of Pure Sodium Penicillin G Inactivated with Alkali. The iodine consumption of a neutral solution of alkali-inactivated sodium penicillin G is a function of time, and under the conditions given below becomes constant after 25 to 30 minutes.

A weighed sample (3 to 5 mg.) of crystalline sodium penicillin G is place in a glass-stoppered flask, dissolved in 5 to 10 cc. of water, and treated with 0.5 cc. of 1 *N* sodium hydroxide. The alkaline solution is allowed to stand for 15 minutes and then neutralized with 0.5 cc. of 1 *N* hydrochloric acid. A measured excess of 0.01 *N* iodine solution (about 10 cc.) is added. After 30 minutes the unconsumed iodine is titrated with 0.01 *N* thiosulfate. It was found that 1 mg. of sodium penicillin G when inactivated will consume under these conditions 2.52 cc. of 0.01 *N* iodine solution, corresponding to 8.97 equivalents of iodine per mole, which is in reasonably good agreement with the range given (*3*) for penicilloates (8.5 to 8.9). This value is well reproducible and independent within fairly wide limits of the penicillin concentrations and the excess iodine used.

Sodium penicillin F (crystalline, anhydrous) after inactivation treated in this manner consumed 2.64 cc. of 0.01 *N* iodine solution per milligram, or 8.8 equivalents per mole, showing that the double bond in this compound is inert to iodine under these conditions. This was confirmed by the blank titration (without prior alkali treatment) which showed an uptake of only 0.07 cc. per milligram. However, it is probable that penicillin X might give abnormally high values in this procedure.

Assay of Unknowns. In the assay of unknowns an amount of material corresponding to approximately 1000 to 5000 units is inactivated with alkali, and the neutralized solution is treated with iodine and back-titrated after 30 minutes as described above for pure sodium penicillin G. A blank titration in which the treatment with alkali is omitted is carried out on a separate sample of the same magnitude. The difference between both titrations can be used, as indicated below, to calculate either international units or micromoles of penicillin.

When relatively pure preparations (800 to 1000 micrograms per mg.) were assayed in this manner, the figures agreed well with the bioassay values regardless of the time allowed for contact of the blank sample with excess iodine (30 minutes as in inactivated sample, or immediate back-titration), the small amounts of iodine-consuming impurities present in such preparations having little influence on the final result. However, with preparations of lower potency containing proportionately greater amounts of iodine-consuming impurities, the iodometric results were considerably lower than the bioassay figures when the 30-minute interval between addition of iodine and back-titration was maintained also in the blank determination. A possible explanation for this is that products formed from these impurities by reaction with iodine catalyze the slow hydrolysis of penicillin to penicilloic acid normally occurring even in neutral reaction, giving rise to an erroneously high blank value, since the latter would include the penicilloic acid thus formed from penicillin. This assumption is supported by the finding that the addition of crystalline penicillin to an impure sample increases the blank titration (after 30 minutes' contact with excess iodine) over the blank value (also determined after 30 minutes standing) given by the impure sample alone.

It was found empirically that the discrepancy between iodometric and bioassay values in the case of low-grade preparations was minimized when the blank value arrived at by immediate back-titration was used in computing the results. In the final

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Rapid Method for Estimation of Penicillin

Andres. Goth and Milton T. Bush

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Note: In lieu of an abstract, this is the article's first page.

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eliminated to a large extent in the modified Low method by dissolving the sample in prefumed sulfuric acid, whereupon the copper precipitates as the anhydrous sulfate in which form it is harmless.

Reduction of antimony, arsenic, and copper with sulfurous acid in strong hydrochloric acid solution is slow, owing to complex formation, but is greatly accelerated by the addition of bromide.

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Rapid Method for Estimation of Penicillin

ANDRES GOTH[1] AND MILTON T. BUSH, Vanderbilt University School of Medicine, Nashville, Tenn.

A rapid method for the estimation of penicillin is based on the observation that penicillin inhibits the production of nitrite in *Staphylococcus aureus* cultures. The test can be carried out in 60 to 90 minutes. The accuracy is greater than that of a standard serial dilution method.

THE commonly used methods for the estimation of penicillin are based on its inhibition of the multiplication of a test organism, usually *Staphylococcus aureus*. With the thought that instead of observation of the multiplication of cells, metabolic processes of these cells could serve for the estimation of antibiotic substances, the authors investigated the production of nitrite from nitrate by actively growing *Staphylococcus aureus* cultures and found that this metabolic property would lend itself to the determination of penicillin. Since nitrite production manifests itself rapidly in an actively growing culture, penicillin could be estimated by this method in a little over an hour.

The method is based on the following facts:

Actively growing *Staphylococcus aureus* cultures produce nitrite from nitrate (Table I).

When penicillin is added to such a culture the authors have observed a gradual decrease in nitrite production, probably as a result of inhibition of multiplication. Within limits the decrease in nitrite production is a function of the concentration of penicillin (Figure 1).

The amount of nitrite produced can be determined by a colorimetric method.

The sensitivity of the test depends on the heaviness of the inoculum. The more concentrated the Staphylococcus suspension, the more penicillin is required to produce a definite decrease in the production of nitrite. A 1 to 4 suspension of a 24-hour culture was found satisfactory for carrying out the test in 60 to 90 minutes. In 5-cc. amounts of this bacterial suspension, 0.5 Oxford unit of penicillin will produce a definite decrease in the production of nitrite. The sensitivity of the test can be increased considerably by reducing the inoculum; however, there is a corresponding increase of the lag phase in the nitrite production and consequently a longer time is required for carrying out the test.

[1] Present address, Southwestern Medical College, Dallas, Texas.

Table I. Reduction of Nitrate to Nitrite by Suspensions of a 24-Hour *Staphylococcus aureus* Culture

Dilution of Culture	Nitrite Produced (Expressed as Sodium Nitrite) 60 minutes	75 minutes	90 minutes
	Mg. per 100 cc.		
1:4	4.3	9.5	21.6
1:4	4.6	10.1	22.4
1:5	2.8	6.9	18.0
1:5	2.8	6.8	18.0
1:6	1.9	4.3	8.6
1:6	1.9	4.0	8.2

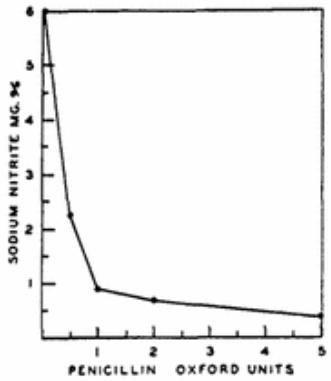

Figure 1. Effect of Penicillin on Nitrite Production by *Staphylococcus aureus*

A standard solution of calcium penicillin is tested together with the unknowns and in this manner the anti-Staphylococcus activity of the unknowns can be expressed in Oxford units. The standard sample of calcium penicillin containing 135 Oxford units per mg. was obtained from the Northern Regional Research Laboratories, U. S. Department of Agriculture, Peoria, Ill.

The microorganism used in these experiments was a *Staphylococcus aureus* strain isolated from a human septicemia. The

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QUANTITATIVE ESTIMATION OF PENICILLIN

Sirs:

Various groups of workers on penicillin concur with the idea that at present an urgent requirement is a sensitive and reliable method for quantitative determination of this and other antibiotic material. Following the discovery that graded doses of penicillin produce quantitatively a proportional inhibition of growth of *Staphylococcus aureus* in nutrient broth, a simple, rapid, and relatively sensitive method of assay has been developed. Growth is measured turbidimetrically as a function of penicillin concentration.

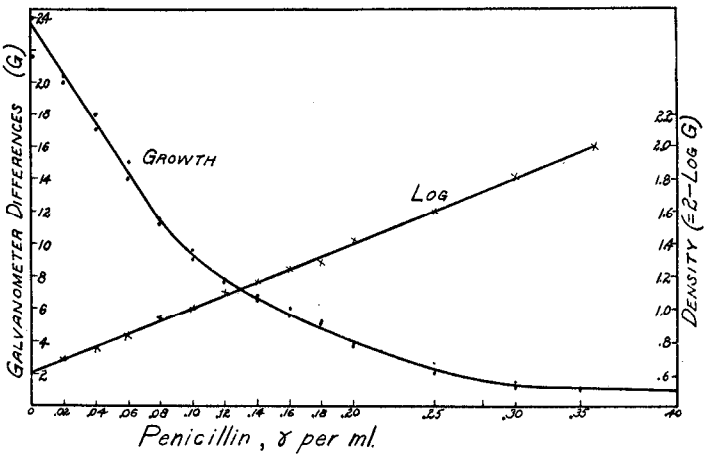

FIG. 1. Inhibition of *Staphylococcus aureus* by penicillin (incubation 16 hours at 37°). Galvanometer differences were obtained by subtracting observed readings from that of uninoculated medium control. The penicillin preparation contained 42 Florey units per mg.

Growth in various dilutions of unknown penicillin samples is compared with a standard curve of inhibition run daily side by side with the unknowns. Computation of the potency of the unknown with respect to the standard reference sample of established potency then is an easy matter. Accuracy is that common to microbiological work; namely, ±10 to 15 per cent.

Fig. 1 shows the growth response as a function of penicillin concentration. The theoretical basis for the inhibition curve of penicillin and the assay method based on this principle seem established by the logarithmic nature of the inhibition.

Use of a stable reference standard is, of course, imperative. Activities

usually are expressed in terms of Florey units, following the lead of the Oxford group. Penicillin preparations of proved stability should be standardized either directly or indirectly against penicillin preparations of known Florey unitage. Use of a standard eliminates variations due to the culture, media, personal factor, etc. In this work the Oxford strain of *Staphylococcus aureus* has been used.

To tubes containing 5 ml. of various dilutions of penicillin samples are added 5 ml. of sterile, double strength nutrient broth, inoculated just prior to apportionment. For inoculum, 0.4 ml. of a 20 hour nutrient broth culture of the test organism is added to 100 ml. of the double strength broth. Dilutions are made as follows: The original samples of penicillin are diluted with ice-cold sterile 0.02 M phosphate buffer at pH 7.2 so as to contain approximately 0.02 unit per ml. These, together with the standard, are kept in an ice bath until all the samples have been treated similarly. Different amounts, namely 0.5, 1.0, 2.0, 3.0, and 5.0 ml., are then added aseptically to tubes previously sterilized with 4.5, 4.0, 3.0, 2.0, and 0 ml. of buffer, respectively.

Aseptic precautions should be observed throughout. In assaying dry preparations of penicillin, contaminants are not a serious factor owing to the ultimate high dilution and short incubation. With *Penicillium* filtrates or penicillin solutions contaminations may, however, be serious. Such liquids, when possible, should be obtained aseptically and maintained sterile. Otherwise they should be kept cold (0–5°), or saturated with ether or chloroform to minimize contamination. These solvents are without effect on the test organism under these conditions.

After 16 hours (overnight) incubation at 37°, the tubes are shaken, the contents poured into calibrated Evelyn tubes, and the turbidimetric readings obtained. Galvanometer differences (per cent transmissible light) are plotted against penicillin concentration. Three to five levels are run on each unknown, depending on how many can be predicted to fall on the central (three-fourths) region of the curve. A short time (4 hour) assay employing this principle has been developed.

In practice the log curve may be used as reference; only 3 or 4 points are required to define the log curve. Other aspects will be reported later.

Research Laboratories
Merck and Company, Inc.
Rahway, New Jersey

JACKSON W. FOSTER

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QUANTITATIVE ESTIMATION OF PENICILLIN

Jackson W. Foster

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REVIEW ARTICLE

THE ESTIMATION OF PENICILLINS AND PENICILLIN DESTRUCTION

By J. M. T. Hamilton-Miller, B.A., J. T. Smith, Ph.D. and R. Knox, M.D., F.R.C.P.

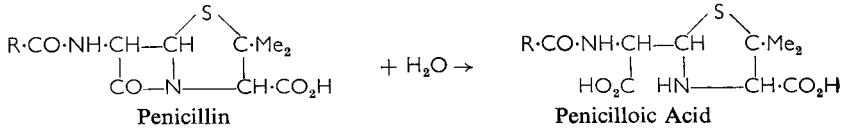
*Department of Bacteriology, Guy's Hospital Medical School, London, S.E.*1

Research into the biological and chemical aspects of penicillin and its production has been actively pursued for almost 35 years; the announcement of the production of the penicillin "nucleus", 6-aminopenicillanic acid, on a commercially attractive scale (Batchelor, Doyle, Nayler and Rolinson, 1959) has given a tremendous stimulus to these studies in the past few years. With the increased range of penicillins now already available for clinical use, and the possibility of further compounds with new specificities in the future, there is a new need for a review of methods for estimating penicillins and penicillin-destroying enzymes. This review covers chemical, physical and microbiological methods and attempts to indicate which of these is most suitable for the varying needs of present day research in this field.

Penicillin-destroying enzymes

Penicillinase

An account of the distribution of these enzymes is given by Abraham (1951). The enzymes act by rupturing the β-lactam ring of penicillins, and perhaps should be more precisely called β-lactamases (Pollock, 1961), as shown in equation 1.



Penicillin — Penicilloic Acid

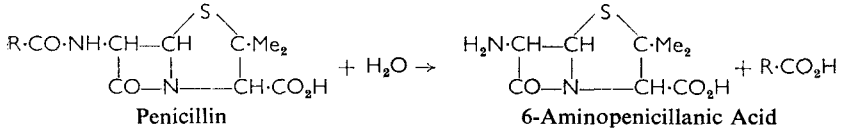
Equation 1

The antibacterial activity of the penicillin is for all practical purposes destroyed by this cleavage, which can also be brought about by alkali treatment. The nomenclature of the penicilloic acids, unlike that of the penicillins (Sheehan, Henery-Logan and Johnson, 1953) has not been strictly defined. "Penicilloic acid" is, strictly speaking, a general name and should be qualified, for example, "benzylpenicilloic acid", or "penicilloic acid G" derived from benzylpenicillin: however, the term has unfortunately been much used to refer specifically to the hydrolysis product of penicillin G. The use of trivial names, such as "methicilloic acid" from methicillin and "penicic acid" (Huang and others, 1960; Murao, 1955) from 6-aminopenicillanic acid, is hardly a satisfactory compromise. It would appear that the best solution to this problem of nomenclature of the penicilloic acids is to qualify the name of each individual compound by the full side-chain, for example, phenylacetamidopenicilloic acid from penicillin G, phenoxypropionamidopenicilloic

acid from phenethicillin, and aminopenicilloic acid from 6-aminopenicillanic acid.

Amidase

The properties and distribution of these enzymes, now of great commercial importance, are described by Batchelor and others (1959), Rolinson and others (1960), Claridge, Gourevitch and Lein (1960) and English, MacBride and Huang (1960). The latter authors call the enzymes "acylases". They act by removing the side-chain attached in the 6-amino-position of the penicillin nucleus.



Penicillin — 6-Aminopenicillanic Acid

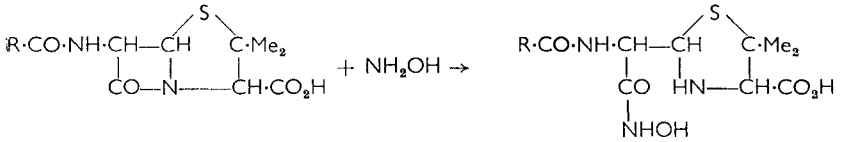
Equation 2

Unlike penicillinases, amidases catalyse both the forward and the backward reaction, and also do not completely destroy antibacterial potency, since the product of the reaction, 6-aminopenicillanic acid, also possesses some limited biological activity (Rolinson and Stevens, 1961).

Quantitative assays

Chemical

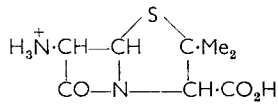
Hydroxylamine (Staab, Ragan and Binkley, 1946). This original method has been modified by Boxer and Everett (1949) and Ford (1947), amongst others. Hydroxylamine reacts with penicillins at pH 7·0 as shown by equation 3.



Equation 3

This reaction is complete within about 10 min. at room temperature and at pH values between 6 and 8. The product is a hydroxamic acid, stable for 2 hr., which forms a coloured internal salt with ferric ions, of unknown structure. This chromogen is stable for about 5 min., but can be further stabilised (Henstock, 1949) by extraction into n-butanol; its absorption is proportional to the concentration of penicillin, between 10–2,000 μg./ml., and can be measured colorimetrically at 500 mμ (Boxer and Everett, 1949; Batchelor, Chain, Hardy, Mansford and Rolinson, 1961). A spectrophotometer can be used, but this is inconvenient because of the short life of the chromogen, and because the method involves the mixing of alcoholic and aqueous solutions, and many gas bubbles are hence formed. The individual penicillins produce, mole for mole, differing colour intensities, and there does not seem to be any

simple relationship between chemical structure and chromogenicity of the hydroxamate-ferric complex. Hydroxylamine will react only with the molecular species having an intact β-lactam ring: hence during penicillinase assays, the substrate which remains rather than the product formed is being measured. Penicillin amidase activity cannot be measured directly by this assay, as the 6-aminopenicillanic acid produced by the hydrolysis still has an intact β-lactam ring. A method for the estimation of this enzyme is described by Batchelor, Chain and Rolinson (1961). At pH 2, 6-aminopenicillanic acid has a net positive charge,



while other penicillins are unionised, and exist as free acids which, being hydrophobic, are extractable into a suitable non-polar solvent, as for example, n-butyl acetate. The hydrophilic 6-aminopenicillanic acid remains in the aqueous phase, where it can be assayed by the hydroxylamine method.

Hydroxylamine does not react only with the penicillins. Hydroxamates are also formed with compounds containing a carbonyl group such as esters, when a purple chromogen is formed with the Fe^{3+}. For example, Lipmann and Tuttle (1945) describe the use of this assay for acyl phosphate determination at acid pH values. Despite this lack of specificity, however, the method is valuable for obtaining absolute rates of breakdown of penicillins, specificity patterns of different enzymes, or Michaelis constants (see Batchelor, Cameron-Wood, Chain and Rolinson, 1961; Knox and Smith, 1962, for examples). The chief disadvantage is the lack of sensitivity, as the lower limit for most penicillins is 20 μg./ml. even under the best conditions. This method has been adapted for automatic operation by Niedermayer, Russo-Alesi, Lendzian and Kelly (1960), for estimating up to 10,000 units/ml. (6 mg./ml.).

Iodometric (Alicino, 1946; Tucker, 1954; Perret, 1954). Penicilloic acids take up iodine; the stoichiometry of the reaction has not been worked out—it is known only that 8–9 atoms of iodine are taken up by each molecule of benzylpenicilloic acid. Penicillins which possess an unsaturated aliphatic side chain, such as penicillin O (allylmercaptomethyl) (Grove and Randall, 1955), and penicillin F (Δ^2-pentenyl) will reduce iodine while the β-lactam ring is intact; *p*-hydroxybenzylpenicillin also behaves in this way (Sneath and Collins, 1961). Iodine is absorbed by many other organic compounds, especially those that are unsaturated, and careful blank estimations must therefore be made. But as most unchanged penicillins do not reduce iodine, the amount of it taken up depends on the amount of penicilloic acid formed. Comparative assays using the hydroxylamine and iodometric methods give closely similar rates of hydrolysis by penicillinases (Boxer and Everett, 1949). The iodometric method cannot be used to follow hydrolysis by amidase, as 6-aminopenicillanic acid does not reduce iodine.

Perret's method estimates iodine uptake by sampling the reaction mixture into excess iodine, and back-titrating with standard thiosulphate. Citri (1958) uses a simplified version, in which the time taken for a standard amount of blue starch-iodine complex to be totally decolorized is used as a measure of reaction velocity. Weiss (1959) has reviewed the factors affecting the accuracy and sensitivity of the macro-iodometric assay.

Newer micro-iodometric assays, depending upon absorption by the I_3^- complex at 360 mμ (Goodall and Davies, 1961) and at 420 mμ (Ferrari, Russo-Alesi and Kelly, 1959), and on the absorption of the starch-iodine complex at 600 mμ, have been developed. The automatic method described by Goodall and Davies (1961), has been modified by Beecham Research Laboratories (Batchelor, F.R., personal communication). Novick (1962a,b) also used the starch-iodine complex as the chromogen, measuring absorption at 620 mμ with a spectrophotometer. He found that his micro-method gave 40 per cent lower activities for penicillinase than those obtained by the macro-method. The methods using the starch-iodine complex are very sensitive; about 1 μg./ml. of penicillin can be accurately estimated. Novick (1962a) has used his assay to determine the K_m of penicillin G for staphylococcal penicillinase, and it can obviously be extended to measure other K_m values for penicillins with especially high affinities for penicillinase.

Other methods. Penicilloic acids, unlike nearly all other organic compounds, will reduce arsenomolybdate at room temperature, in the presence of traces of mercuric chloride, to a chromogen "molybdenum blue" which is measurable colorimetrically (Pan, 1954). A semi-automatic modification has been described (Green and Monk, 1959).

Hiscox (1949) developed a method whereby the amount of ferricyanide reduced to ferrocyanide by penicillins is measured by back-titration with ceric sulphate. The assay can be used only for crystalline penicillins.

Scudi (1946) found that penicillins react with *N*-(1-naphthyl-4-azobenzene)ethylenediamine to form a red chromogen, which is measured at 540 mμ. The same author has also developed a very sensitive fluorimetric procedure (Scudi and Jellinek, 1946).

Acidimetric

When penicillins are hydrolysed by penicillinase a new carboxyl and a secondary amino-group are generated (equation 1). The latter, being weak, has little or no effect on the net production of hydrogen ions and hence the pH falls in proportion to the amount of hydrolysis. Benedict, Schmidt and Coghill (1945) found, by electrometric titration, that the pK of the newly-formed carboxyl group, in the case of benzylpenicillin, was 4·7 (compare the pK of the original carboxyl group, 2·16). Batchelor, Chain, Hardy, Mansford and Rolinson (1961) similarly found the pK of the penicilloic acid of 6-aminopenicillanic acid to be 3·7.

When the side chain is removed from a penicillin by hydrolysis with an amidase (equation 2), a new carboxyl and a primary amino-group are formed. Here the amino-group is sufficiently strong to absorb hydrogen

ions and hence the pH falls progressively more slowly as the reaction proceeds. Initial velocities can be taken by the usual method of drawing tangents, and hence reaction velocities, and Michaelis constants, could be calculated.

Comparison of rates of hydrolysis of different penicillins by penicillinase cannot be directly undertaken by these methods as different penicilloic acids are likely to have different pK values so that the fall in pH per molecule hydrolysed will differ for each penicillin.

The rate of production of hydrogen ions, and hence the rate of hydrolysis of penicillin, can be determined in the following ways.

Manometric assay (Foster, 1945). This method was standardised by Henry and Housewright (1947) and was modified further by Pollock (1952); it depends on the fact that the volume of CO_2 liberated from a bicarbonate buffer as penicillin is hydrolysed to penicilloic acid is proportional to the rate of hydrolysis. Manometric methods are time-consuming, especially where gassing is necessary; but many workers (Pollock, 1952; Pollock and Torriani, 1953; Steinman, 1961a, b; Crompton and others, 1962; Leitner and Cohen, 1962; and others) still use this assay.

Retention of either CO_2 or hydrogen ions will result in diminished gas evolution and thus cause misleading results. The results of Steinman (1961a) show that the hydrolysis of 6-aminopenicillanic acid by penicillinase cannot be estimated manometrically. Hydrolysis of 6-aminopenicillanic acid results in the formation of not only a carboxyl group but also of an α-amino group, and hence there is no net production of hydrogen ions. Furthermore Batchelor, Gazzard and Nayler (1961) showed that CO_2 reacts with 6-aminopenicillanic acid forming first a carbamate then a penillic acid.

The method is mainly used for the assay of penicillinases, although originally described also for penicillin assay. It appears that, at present, this assay method is in the process of being superseded by other methods.

Alkaline titrimetric methods. Patterson and Emery (1948) described a method for the estimation of benzylpenicillin in which the amount of standard alkali required to split the β-lactam ring was measured by back-titration with acid. Murtaugh and Levy (1945) used a pH stat for the same purpose, and Jeffrey, Abraham and Newton (1961) used this principle to follow the alkaline hydrolysis of cephalosporin C. The pH of the reaction mixture is kept constant by the automatic addition of sodium hydroxide solution, the rate of addition being proportional to the rate of hydrolysis. The latter method has also been used by Crompton and others (1962) to follow the course of alkaline hydrolysis of penicillin G, methicillin and deacetyl cephalosporin C.

Indicator method. The rate of change in pH is followed, in this method, by means of an indicator. The absorption of the alkaline form of the indicator at a certain wavelength will decrease as more penicilloic acid is formed. The indicator of choice is that whose pK coincides roughly with the pH at which the reaction is carried out (for example, phenol red pK = 7·8 at pH 7·6; bromothymol blue pK = 7·0 at pH 7·0; bromcresol

purple pK = 6·2 at pH 6·5, etc.). The method of Nirenberg (cited by Saz, Lowery and Jackson, 1961) measures the decrease in the red form of phenol red by determining the absorption at 558 mμ with a spectrophotometer. Work in this laboratory with bromothymol blue and bromcresol purple has shown the maximum absorption of the alkaline forms of these indicators to be at 645 mμ and 615 mμ respectively: the sensitivity of the method is shown by the fact that, in the presence of M/240 phosphate buffer and 0·0013 per cent (w/v) aqueous phenol red, a change in absorption of 0·001 unit (the smallest change that can be accurately detected) represents a change in pH of 0·0012 units. The absorption of the indicators changes in an almost linear fashion with pH over a reasonable range (for example between pH values of 7·8 and 7·1 for phenol red), and when following hydrolytic reactions, straight line progress plots are obtained. The method can also be used with a colorimeter to follow the reaction: this increases the flexibility of the assay.

Michaelis constants can be determined conveniently with this assay, but probably its greatest use is in detecting changes in reaction velocity (see Saz and others, 1961, for action of activators on staphylococcal penicillinase), particularly in relation to inhibition studies, which, up to now, have been studied mainly by the manometric method (Abraham and Newton, 1956). By virtue of its far greater convenience and increased ease of operation, it is possible that the indicator method may take the place of manometric methods in this respect.

Miscellaneous

Microbiological assay. The "Oxford cup method" was first described by Abraham and others (1941) and further developed by Heatley (1944). It has been modified by Schmidt and Moyer (1944), McKee, Rake and Menzel (1944), and Foster and Woodruff (1943b, 1944). The latter (1943a) also gave a carefully reasoned critique of various microbiological methods and finally recommend the Oxford cup method; in addition, Heatley (1948), Lees and Tootill (1955) and Kavanagh (1960) have reviewed this topic. The theory of diffusion of substances through agar has been worked out by Cooper and his associates (Cooper, 1955; Cooper and Linton, 1952; Cooper and Woodman, 1946).

The method is valuable for the determination of the antibacterial activity of clinical specimens and particularly useful for the investigation of the inactivation of penicillins by proteins, and it is more sensitive than any chemical method to date. Microbiological assay methods, however, are not particularly accurate, require many controls and are time-consuming. In addition, enzymic destruction of penicillins cannot be studied since there is no method available to stop the reaction which would not also interfere with the biological assay.

The choice of test organism is important when dealing with penicillins active against organisms which are relatively resistant to penicillin G. *Sarcina lutea* is widely used because of its high sensitivity to penicillin G and *B. subtilis* and *Staph. aureus* are also often used. But with ampicillin, which possesses activity against Gram-negative organisms, perhaps it

would be better to use a Gram-negative organism, and with penicillinase-resistant penicillins a penicillinase-producing organism may be a better choice as indicator strain. This could be vital when a penicillin is altered in the body (see Rollo, Somers and Burley, 1962) to become a compound which may be active against a badly chosen indicator organism but not active against the organism for which the penicillin was designed.

Spectrophotometric methods. Various direct spectrophotometric methods were described in the early days of large-scale penicillin production, mainly for estimating the amount of penicillin in fermentation liquors. Methods utilising absorption in the ultra-violet range have been reviewed by Colon, Herpich, Neuss and Frediani (1949) and Twigg (1949). The latter also discusses infra-red methods.

Polarimetric method. Abraham and Newton (1956) followed the alkaline hydrolysis of penicillin G by the change in optical rotation as sodium penicillin G was converted into sodium D (+)-α-benzylacetamidopenicilloate. The method is complicated by the fact that mutarotation of the product occurs.

Qualitative assays

Filter membrane method (Knox and Smith, 1961). This is a simple qualitative test for the production of acid by colonies when incubated for a short time with penicillin G and is a modification of the pour-plate method of Manson, Pollock and Tridgell (1954). Organisms grown on membranes are transferred to a solution of penicillin G containing Andrade's indicator: acid-producing colonies take up a red colour; controls can be tested without penicillin. Colonies producing penicillinase or amidase are hence positive. The results can be confirmed microbiologically as later described by Knox and Smith (1961). A modification has been described (Novick, R.P., personal communication) using *N*-phenyl-1-naphthylamine azocarboxybenzene as indicator; this compound precipitates as it changes to the purple coloured form at acid pH values, and thus diffusion of the dye does not occur; this modification is of use in the study of mixed populations.

Iodometric method. Foley and Perret (1962) have described a method based on the principle of the iodometric assay (Perret, 1954): penicillinase-producing colonies decolorise starch iodine impregnated filter paper, whilst other colonies do not.

Both these methods can be used to "screen" large numbers of organisms for penicillinase activity. In addition, the filter membrane method will indicate the amidase producers.

Haight-Finland (1952) *method.* This procedure, a modification of the method of Gots (1945), is recommended by Woolff and Hamburger (1962). An agar plate containing *Sarcina lutea*, and a just inhibitory amount of penicillin G, is inoculated with the organism under investigation and incubated. Satellite colonies of *S. lutea* grow in the region where penicillinase has destroyed the penicillin G.

The method is time-consuming and uneconomical as only five or six organisms can be investigated on each plate. However, by using a filter

membrane (Knox and Smith, 1961) many colonies of organisms can be investigated simultaneously using the same principle. The organisms are grown overnight on a filter membrane on nutrient agar containing 0·05 μg./ml. of penicillin G. The filter membrane is then discarded and the penicillin G-containing plate is inoculated with a confluent growth of *Sarcina lutea* and incubated. The *Sarcina lutea* then grows in the areas where penicillin G has been destroyed.

Chromatographic methods. Early workers using chromatographic procedures were concerned only with discovering the different number of penicillins present in fermentation liquors; chromatograms can be developed with biological systems (Goodall and Levi, 1946; Winsten and Spark, 1947); Baker, Dobson and Martin (1950) developed a colour reaction—they applied the hydroxamates of penicillins to the paper, ran in isopropyl ether + 15 per cent (w/v) isopropanol saturated with 0·1M potassium hydrogen phthalate, and sprayed with ferric chloride solution. The brown spots were eluted into n-butanol and the extinction read with an EEL colorimeter. Thus both quantitative and qualitative assays were performed simultaneously. The sensitivity of their method was 100 μg. Solvent systems that have been used are: water saturated diethyl ether (Glister and Grainger, 1950); n-butanol 4:ethanol 1:water 5; n-butanol 12:acetic acid 3:water 5; n-butanol 1:pyridine 1:water 1 (Cole and Rolinson, 1961).

Colour reactions, based on the iodometric method, have recently been developed (Thomas, 1961; Sneath and Collins, 1961). 6-Aminopenicillanic acid has been detected by phenylacetylation (Batchelor and others, 1959; Cole and Rolinson, 1961; Uri and Sztaricskai, 1961). Work in this laboratory has shown that ninhydrin used with the n-butanol 4:acetic acid 1:water 5 solvent, or the xylose-aniline spray of Saarnio Niskasaarki and Gustafsson (see Hulme, 1961) with the solvent system n-propanol 6:ammonia 3:water 1 (Hanes and Isherwood, 1949) will detect about 25 μg. of penicillins, penicilloic acids and 6-aminopenicillanic acid, in enzymic hydrolysis mixtures; excellent separation is obtained with both systems. Huang and others (1960) have devised a method for estimating aminopenicilloic acid by means of quantitative paper chromatography using ninhydrin as the colour developer.

Thus chromatographic methods have obvious applications for the differentiation between penicillinases and amidases.

Enzyme Units

Pollock and Torriani (1953) defined the unit of penicillinase as that amount of enzyme that hydrolyses 1 μmole of penicillin G per hr. at pH 7·0 and 30°. The figures were chosen because these workers were using the manometric assay. Other assay methods use different physical conditions, and so other definitions of the enzyme unit have appeared. A commonly used definition quotes pH 7·46 and 37°. (See for instance Leitner and Cohen (1962) who attribute this definition to Pollock and Torriani.) Perret (1954) defines his unit at pH 6·5 and 30°, and Novick (1962a) introduces another set of physical conditions, pH 5·8 and 30°,

and also attributes the definition to Pollock and Torriani. The position hence remains unsatisfactory; a relevant discussion of the experimental conditions and the units that should be used for enzymes is given by Thompson (1962).

CONCLUSIONS

Methods for Determining Penicillin Destruction

It has been emphasised that not all methods for estimating penicillin breakdown can be used with all penicillins. The hydrolysis of 6-aminopenicillanic acid by penicillinase cannot be followed either by the acidimetric or the manometric assays; it is better to avoid iodometric methods for penicillins that absorb iodine with their β-lactam ring intact.

By means of a screening technique, such as the filter membrane method, many organisms can be investigated simultaneously for penicillin destruction. In the light of modern developments, it is important to include a test for amidase activity on organisms positive in the filter membrane test, and for this purpose the butyl acetate extraction technique must be used. Furthermore, chromatography at an early stage in the investigation will also elucidate the precise nature of the hydrolytic cleavage of the penicillin. Quantitative amidase estimation can be carried out by the butyl acetate extraction technique or by chromatography, but neither is completely satisfactory for this purpose.

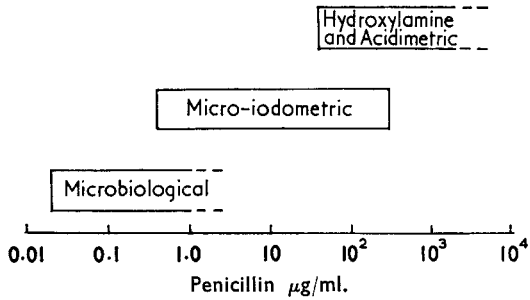


FIG. 1. The relation between the various methods and their use with varying concentrations of penicillins.

Methods for Estimating Penicillins

For assaying minute amounts of penicillins, microbiological methods are the only ones available, and by careful choice of experimental conditions and indicator organism, levels as low as 0·02 μg./ml. of penicillin G can be detected. For higher concentrations of penicillin (for example above 20 μg./ml.) the hydroxylamine assay is the most useful method because it gives a direct estimate of penicillin concentration. All the remaining methods are indirect, since it is necessary first to convert the penicillin to its corresponding penicilloic acid and then estimate this. However, when a chemical method is needed for concentrations less than 20 μg./ml. the iodometric method is the most suitable. Of all the methods

described above, the hydroxylamine and iodometric methods are the most useful for quantitative estimation of penicillins and penicillinase. The hydroxylamine method, being specific for the β-lactam ring, is the method of choice for relatively large concentrations of substrate and penicillinase. The micro-iodometric method is then available for measuring low levels of penicillinase, although it must be borne in mind that it gives results 40 per cent lower than those obtained with other assays. The relation between the various methods and their use with varying concentrations of penicillins is represented diagrammatically in Fig. 1.

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Free Biotechnology Notes

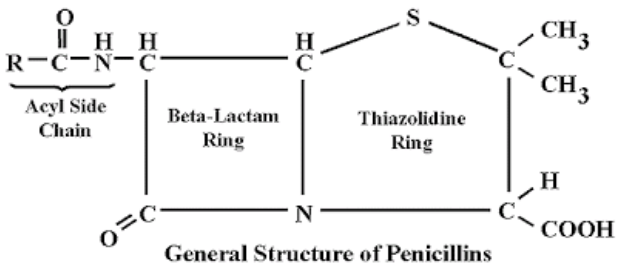
This blog is a little effort to make biotechnology concepts simplified to all the students in the degree classes from all the universities.I took the initiative to make concise and well formulated notes supported with the diagrams.I have struggled a lot in making notes and searching for the concepts by digging library books and surfing various sites. All i want is to create one place where you can look up to biotechnology notes and rely upon them as i have put up a lot of effort on them.

Tuesday, 6 December 2011

PENICILLIN PRODUCTION

PENICILLIN PRODUCTION:

Penicillin was the first naturally occurring antibiotic discovered. It is obtained in a number of forms from Penicillium moulds. Penicillin is not a single compound but a group of closely related compounds, all with the same basic ring-like structure (a β-lactam) derived from two amino acids (valine and cysteine) via a tripeptide intermediate. The third amino acid of this tripeptide is replaced by an acyl group (R) and the nature of this acyl group produces specific properties on different types of penicillin.



There are two different types of penicillin.

Biosynthetic penicillin is natural penicillin that is harvested from the mould itself through fermentation.

Semi-synthetic penicillin includes semi synthetic derivatives of penicillin - like Ampicillin, Penicillin V, Carbenicillin, Oxacillin, Methicillin, etc. These compounds consist of the basic Penicillin structure, but have been purposefully modified chemically by removing the acyl group to leave 6-aminopenicillanic acid and then adding acyl groups that produce new properties.

These modern semi-synthetic penicillins have various specific properties such as resistance to stomach acids so that they can be taken orally, a degree of resistance to penicillinase (or β-lactamase) (a penicillin-destroying enzyme produced by some bacteria) and an extended range of activity against some Gram-negative bacteria. Penicillin G is the most widely used form and the same one we get in a hypodermic form.

About Me

biotechaddict

Myself a researcher interested in the field of genomics and biotechnology. Apart from following my dreams i also like to engage myself in helping other newbies in the field of life sciences. This blog is just a beginning to help budding scientists; who can get full fledged notes on biotech concepts. i have tried to make concise, thoroughly explained and supported with diagrams wherever necessary.This blog helps you avoid surfing websites for the conceptual notes. I am open to the suggestions and improvements!!! Good luck and Happy learning!!!

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PENICILLIN G

Penicillin G is not stable in the presence of acid (acid-labile). Since our stomach has a lot of hydrochloric acid in it (pH2.0), if we were to ingest penicillin G, the compound would be destroyed in our stomach before it could be absorbed into the bloodstream, and would therefore not be any good to us as a treatment for infection somewhere in our body. It is for this reason that penicillin G must be taken by intramuscular injection - to get the compound in our bloodstream, which is not acidic at all. Many of the semi-synthetic penicillins can be taken orally.

Penicillium chrysogenum that produce antibiotics, enzymes or other **secondary metabolites** frequently require precursors like purine/pyrimidine bases or organic acids to produce said metabolites. Primary metabolism is the metabolism of energy production for the cell and for its own biosynthesis. Typically, in aerobic organisms (*Penicillium chrysogenum*) it involves the conversion of sugars such as glucose to pyruvic acid2 and the production of energy via the TCA cycle. Secondary metabolism regards the production of metabolites that are not used in energy production for example penicillin from *Penicillium chrysogenum*. In this case the metabolite is being utilized as a defence mechanism against other microorganisms in the environment. In essence *Penicillium chrysogenum* can kill off the competition to allow itself to propagate efficiently. It should be noted that these secondary metabolites are only produced in times of stress when resources are low and the organism must produce these compounds to kill off its competitors to allow it to survive.

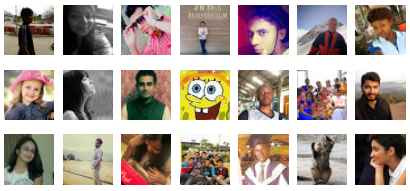
MEDIA FORMULATION:

Lactose: 1%
Calcium Carbonate: 1%
Cornsteep Liquor: 8.5%
Glucose: 1%
Phenyl acetic acid: 0.5g
Sodium hydrogen phosphate: 0.4%
Antifoaming Agent: Vegetable oil

FERMENTATION

To begin the fermentation process, a number of these spores will be introduced into a small (normally 250-500ml) conical flask where it will be incubated for several days. At this stage, explosive growth is the most desired parameter and as such the medium in the flask will contain high amounts of easily utilisable carbon and nitrogen sources, such as starch and corn-steep liquor. At this stage, the spores will begin to revive and form vegetative cells. Temperature is normally maintained at 23-280C and pH at ~6.5, although there may be some changes made to facilitate optimum growth. The flask will often have baffles in it and be on a shaking apparatus to improve oxygen diffusion in the flask.

Once the overall conditions for growth have been established and there is a viable vegetative culture active inside the flask, it will be transferred to a 1 or 2 litre *bench-top reactor*. This reactor will be fitted with a number of



instruments to allow the culture to be better observed than it was in the shake flask. Typical parameters observed include pH, temperature, and stirrer speed and dissolved oxygen concentration. This allows tweaking of the process to occur and difficulties to be examined. For example, there may not be enough oxygen getting to the culture and hence it will be oxygen starved. At this point, the cells should be showing filamentous morphology, as this is preferred for penicillin production. As before, cell growth is priority at this stage. At this stage, growth will continue as before, however, there are often sudden changes or loss in performance. This can be due to changes in the morphology of the culture (*Penicillium chrysogenum* is a filamentous fungi and hence pseudoplastic) that may or may not be correctable.

At this stage the medium being added to the reactor will change. Carbon and nitrogen will be added sparingly alongside precursor molecules for penicillin fed-batch style. Another note is that the presence of penicillin in the reactor is itself inhibitory to the production of penicillin. Therefore, we must have an efficient method for the removal of this product and to maintain constant volume in the reactor. Other systems, such as cooling water supply, must also be considered. If all goes well we should have penicillin ready for downstream processing. From here it can be refined and packaged for marketing and distribution to a global market.

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Posted by biotechaddict at 19:40

Reactions: funny (0) interesting (2) cool (1)

Labels: BETA LACTAM, PENICILLIN, PENICILLIN FERMENTATION

1 comment:

Vishakha Parijatham 30 August 2017 at 19:38

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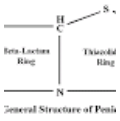

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Penicillin: Biosynthesis, Structure, Fermentation Process and Uses

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In this article we will discuss about:- 1. Introduction to Penicillin 2. Biosynthesis of Penicillin 3. Structure 4. Fermentation Process 5. Uses.

Introduction to Penicillin:

Chemically the natural penicillin is 6-amino penicillanic acid (6 – APA), which consists of thiazolidine ring with a condensed β-lactum ring. The various penicillins differ primarily in the nature of R-side chain which are attached by an amido linkage to the chemical nucleus of the molecule. Fleming's original Penicillium notatum strain, when grown on his medium produced penicillin-F, which is known as 2-pentinyl penicillin.

Subsequently P. chrysogenum proved to be better fungus and more suitable for submerged fermentation. The basic structure of penicillin and different types of natural penicillin's differing in the composition of side chain are shown in Fig. 6.2.

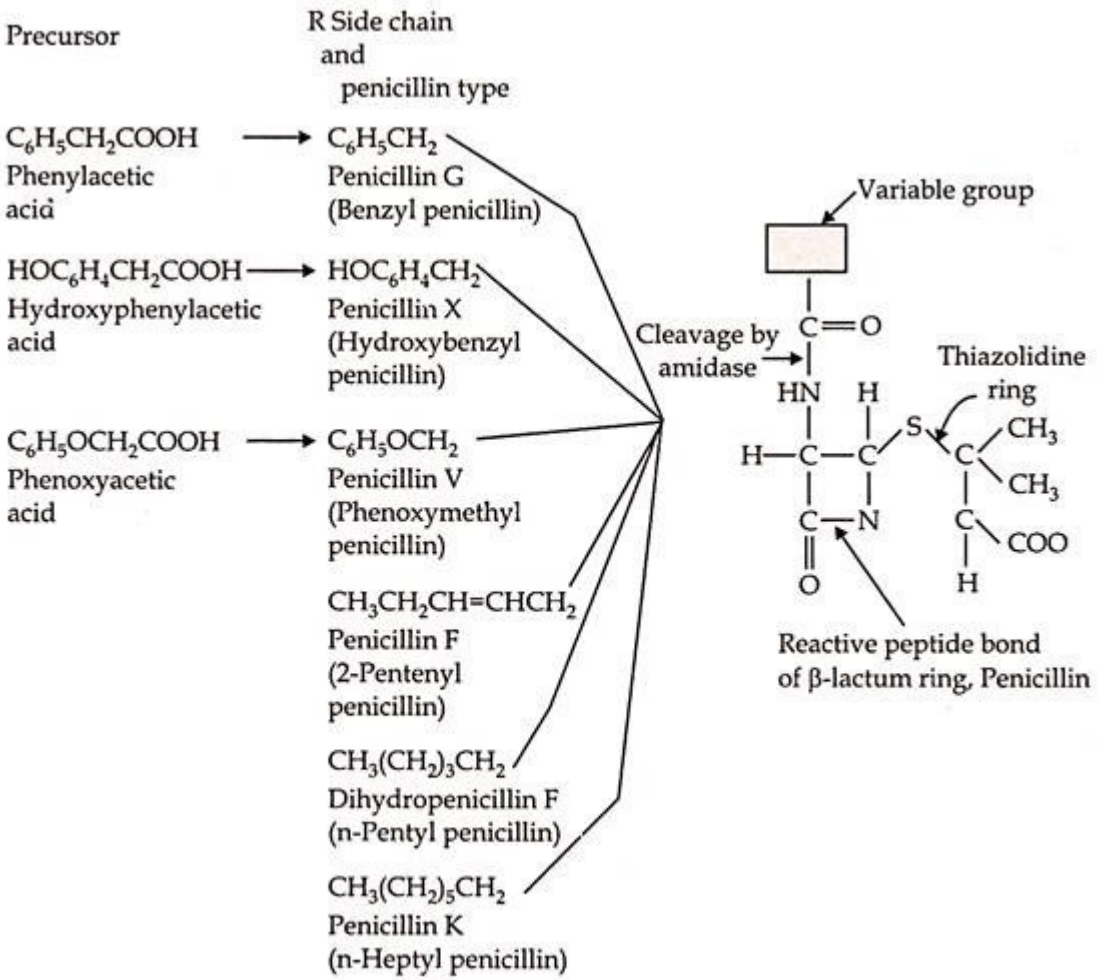

Fig 6.2: Different Penicillins with their precursor and R side chain

If penicillin fermentation is carried out without the addition of side chain precursor, the natural penicillins are formed from which only benzyl penicillin can be isolated. However, the desired penicillin can be obtained by adding suitable side chain precursor into the medium. Such penicillins are called as semi-synthetic penicillins.

Penicillin-G and Penicillin-V are generally produced commercially. When compared to natural penicillins, semisynthetic penicillins have improved characters viz, acid stability, resistance to plasmid or chromosomally coded β-lactamases, expanded antimicrobial effectiveness and are therefore, extensively used in therapy.

Biosynthesis of Penicillin:

The β-lactum thiazolidine ring of penicillin is formed by the condensation of L-cystine and L-valine. The biosynthesis occurs in a non-ribosomal process by means of dipeptide composed of (α – α – AAA) and α-cystine or a breakdown product of cystothiamine. Subsequently L-valine is connected via epimerization reaction resulting in the formation of tripeptide. The first product of cyclization of the tripeptide which can be isolated is isopenicillin N but the biochemical reactions leading to this intermediate is not understood. Benzyl penicillin is produced in exchange of α -α-AAA with activated phenylacetic acid (Fig. 6.3).

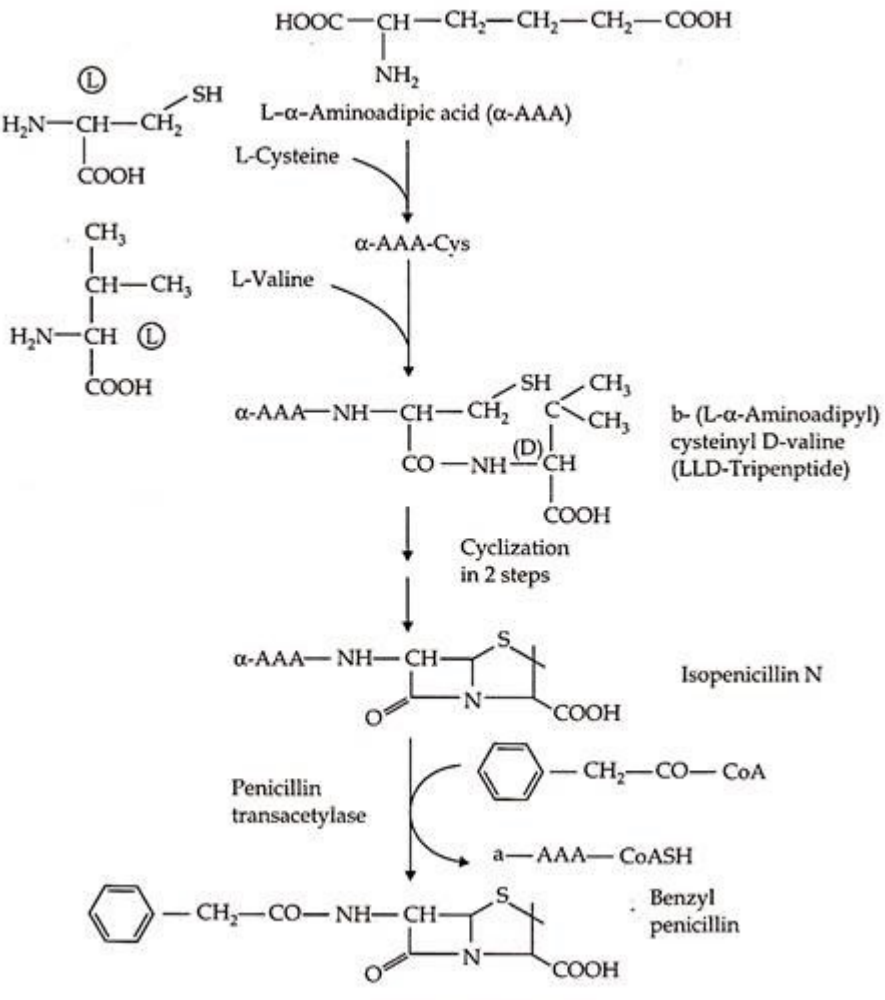

Fig. 6.3: Biosynthesis of Penicillin

About 38% of the penicillins produced commercially are used as human medicine, 12% in veterinary medicine and 43% as starting materials for the production of semi-synthetic penicillins.

Structure of Penicillin:

Some of such semisynthetic penicillins along with structure and biological activity are presented in table 6.4 and Fig. 6.4.

Table 6.4: Characteristics of clinically used semi-synthetic Penicillins

		Specific activity against			
Name	**Method of preparation**	**Gram (+) cocci**	**Gram (–) rods**	**Acid stability**	**Resistance to staphylococcal penicillinase**
Amoxicillin	Semi synthetic	+	+	+	Low
Ampicillin	Semi synthetic	+	+	+	Low
Azidocillin	Semi synthetic	+	+	+	Low
Azolocillin	Semi synthetic	+	Some	+	Low
Bacampicillin	Semi synthetic	+	+	+	Low
Carbenicillin	Semi synthetic	+	Some	-	Low
Cloxacillin	Semi synthetic	+	Nil	+	High
Cyclacillin	Semi synthetic	+	+	-	High
Dicloxacillin	Semi synthetic	+	Nil	+	High
Epicillin	Semi synthetic	+	+	++	Low
Flucloxacillin	Semi synthetic	+	Nil	+	High
Hetacillin	Semi synthetic	+	+	+	Low
Mecillinam	Semi synthetic	+	+	+	High
Metamacillin	Semi synthetic	+	+	+	Low
Methicillin	Semi synthetic	+	-	+	High
Mezlocillin	Semi synthetic	+	+	+	Low
Nafcillin	Semi synthetic	+	Nil	+	High
Oxacillin	Semi synthetic	+	Nil	+	High
Penicillin G	Natural	+	Nil	–	Nil
Penicillin V	Natural	+	Nil	+	Nil
Phenethicillin	Semi synthetic	+	Nil	+	Nil
Pirbenicillin	Semi synthetic	+	+	+	Medium
Pivampicillin	Semi synthetic	+	+	+	Low
Pivmecillinam	Semi synthetic	+	+	+	High
Propicillin	Semi synthetic	+	Nil	+	Low
Sulbenicillin	Semi synthetic	+	Some	+	Low
Talampicillin	Semi synthetic	+	+	+	Low
Ticarcillin	Semi synthetic	+	Some	+	Low

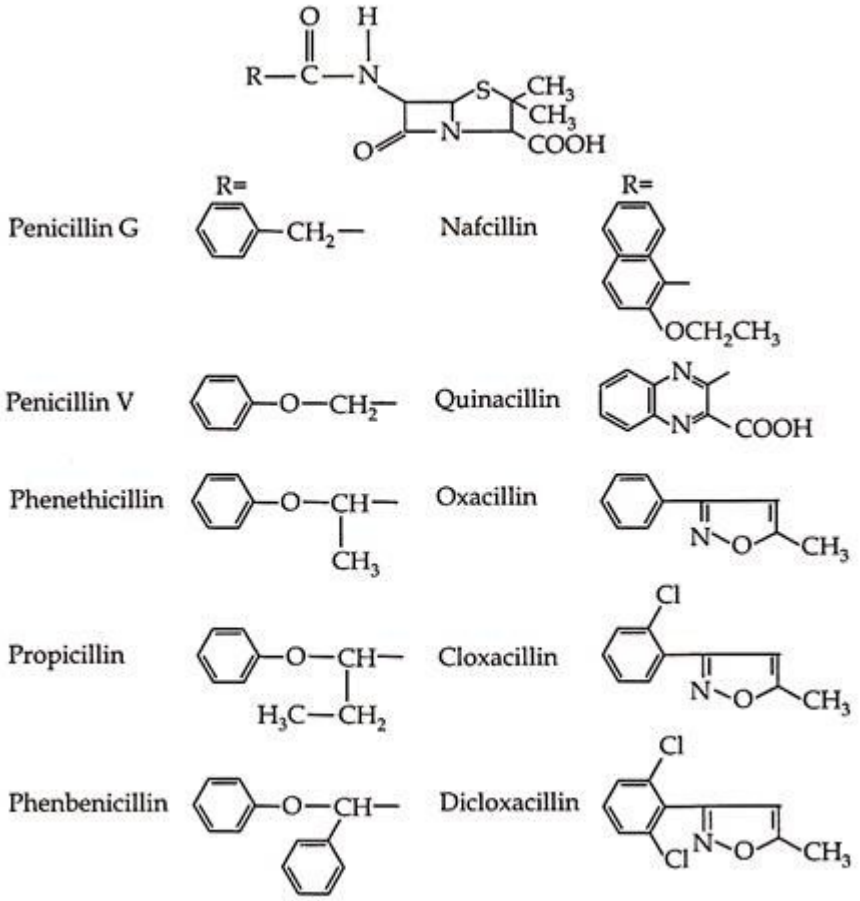

Fig. 6.4: Structure of some clinically useful penicillins

Hetacillin, bacampicillin, epicillin, pivampicillin, and talampicillin are converted to ampicillin in the body. These penicillins exhibit various improvements including resistance to stomach acids to allow oral administration, to pencillinase and an extended range of activity against gram (+) positive bacteria.

It has been reported that most of the high yielding strains of P. chrysogenum are genetically unstable. Genetic unstability increases with the increase in the yield. However, it can be controlled to some extent by following suitable preservation methods. The following preservation methods are generally adopted for storing high yielding strains of P. chrysogenum.

1. A spore suspension is stored in a frozen state under liquid nitrogen.

2. A spore suspension can be lyophilized in an appropriate medium.

3. A spore suspension is mixed with a sterile finely divided inert material like soil or sand and desiccated.

Fermentation Process of Penicillin:

Penicillin fermentation is an aerobic process with a volumetric oxygen absorption rate of 0.4 -0.8mm min^{-1}. The required aeration rate varies according to the strain, the type of fermenter used and on the impellor system. However, the aeration rate varies between 0.5 and 1.0 vvm. It is produced by fed batch submerged fermentation in a stirred tank fermenter.

This process can be described under following headings:

1. Strain development,

2. Inoculum production,

3. Inoculation,

4. Extraction and purification

1. Strain Development:

The variety of molds which yield greater amount of penicillin is called as high yielding strain. They are generally developed from the wild P. chrysogenum by a process called sequential genetic selection. This process consists of stepwise development of improved mutant by treating the wild strain of P. chrysogenum with a series of mutagenic agents or exposing to ultraviolet radiation either individually or in combination, such as X-rays and chemical mutagens, is called as strain improvement.

Strain development is a laborious and time-consuming process. The selected mutant possesses greater capacity for antibiotic production than the wild type.

Table 6.3: Significant stages in strain improvement programme in *P. chrysogenum*

Strain	Method	Penicillin (mg ml^{-1})	Characteristics
P. notatum (Fleming)	**P. chrysogenum**	3	
NRRL 1951	Original isolate	60	Yellow pigment
NRRL 1951.B25	Selection	125	Yellow pigment
X-1612	X rays	300	Yellow pigment
WIS Q 176	UV-light	800–1000	Yellow pigment
B 13-D10	UV-light	-	Pigment free
WIS 47-638	Selection	-	Pigment free
WIS 47-1564	Selection	800–1000	Pigment free, 95-100% G*
WIS 48-701	Selection	-	Pigment free, 95-100% G*
WIS 49-133	Nitrogen mustard gas	1500–3000	Pigment free, 95-100% G*
WIS 49-2166	Nitrogen mustard gas	1500–3000	Pigment free, 95-100% G*
WIS 50-535	Nitrogen mustard gas	1500–00	Pigment free, 95-100% G*
WIS 51-20	Selection	2000–3000	Pigment free, 95-100% G*
EIS.1	NM & Selection	7000	Pigment free, Penicillin G

The expanded role for penicillins came from the discovery that different biosynthetic penicillins can be formed by the addition of side chain precursors to the fermentation medium and that natural penicillins can be modified chemically to produce penicillins with improved characteristics. Most penicillins are now semisynthetic produced by chemical modification of natural penicillin obtained by fermentation using strains of P chrysogenum.

Modification is achieved by removing their natural acyl group, leaving 6 APA to which other acyl groups can be added to confer new properties. This is achieved by passage through a column of immobilized penicillin acylase usually obtained from E.coli at neutral pH. Penicillin G for example converted to 6-APA and phenylacetic acid. The 6-APA is then ethically acylated with an appropriate side chain to produce a semi-synthetic penicillin.

2. Inoculum Production:

The microorganism which is used in a fermentation process is called as the inoculum. A high yielding strain of P. chrysogenum is generally employed as

inoculum.

A strain of the fungus is sub-cultured from stock culture for inoculum development. Spores from primary source are suspended in water or in a dilute solution of a nontoxic wetting agent such as 1:10000 sodium lauryl sulfate. The spores are then added to flasks or bottles of wheat bran plus nutrient solution and these are incubated for five to seven days at 24°C so as to provide heavy sporulation. The entire process is repeated several times in order to have more sporulation.

The resulting spores are used directly to inoculate inoculum tanks or stirred fermenters. The incubation temperature is maintained at 24-27°C for 2 days with agitation and aeration in order to facilitate heavy mycelial growth, which may be added to a second or even a third stage fermentation.

The resulting inoculum which is employed in a production tank is tested both by microscopic examination and by sub-culturing method. Many sporulation media have been designed to obtain large number of spores. The one developed by Moyer and Coghill (1946) is most extensively used and given below (table 6.5).

Table 6.5: Composition of Moyer and Coghill (1946) sporulation medium

Component	Concentrating (gl^{-1})
Glycerol	7.5
Cane molasses	7.5
Corn steep liquor	2.5
$Mg\ SO_4.\ 7H_2O$	2.5
KH_2PO_4	0.050
Peptone	0.060
NaCl	5.00
Fe-tartarate	0.005
$CuSO_4.5H_2O$	0.004
Agar	2.50
Distilled water	1.0

3. Inoculation:

Introduction of pure inoculum into the production tanks or fermenters is called as inoculation.

This is done by any one of the following three methods:

1. Dry Spores may be used as Inoculum:

Since the spores of P. chrysogenum are hydrophobic, either spores are blown deep into the medium or a wetting agent such as sodium lauryl sulphate is used.

2. Suspension of Ungerminated Spores:

This suspension is made by using 1:10000 sodium lauryl sulfate solution. This suspension is fed to the fermenter by suitable techniques like spray guns or pipettes. This is followed by agitation and aeration of the fermentation medium in order to achieve equal and uniform distribution of the spores in the entire medium.

3. Feeding the fermentation tanks with pre-germinated spores or mycelial pellets which are prepared by the germination of spores. Pellets are generally fed to the fermentation medium after two or three days of spore inoculation.

Fermenters with a capacity of 40,000 to 2 lakhs liters are generally employed for the production of penicillin. Due to difficulties with the oxygen supply larger tanks are not employed. Some manufacturer's use of Waldh of fermenters or air lift fermenters, but this is only possible in mutants which generate low viscosity. Depending upon the production strain, the operational temperature is maintained between 25°-27°C. A typical flow chart for penicillin production is given in Fig. 6.5.

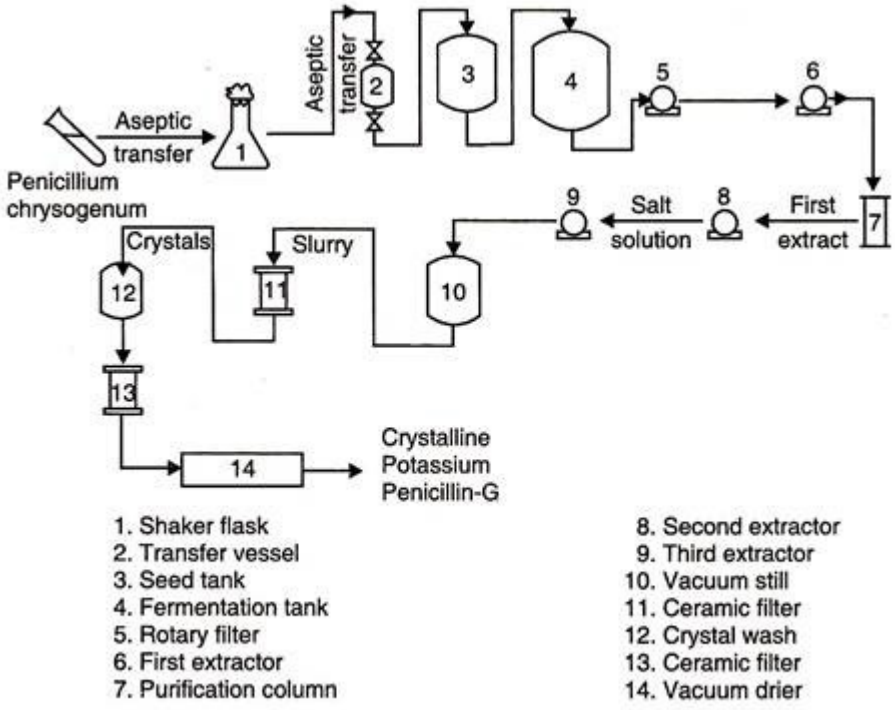

Fig. 6.5: Flow sheet for large-scale production of Penicillin

(ii) Medium:

The medium employed for penicillin production should be suitable to achieve:

1. An abundant growth of the mycelium.

2. Maximum accumulation of the antibiotic.

3. Easy and inexpensive extraction and purification of the antibiotic.

Carbon source is generally supplied in the form of lactose. Glucose, sucrose, glycerol and sorbitol can also be employed as carbon source. Nitrogen source is generally supplied in the form of ammonium sulphate or ammonium acetate or ammonium nitrate. Abundant formation of mycelium and spores takes place when a medium contains corn-steep liquor because it contains important amino acids required for mycelial growth.

Potassium, phosphorus, magnesium, sulphur, zinc and copper are supplied in the form of salts. Potassium and phosphorus are supplied in the form of

potassium dihydrogen phosphate, magnesium, iron and copper are supplied in the form of sulphates. All these elements may be present in corn steep liquor.

Penicillin-F and penicillin-K are the naturally produced penicillins synthesized by P. notatum and P. chrysogenum, respectively, in the absence of precursor. But, if phenylacetic acid is supplied in the medium P. chrysogenum produces penicillin-G instead of penicillin-K. Similarly, desired synthetic penicillins can be obtained by adding the medium with suitable precursor.

A medium designed by Jackson (1958) which has the following composition, is generally used in fermentative production of penicillin (table 6.6).

Table 6.6: Composition of Jackson's (1958) medium

Component	Concentration (In Percentage)
Corn steep liquor	3.5
Lactose	3.5
Glucose	1.0
Calcium carbonate	1.0
Potassium dihydrogen phosphate	0.4
Edible oil	0.25

Penicillin yields with time are linear from approximately 48 to 96 hours. The final penicillin yield is in the range of 3 to 5% which largely depends upon the amount of carbohydrate consumed during fermentation process, which is approximately equal to 1500 international units per milliliter. Sylvester and Coghill (1954) have estimated that to produce 1000 gallons of fermented culture, which is capable of yielding 2.2-2.7 kg of penicillin by the submerged culture method requires approximately 227 kg of nutrients, 3400 kg of steam, 45460 lt of water, 1000 kWh of electricity and 7075 m^3 of air.

Penicillin easily get carboxylated to form penicillianic acid which is biologically inactive by the action of enzyme penicillinase. The enzyme penicillinase is widely distributed among different microorganisms. These organisms may

enter into the fermenter at any stage and may convert penicillin into penicillianic acid (Fig. 6.6).

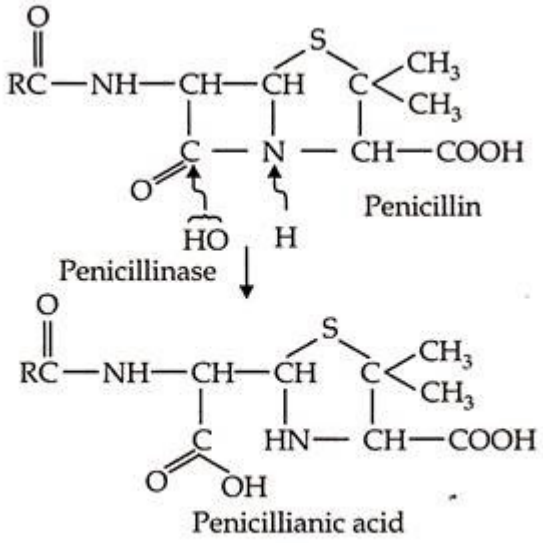

Fig. 6.6: The carboxylation of penicillin by the action of penicillinase

Thus, in penicillin fermentation contamination is a main constraint. Hence, one has to be careful in preventing contamination. This was the one of the main problems during early times of penicillin production, when fermentation was carried out in bottles and contamination in one bottle may destroy penicillin in entire batch of bottles.

In the typical penicillin fermentation there is a growth of 10 hrs duration with a doubling time of 6 hrs during which the greater part of the cell mass is formed. The oxygen supply in the growing culture is critical since the increasing viscosity hinders oxygen transfer. After growth phase, the culture proceeds to actual penicillin production. The growth is sharply reduced by feeding with various culture medium components.

The production phase can be extended to 120- 180 hrs. Penicillin production by continuous fermentation has been attempted but it has been difficult due to instability of the production strains. A batch fill and draw system has been suggested as an alternative. In this process 20-40% of the fermentation contents is drawn off and replaced with fresh nutrient solution. This process may be repeated up to 10 without affecting yield.

4. Extraction and Purification:

After it is assessed that sufficient amount of penicillin has been produced during fermentation process, it is extracted and then purified.

The entire process is carried out in three different stages.

They are:

(a) Separation of mycelium

(b) Extraction of penicillin and

(c) Treatment of crude extract

(a) Separation of Mycelium:

Mycelium is separated from the medium by employing rotatory vacuum filter. This process should be performed carefully in order to avoid contaminating microorganisms which produce penicillinase enzyme, degrading the penicillin.

(b) Extraction of Penicillin:

The penicillin is excreted into the medium and less than 1% remains as mycelium bound. Extraction of penicillin is carried out by employing counter current extraction method. The pH of the liquid after separation of the mycelium is adjusted to 2.0 to 2.5 by adding phosphoric or sulphuric acid. This treatment converts penicillin into anionic form.

The liquid is immediately extracted with an organic solvent such as amylacetate or butylacetate or methyl isobutyl ketone. This step has to be carried out quickly because penicillin is quite unstable at low pH values. Podbielniak counter current extractor is used for this purpose. The penicillin is then back extracted into water from the organic solvent by adding enough

potassium or sodium hydroxide which also results in the elevation of pH to 7.0 to 7.5.

The resulting aqueous solution is again acidified and re-extracted with organic solvent. These shifts between the water and the solvent help in the purification of the penicillin. Finally, the penicillin is obtained in the form of sodium penicillin. The spent solvent is recovered by distillation for reuse.

(c) Treatment of Crude Extract:

The resulted sodium penicillin is treated with charcoal to remove pyrogens (fever causing substances). It is also, sometimes, sterilized to remove bacteria by using Seitz filter. Then, the sodium penicillin is prepared in crystalline form by crystallization. It may be packed as powder in sterile vials or prepared in the form of tablets or in the form of syrups for oral usage. The pharmaceutical grade may be used in the production of semi synthetic penicillin.

Uses of Penicillin:

1. Most of the penicillin's are active against Gram-positive bacteria, in which they inhibit the cell wall synthesis leading to the death of bacteria.

2. Used therapeutically in the treatment of infectious diseases of humans caused by Gram (+) positive bacteria.

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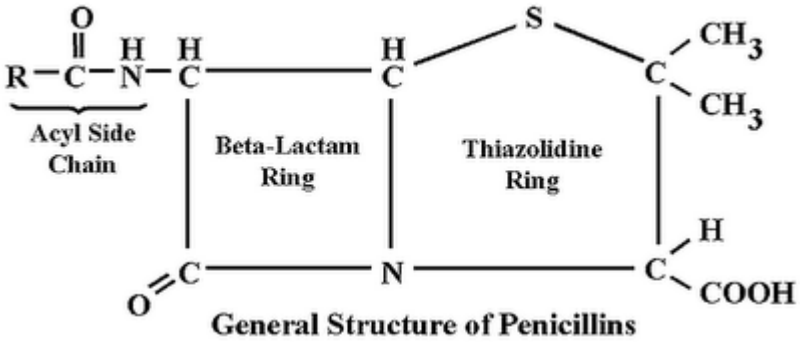
Module-26: Penicillin Fermentation

Introduction:

- The term antibiotic has been defined by Selman Waksman as being an organic compound produced by one microorganism that, at great dilutions, inhibits the growth of or kills another or even group of other harmful microorganisms.
- Antibiotics are available in various forms.
- For example, to treat bacterial infection on the surface, an antibiotic should be there in an ointment or cream form.
- But to treat internal infection it can be directly injected into the bloodstream which finally distributed throughout the body.
- Antibiotics are produced primarily by bacteria, Streptomycetes, Nocardia and fungi.
- However, antibiotics produced by Streptomycetes spp. Have found greatest commercial applications.
- On the basis of mode of action antibiotics are divided into various groups like,
  - i Antibiotics affecting cell wall
  - ii Antibiotics damaging cell membrane
  - iii Antibiotics interfering with protein synthesis
  - iv Antibiotics inhibiting nucleic acid synthesis
  - v Antibiotics blocking cell metabolism
- Fermentation is the process used for the large-scale production of an antibiotic.
- The first discovered natural antibiotic was **Penicillin**.
- Penicillin was obtained from multicellular fungi, "Penicillium molds".
- Penicillin is a group of compounds having common basic nucleus, 6-amino penicillinic acid (6-APA).
- 6-APA contains ring like structure termed as a β-lactam ring.
- Penicillin are of two different types,
  - i Natural Penicillin
  - ii Semi-synthetic Penicillin
- **Natural penicillin** is directly harvested from the *penicillium mold.*
- **Semi-synthetic penicillin** consists of the basic Penicillin nucleus (6-APA), but with new side chain that change properties of natural penicillin.
- Semi synthetic penicillin includes Ampicillin, Methicillin, Penicillin V, etc.
- They are produced by modifying natural penicillin by removing the natural acyl group to leave 6-APA and then adding new acyl groups having different properties like,
  - i resistance to stomach acids so it can be taken orally
  - ii a degree of resistance to penicillinase (penicillin degrading enzyme)
  - iii an extended range of activity against some Gram-negative bacteria.



History

- In 1928, Sir Alexander Fleming made one of the most important contributions to the field of antibiotics.
- He first observed the antibiotic properties & therapeutic value of penicillin.
- In an experiment, he observed that air born contaminant, later shown to be *Penicillium notatum,* inhibited the growth of a culture of *S.aureus* on an agar plate.
- He called this material penicillin after the mold that had produced it.
- By that the progress of the first modern era antibiotic, penicillin gets started.
- In 1932, he published paper, which proposed a method for use of penicillin in treatment of infected wounds.
- But early samples of penicillin were not purified, and further refinements were needed.
- Howard Florey and associates discovered a new high yielding strain of *Penicillium* in the 1940s.
- This allowed extensive production of penicillin, which helped launch the modern antibiotics industry.
- Dr. Ernst B. Chain was there with Florey's team, who initiated extracting penicillin into a purified and powerful antibiotic.
- Later on, different scientists had work on in detail regarding different types of penicillin and their production on large scale.
- Penicillin is acting against many Gram positive bacteria, Nocardia, and Actinomycetes, but not against most Gram negative bacteria except at higher dosage level.
- It interferes with cell wall synthesis of actively growing sensitive organisms.
- It mainly inhibit the cross linking steps of peptidoglycan synthesis in the cell wall.
- The World War II had brought a demand for penicillin on a large scale for the treatment of burns and wounds.
- By the end of the war (late 1943), mass production of the penicillin had started by many drug manufacturing companies.
- In 1945 Fleming, Florey and Chain were awarded the Nobel Prize in Physiology and Medicine.

Penicillin Fermentation

Microorganisms

- Out of various species of the fungus Penicillium mainly two species are used in the fermentation.
- These are *P. notatum* & *P. chrysogenum*.
- Even from these two *P. chrysogenum* is high yielding strain and therefore most widely used as production strain.
- The production strain is improved by mutation with the help of X-rays or any other agents to give high yield.
- After strain improvement the production strain should be carefully maintained because *P. chrysogenum* is genetically unstable.
- Different preservation techniques are used like,
    1. A spore suspension may be mixed with a sterile, finely separated inert support like soil or sand and then desiccated.
    2. The spore suspension can be stored under liquid nitrogen (-196°C) i.e. in a frozen state.
    3. The spore suspension can be lyophilized in appropriate media.

Inoculum preparation

- Here the chief purpose is to develop a pure inoculum in an adequate amount and in the fast growing phase for the production stage fermenter.
- To do so various sequential steps are necessary like,
    1. A starter culture which is available in cold-stored form is transferred to an agar-containing plate to allow growth.
    2. After getting growth on solid media, one or two growth stages should allowed in shaken flask cultures to create a suspension, which can be transferred to seed tanks for further growth.
    3. The seed tanks are made up of stainless steel which is designed to provide an ideal environment to production strain. They contain all the nutrients including Growth factors like vitamins & amino acids. The seed tanks are equipped with agitators, which allow continuous mixing of growth medium, and a pump to deliver sterilized, filtered air.
    4. After about 24-28 hours, the content of the seed tanks is transferred to the primary fermentation tanks.
    5. The main fermentation tank is a larger version of the seed tank, which contains same growth media and also provides proper growth promoting environment. During this process, they excrete huge amounts of the desired antibiotic.
    6. All the bio parameters like temperature, pH, aeration, agitation etc. should be properly maintained.

Bio parameters

- PH: near 6.5
- Temperature: 26°C to 28°C
- Aeration: a continuous stream of sterilized air is pumped into it.
- Agitation: have baffles which allow constant agitation.

Raw Materials

- Raw materials are primary requirement to design the fermentation broth for antibiotic production.
- Fermentation broth contains all the necessary elements required for the proliferation of the microorganisms.
- Generally, it contains a carbon source, nitrogen source, mineral source, precrsors and antifoam agents if necessary.

Carbon Source

- Lactose acts as a very satisfactory carbon compound if it is used in a concentration of 6%.
- Other carbohydrates like glucose & sucrose may be used but it has to provide with slow feeding rate.
- Complex as well as cheap sources like molasses, or soy meal can also be used which are made up of lactose and glucose sugars.
- These materials are desired as a food source for the organisms.

Nitrogen Source

- Another essential compound for metabolism of organisms is nitrogen.
- Ammonium salts such as ammonium sulfate, ammonium acetate, ammonium lactate or ammonia gas are used for this reason.
- Sometime corn steep liquor may be used.

Mineral Source

- Additionally, some minerals are necessary for the proper growth of these organisms. are included.
- These elements include phosphorus, sulfur, magnesium, zinc, iron, and copper which generally added in the form of water soluble salts.

Precursors

- Various types of precursors are added into production medium to produce specific type of penicillin.
- The most important naturally occurring penicillin is penicillin-G.

- But depending upon the precursors added, the type of penicillin going to produced can be changed.
- For example, if phenyl acetic acid is provided then only penicillin-G will be produced but if hydroxy phenyl acetic acid is provided then penicillin-X will be produced.
- Phenoxy acetic acid is provided as precursor for penicillin-V production.
- When corn steep liquor is provided as nitrogen source, it also provides phenyl acetic acid derivatives; therefore it is widely used in the production of penicillin-G.

Anti-foam agents

- Anti-foaming agents such as lard oil, octadecanol and silicones are used to prevent foaming during fermentation.
- Following three points should be kept in mind before choosing raw materials for manufacture of penicillin,

1. An abundant growth of mycelium
2. Maximum accumulation of penicillin
3. Ease of extraction and purification of antibiotics.

Production medium

Components	**Percent (%)**
Lactose:	3.5 to 6
Calcium Carbonate:	1.0
Cornsteep Liquor:	3.5
Glucose:	1.0
Phenyl acetic acid:	0.5
Sodium hydrogen phosphate:	0.4
Antifoaming Agent: Edible oil:	0.25

- In inoculum medium lactose is generally absent because it induces penicillin production and retarding the growth of production strain.

Recovery

- The recovery of penicillin is carried out in three successive stages:

1. Removal of mycelium
2. Counter current solvent extraction of penicillin
3. Treatment of crude extracts

- At harvest the fermentation broth is filtered on a rotatory vacuum filter to remove the mycelium and other solids.

- Phosphoric or sulfuric acids are added to lower the pH (2 to 2.5) in order to transform the penicillin to the anionic form.
- Then the broth is directly extracted in a Podbielniak Counter Current Solvent Extractor with an organic solvent such as methyl isobutyl ketone, amyl acetate or butyl acetate.
- The filtration is carried out under such conditions which avoid contamination of the filtrate with penicillinase producing organisms which otherwise may allow serious or full loss of an antibiotic.
- This step has to be carried out rapidly because penicillin is very unstable at low pH values.
- Penicillin is then again extracted into water from the organic solvent by adding an adequate amount of potassium or sodium hydroxide to form a salt of the penicillin.
- The resulting aqueous solution is again acidified & re-extracted with methyl isobutyl ketone.
- This shifts between water and solvent help in purification of the penicillin.
- The solvent extract is carefully back extracted with NaOH and from this aqueous solution; various procedures are utilized to cause the penicillin to crystalize as sodium or potassium penicillinate.
- The resulting crystalline penicillin salts are then washed and dried.
- Final product must pass rigorous government standards.
- Spent solvents resulting from the above procedure are recovered for re use.
- Sometimes the crude extract of penicillin is passed out from charcoal treatment to eliminate pyrogens; even sterilization can also be done.
- Sterile vials are used for packaging of an antibiotic either as a powder or suspension.
- For oral use it is tableted usually with a film coating.
- It must satisfy all the criteria of the government standards before being marketed.

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Quantitative Estimation of Bioethanol Produced From Lignocellulosic & Household Wastes

Mrs. Bindu Elizabeth Koshy[1], Dr. Fanish Kumar Pandey[2], Dr. Tripti Bhatnagar[3]

[1,2,3] Department of Science and Technology, Noida International University, India

Abstract: **Bio ethanol is a liquid bio fuel which can be produced from several different biomass feed stocks and conversion technologies. Ligno-cellulosic waste materials such as fruit waste, agricultural waste, biodiesel waste glycerol and bagasse can be used to produce bio ethanol. Bio ethanol is identical to ethanol from other sources but has the advantage that the raw materials required to produce bio ethanol are highly abundant, diverse, and renewable. Bio ethanol produced from ligno-cellulosic materials, shows energetic, economic and environmental advantages in comparison to bio ethanol from starch or molasses. Ligno-cellulosic waste materials can be processed to rupture cell walls and liberate the sugars needed to produce ethanol. Cellulose molecules are similar to starch and contain long chains of glucose molecules. However, their structural configuration and the encapsulation by lignin make cellulosic materials comparatively more difficult to hydrolyze than starchy materials. Cellulose requires hydrolysis to liberate the cellulose and hemicelluloses from the lignin encapsulation and access its crystalline structure. This can be performed by chemical and enzymatic hydrolysis of ligno-cellulosic material. The extracted sugar molecules from the ligno-cellulosic material need to be converted into ethanol by submerged batch fermentation. Quantitative estimation of bio ethanol produced was carried out by biochemical method and by using a gas chromatograph.**

Keywords: **Molasses, Lignin, Cellulose, Ethanol, Glycerol, Gas chromatography.**

I. INTRODUCTION

An advantage of ethanol (CH_3CH_2OH *or* C_2H_6O) is that it has a higher octane rating than ethanol-free gasoline available at roadside gas stations, which allows an increase of an engine's compression ratio for increased thermal efficiency. In high-altitude locations, some Ethanol also called *ethyl alcohol , pure alcohol, grain alcohol, or drinking alcohol*, is a *volatile, flammable, colorless liquid*. Bio ethanol is an alcohol made by fermentation, mostly from carbohydrates produced in sugar or starch crops such as corn, sugarcane, or sweet sorghum. Cellulosic biomass, derived from non-food sources, such as trees and grasses, is also being developed as a feedstock for ethanol production. Ethanol can be used as a fuel for vehicles in its pure form, but it is usually used as a gasoline additive to increase octane and improve vehicle emissions. Current plant design does not provide for converting the lignin portion of plant raw materials to fuel components by fermentation.

Ethanol fuel is the most common bio fuel worldwide, particularly in Brazil. Alcohol fuels are produced by fermentation of sugars derived from wheat, corn, sugar beets, sugar cane, molasses and any sugar or starch from which alcoholic beverages such as whiskey, can be made . The ethanol production methods used are enzyme digestion , fermentation of the sugars, distillation and drying. The distillation process requires significant energy input for heat (often unsustainable natural gas fossil fuel, but cellulosic biomass such as bagasse, the waste left after sugar cane is pressed to extract its juice, can also be used more sustainably).

Ethanol is also used to fuel bio ethanol fireplaces. As they do not require a chimney and are *"flue less"*, bioethanol fires are extremely useful for newly built homes and apartments without a flue. The downsides to these fireplaces is that their heat output is slightly less than electric heat or gas fires, and precautions must be taken to avoid carbon monoxide poisoning.

Ethanol (ethyl alcohol, grain alcohol) is a clear, colorless liquid with a characteristic, agreeable odor. In dilute aqueous solution, it has a somewhat sweet flavor, but in more concentrated solutions it has a burning taste. The word *alcohol* derives from Arabic *al-kuhul*, which denotes a fine powder of antimony used as an eye makeup. *Alcohol* originally referred to any fine powder, but medieval alchemists later applied the term to the refined products of distillation, and this led to the current usage.

Ethanol has been made since ancient times by the fermentation of sugars. All beverage ethanol and more than half of industrial ethanol is still made by this process. Simple sugars are the raw material. Zymase, an enzyme from yeast, changes the simple sugars into ethanol and carbon dioxide.

In the production of beverages, such as whiskey and brandy, the impurities supply the flavor. Starches from potatoes, corn, wheat, and other plants can also be used in the production of ethanol by fermentation. However, the starches must first be broken down into simple sugars. An enzyme released by germinating barley, diastase, converts starches into sugars. Thus , the germination of barley, called malting, is the first step in brewing beer from starchy plants, such as corn and wheat.

The ethanol produced by fermentation ranges in concentration from a few percent up to about 14 percent. About 14 percent, ethanol destroys the zymase enzyme and fermentation stops. Ethanol is normally concentrated by distillation of aqueous solutions, but the composition of the vapor from aqueous ethanol is 96 percent ethanol and 4 percent water. Therefore, pure ethanol cannot be obtained by distillation. Commercial ethanol contains 95 percent by volume of ethanol and 5 percent of water. Dehydrating agents can be used to remove the remaining water and produce absolute ethanol.

Balat et al(2008) highlighted the production of ethanol (bio ethanol) from biomass to reduce both consumption of crude oil and environmental pollution. The study revealed that bio ethanol is appropriate for the mixed fuel in the gasoline engine because of its high octane number, and its low ketene number and high heat of vaporization impede self-ignition in the diesel engine. The study also implied the disadvantages of bio ethanol which include its lower energy density than gasoline, its corrosiveness, low flame luminosity, lower vapor pressure (making cold starts difficult), miscibility with water, and toxicity to ecosystems.

II. MATERIALS AND METHODS

2.1 Prepration of PDA culture media

Material Required:- Potato, dextrose, sugar, agar, peptone, beef extract, beaker, measuring cylinder, funnel, filter paper, muslin cloth, mortar and pestle, heater, autoclave, laminar air flow, chamber, test tubes, petriplates, al foils, cotton rolls etc, knife, etc.

2.1.1 Procedure-PDA media Preparation

Take potatoes peel off and weigh 50g.Cut it into small pieces with a knife and boil it in a beaker containing water for 20-30 min, fill the pieces give a whitish appearance or easily penetrated by a glass rod. Decant the excess supernatant Mash the potatoes and filter the sol through a muslin cloth. Take the potato extract in a beaker and add 5g of dextrose and 5g of agar. Make up the vol. to 250ml by adding distilled water to it. Boil the flask a little to dissolve the ingredients .Cotton plug it and cover it with a foil. Autoclave the flask along with petri plates at 121˚C for 20 min at 15 Psi pressure.

2.1.2 Preparation of PDA plates

Take the entire material in a laminar air flow cabinet which is previously sterilized for 30 min in u.v. light.When temp cools down a little pour about 15-20 ml of medium aseptically into the bottom half of Petriplates. Place the plates on a horizontal base and after 20-30 min, it solidifies to form PDA plates.

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Inoculate the different source on PDA plates:-Taking the PDA plates and inoculate the soil sample as a pour plate method. With a different PDA plate curd pour on the it. Incubate these plates under the BOD incubator for 48 hours for its growth. After that recognized the colonies with the help of gram staining.

2.2 Collection of the raw material

Collect the lignocelluloses waste material from different sources like house-hold wastes, agricultural wastes, fruit waste, and industrial wastes. E.g.- baggase, pea *waste*,, orange peels, molasses.

2.3 Hydrolysis of ligno cellulosic material

The ligno-cellulosic waste was collected and dried overnight. The dried waste was powdered and was hydrolysed using chemical and enzymatic method. Chemical hydrolysis was carried out using dilute H_2SO_4.Dilute sulphuric acid solutions of 0.3M and 0.5M were prepared and 5 grams of powdered ligno-cellulosic waste was added in each acid solution. The acid solutions were kept for 24 hours for hydrolysis. The acid solutions were then kept at high temperature of 121^oC and high pressure of 15 psi for 1 hour.

2.3.1 Enzymatic Hydrolysis

Another basic method of hydrolysis is enzymatic hydrolysis. Enzymatic are naturally occurring plant proteins that cause chemical reactions to occur. The most commonly used micro-organism to produce ethanol is *Saccharomyces cerevisiae* is a species of yeast. It is perhaps the most useful yeast, having been important to wine making, baking and brewing since ancient times.

Enzymatic hydrolysis was done using enzyme amylase. The enzyme solution was prepared at a concentration of 1 mg/ml. 5 grams of lingo-cellulosic waste was added in 20 ml enzyme solution and volume was made up to 100 ml using distilled water. The solution containing lingo-cellulosic waste and enzyme was kept overnight for hydrolysis.

2.4 Calorimetric determination of glucose by dinitro salysilic acid (DNS) method

3,5-Dinitrosalicylic acid is an aromatic compound which reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, that 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 the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions.

2.4.1 Standard Maltose solution

Weigh 20 mg of maltose and transfer it into a volumetric flask dissolve and make up the volume 10 ml with distill water

2.4.2 DNS Reagent

1g of DNS dissolved, 200 mg of phenol and 50 mg of sodium sulphide in 100 ml of 1% (w/v) NaOH. Store this reagent in refrigerator.

2.4.3 Pottasium-sodium tartrate tetrahydrate (40% w/v)

Standard Maltose solution:-

Component	Volume
Maltose	20mg
Distill water	10ml

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DNS Reagent:-

Components	Volume
DNS	0.5 g
Phenol crystal	100 mg
Sodium sulphite	25 mg
NaOH	0.5 g
Distill water	50 ml

Procedure:-

1 ml of sugar solution and 2 ml of DNS was added and vortexed. The tubes were heated in water bath for 5 min and then 1 ml of 40% K-Na tartarate was added. Tubes were cooled at room temp and 6 ml distill water added. Absorbance was measured at 540 nm using spectrophotometer.

2.5 Fermentation

Anaerobic batch fermentation of 100 ml broth media consisting of and hydrolysed lingo-cellulosic waste was carried out in order to convert the released sugars into ethanol, the conversion process being accomplished by the enzymes released by *Saccharomyces cerevisiae*. The pH of the solution was brought to 5 -6 by adding required amount of NaOH to accommodate yeast growth. The fermentation media was prepared by addition of yeast extract, urea and dextrose in the following amount per 100 ml of fermentation media.

Yeast extract = 0.2 grams

Urea = 1 gram

Dextrose = 15 grams

The fermentation media containing hydrolysed material was completely sterilized by autoclaving (120°C, 15 psi pressure and 60 mins) before inoculating the yeast.

2.6 Quantitative Estimation Of Bio-Ethanol

Samples were regularly collected from each of the four fermentation broths for three days for quantitative estimation of ethanol. Quantitative estimation was done by both biochemical method and by using a gas chromatograph.

2.6.1 Biochemical Method

Quantitative estimation of ethanol using biochemical method was done by potassium dichromate method. Most of the chemical oxidation methods are based on the complete oxidation of ethanol by dichromate in the presence of sulphuric acid with the formation of acetic acid. This reaction is popular because potassium dichromate is easily available in high purity and the solution is indefinitely stable in air. The theoretical reaction stoichiometry is shown below:

2Cr2O7-- + 3C2H5OH + 16H+ -----> 4Cr+++ + 3CH3COOH + 11H2O

2.6.1.1 Acid dichromate solution: 125 ml of water was added to a 500 ml conical flask. Then 325 ml of concentrated sulphuric acid was carefully added. The flask was cooled under cold water tap and 34 grams of potassium dichromate was added. Dilute to 500 ml with distilled water.

2.6.1.2 2 M Sodium Hydroxide Solution: Add 40 grams of NaOH in 1000 ml of distilled water.

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Procedure:-

10-50 microlitres of absolute alcohol was taken in different test tubes and the volume was made up to 500 microliters by adding distilled water in each test tube.30 microlitres of test sample was taken and the volume was made up to 500 microliters by adding distilled water in test tube.1 ml of potassium dichromate reagent was added in each test tube. Then 2 ml of sodium hydroxide solution was added in each test tube. The test tubes were incubated at 50^0C for 30 minutes. The absorbance was measured at 600 nm by using a spectrophotometer.

2.7 Gas Chromatography

Gas chromatography- specifically gas-liquid chromatography-involves a sampling being vaporized and injected on to the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. It exploits differences in the partition coefficients between a stationary liquid phase and a mobile gas phase of the volatilized analyzed they are carried through the column by the mobile gas phase .the temperature of the column is raised to 50-300 C volatilization.

III. RESULTS

The various lingo cellulosic waste materials have complex carbohydrate in them which cannot be directly converted to ethanol by yeast cells. Thus different Hydrolysis methods with acid, autoclave and enzyme were used to break down the complex sugars into simple monosaccharide units. The total sugar content of the hydrolyzed sugar was measured by DNS method.

Table: 1: DNS Test Observation & Graph (Standard)

S.NO	STANDARD MALTOSE	CONCENTRATION OF MALTOSE (mg/ml)	D.W (ml)	DNS (ml)	POTASSIUM SODIUM TATRATE (ml)	D.W (ml)	O.D
1	BLANK	0	1	2	1	6	0
2	0.1	0.2	0.9	2	1	6	0.16
3	0.2	0.4	0.8	2	1	6	0.32
4	0.3	0.6	0.7	2	1	6	0.48
5	0.4	0.8	0.6	2	1	6	0.62
6	0.5	1.0	0.5	2	1	6	0.78
7	0.6	1.2	0.4	2	1	6	0.94
8	0.7	1.4	0.3	2	1	6	1.09
9	0.8	1.6	0.2	2	1	6	1.24
10	0.9	1.8	0.1	2	1	6	1.39
11	1.0	2.0	0	2	1	6	1.55

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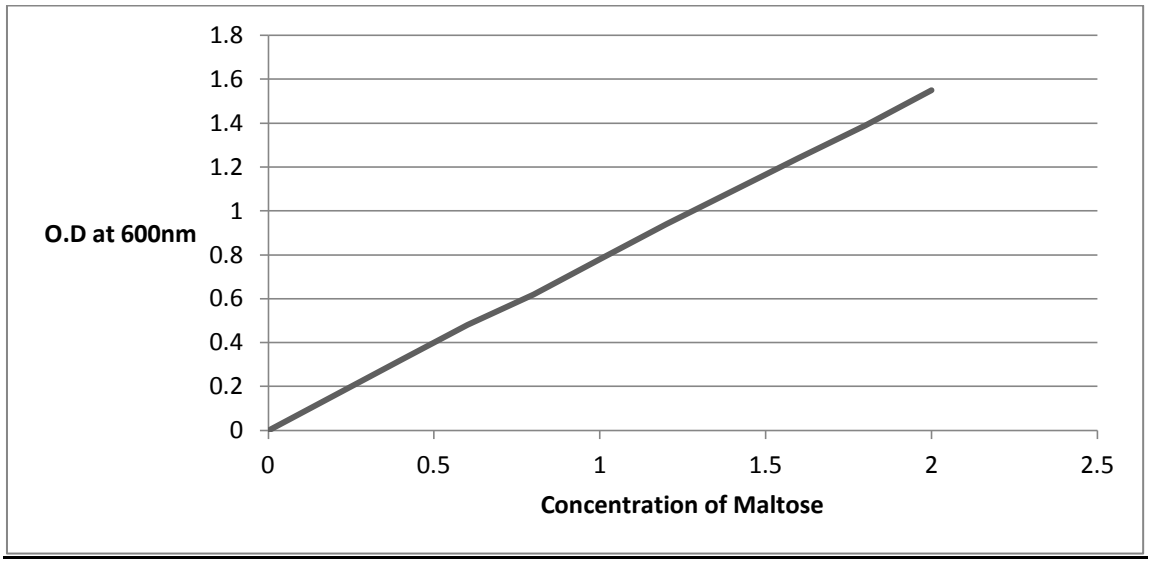

Fig: 1: DNS Test Observation & Graph of standard

Table: 2: Observation of Sugar concentration of 0.3 M hydrolyzed.

S.NO	SAMPLE	O.D Before autoclave	O.D After autoclave	Concentration of sugar (mg/ml) after Autoclave
1	BLANK	0.00	0.00	
2	Bagasse	1.22	1.54	2.05
3	Pea waste	0.26	0.38	0.49
4	Orange Pulp	1.00	1.02	1.16
5	Molasses	1.49	1.69	3.16

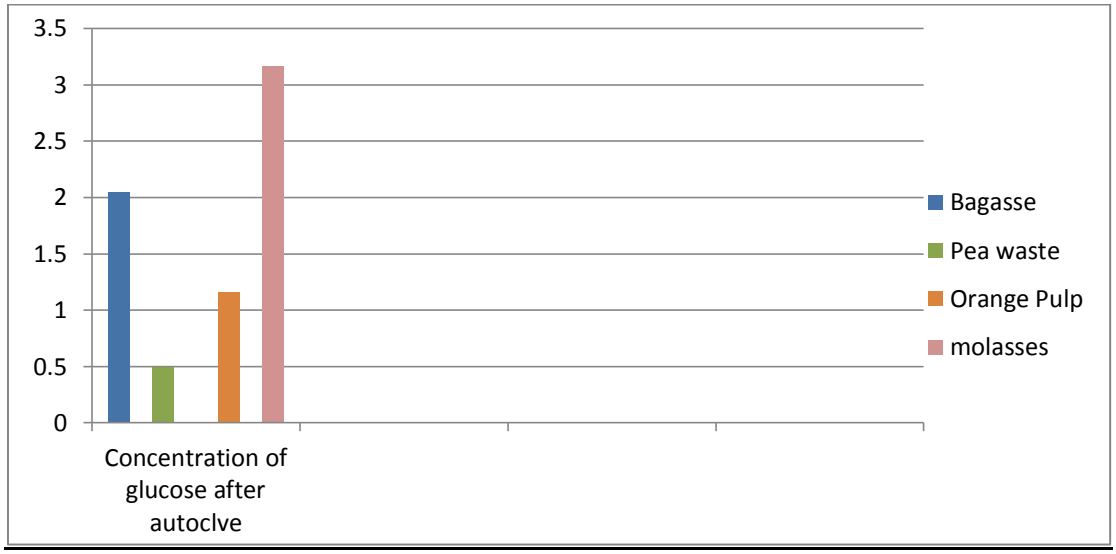

Fig: 2: Concentration of sugar in 0.3 M hydrolyzed compound

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Table: 3: For 0.5 M hydrolyzed compound Sugar concentration.

S.NO	SAMPLE	O.D Before autoclave	O.D After autoclave	Concentration of sugar (mg/ml) after Autoclave
1	BLANK	0.00	0.00	
2	Bagasse	1.42	1.70	2.45
3	Molasses	1.89	1.99	3.56

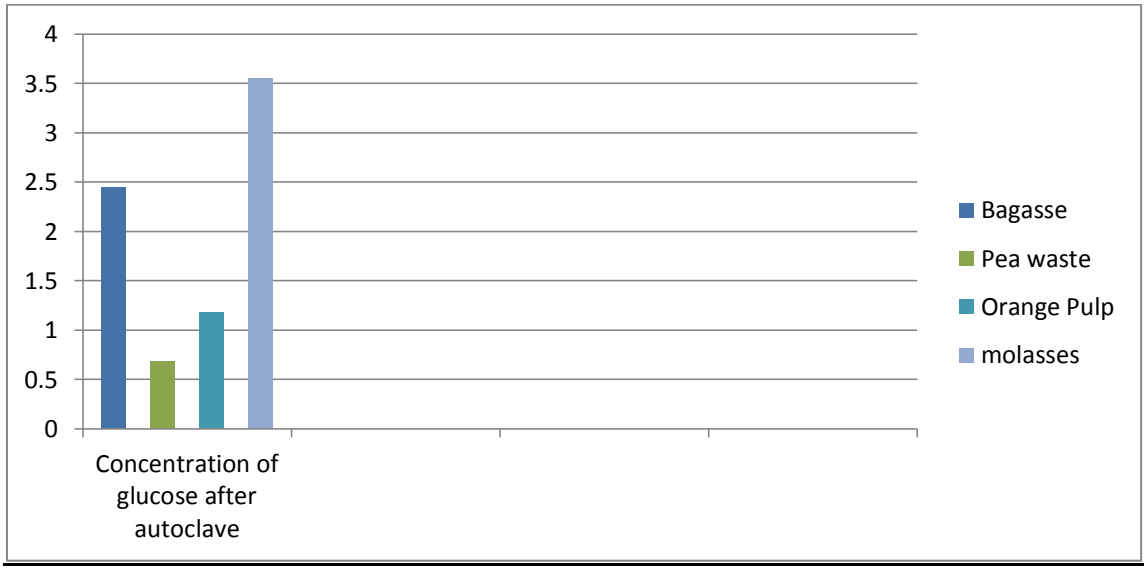

Fig.:3 Graphical representation of concentration of Glucose of 0.5 M hydrolyzed compound DNS result.

Table:4 For Enzyme hydrolyzed compound Sugar concentration.

S.NO	STANDARD MALTOSE	O.D Before autoclave	O.D After autoclave	Concentration of sugar (mg/ml) after Autoclave
1	BLANK	0.00	0.00	
2	Bagasse	1.03	1.45	1.87
3	Pea waste	1.45	1.51	1.94
4	Orange Pulp	0.95	1.12	1.44
5	Molasses	1.68	1.84	2.37

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Fig: 4: Graphical representation of Enzyme hydrolyzed compound sugar concentration in various samples

Table: 5: Absorbance of standard ethanol

Potassium Dichromate (Standard):

Sample Concentration	Amount of sample(Absolute alcohal)	Distilled Water (µl)	$Kr_2Cr_2O_7$ +D.W+H_2SO_4 (ml)	2M NaOH (ml)	Absorbance at 600 nm
10%	50	450	2	1	0.35
20%	100	400	2	1	0.38
30%	150	350	2	1	0.41
40%	200	300	2	1	0.45
50%	250	250	2	1	0.50
60%	300	200	2	1	0.54
70%	350	150	2	1	0.57
80%	400	100	2	1	0.61
90%	450	50	2	1	0.66
100%	500	00	2	1	0.72

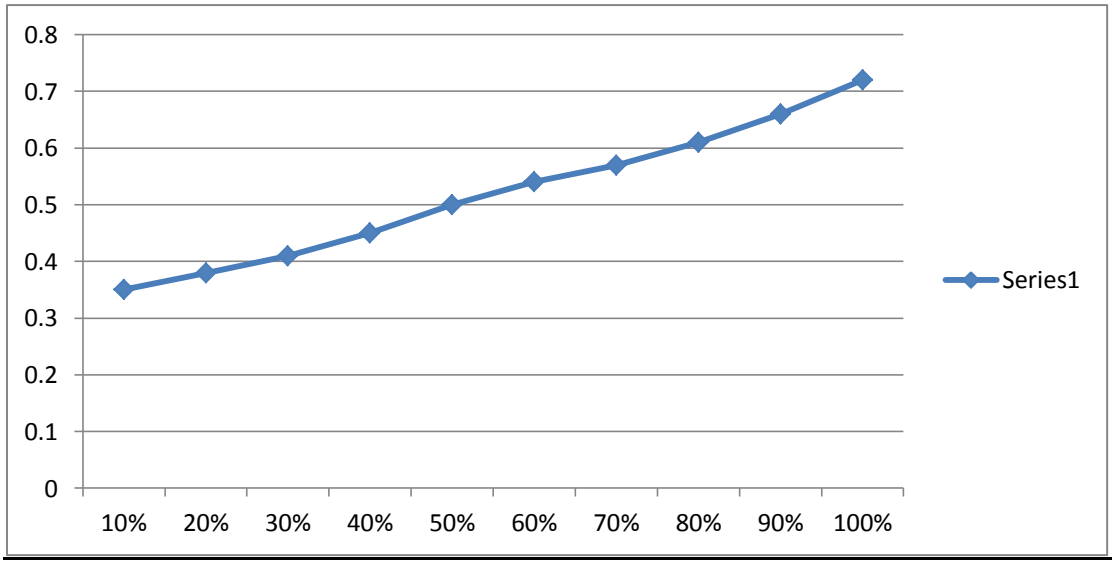

Fig.5 Graphical presentation of Standard alcohol by potassium dichromate test

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Table: 6: Dichromate test of Sample hydrolyzed by 0.3 M Sulphuric acid-

S.No.	SAMPLE NAME	AMOUNT OF PRODUCED ETHANOL IN % age
1	Bagasse	63 %
2	Potato	60%
3	Pea	58%
4	Molasses	95%
5	Orange Peel	58%
6	Orange Pulp	63%

Table: 7: Potassium dichromate test of ethanol produced in different samples hydrolyzed using 0.3 M Sulphuric acid

Sl. no.	Sample Name	OD at 600 nm				Ethanol
		Day 1	Day 2	Day 3	Day 4	
1	Bagasse	0.52	0.6	0.45	0.29	0.6
2	Potato	0.4	0.58	0.42	0.31	0.58
3	Pea	0.52	0.56	0.42	0.26	0.56
4	Molasses	0.46	0.9	0.67	0.49	0.9
5	Orange peel	0.46	0.56	0.45	0.4	0.56
6	Orange pulp	0.44	0.53	0.46	0.31	0.53

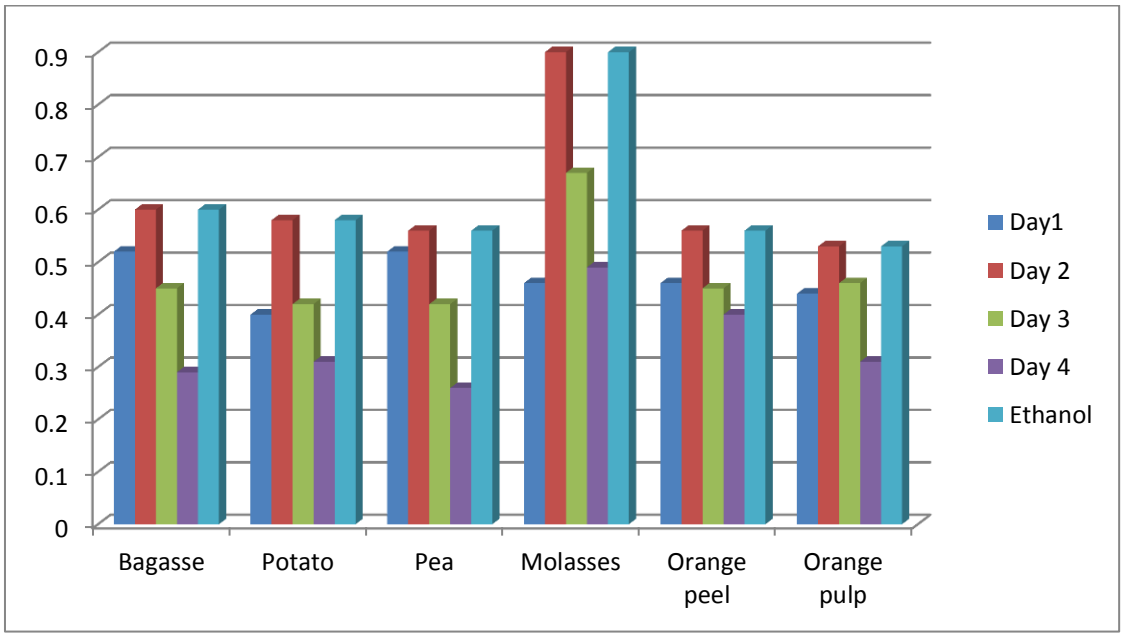

Fig: 6 : Dichromate test of Samples Hydrolysed by Enzyme

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Table: 8: Potassium dichromate test of ethanol produced in different samples hydrolyzed using Enzyme on 2 nd day.

S.No.	SAMPLE NAME	AMOUNT OF PRODUCED ETHANOL IN % age
1	Bagasse	42%
3	Pea waste	24%
4	Molasses	85%
5	Orange Peel	56%
6	Orange Pulp	53%

Table: 9: Estimation of the amount of ethanol produced by using Potassium Dichromate test of 0.5M acid hydrolyzed samples.

Sample	Potassium dichromate (ml)	2M NaOH (ml)	Amount of sample (µl)	D/W (µl)	O.D. at 600nm			
					First Day	Second day	Third day	Fourth day
Agricultural Wastes (Mixture of all lignocellulosic & household waste)	1	2	250	250	0.74	**0.80**	0.65	0.40

SAMPLE NAME	AMOUNT OF PRODUCED ETHANOL IN % age
Agricultural Wastes	**80%**

Table: 10, 11, 12& 13. Estimation of ethanol in various samples using Gas Chromatography Method

				Ethanol Concentration (gm/l)		
Sl no	Sample	Hydrolysis	R.T (Peak Obtnd.)	Day 1	Day 2	Day 3
1	Bagasse	0.3 M H2SO4	2.1	10.78	13.17	11.69
2		0.5 M H2SO4	2.2	16.14	25.3	23.66
3		Amylase Enzyme	2.15	19.14	21.16	20.54

Table:10

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Sl no	Sample	Hydrolysis	R.T (Peak Obtnd.)	Day 1	Day 2	Day 3
1	Pea waste	0.3 M H2SO4	2.15	17.13	18.42	11.69
2		0.5 M H2SO4	2.31	19.6	13.17	20.1
3		Amylase Enzyme	2.2	13.56	20.54	15.43

Table 11

Sl no	Sample	Hydrolysis	R.T (Peak Obtnd.)	Day 1	Day 2	Day 3
1	Orange Pulp	0.3 M H2SO4	2.15	2.29	10.12	11.69
2		0.5 M H2SO4	2.01	10.78	21.68	12.33
3		Amylase Enzyme	2.2	4.3	11.5	9.85

Table 12

Sl no	Sample	Hydrolisis	R.T (Peak Obtnd.)	Day 1	Day 2	Day 3
1	Molasses	0.3 M H2SO4	2.16	20.73	21.71	20.89
2		0.5 M H2SO4	2.42	28.59	30.25	28.9
3		Amylase Enzyme	2.15	21.5	23.25	22.31

Table: 13

Table: 14: Estimation of the amount of ethanol produced by using a gas chromatograph in Bagasse.

S. No.	SAMPLE	HYDROLYSIS	R.T.(PEAK OBTAINED)	DAY 1 ETHANOL COCN (gm/l)	DAY 2 ETHANOL COCN (gm/l)	DAY 3 ETHANOL COCN (gm/l)
1.	Bagasse	0.3 M H_2SO_4	2.10	10.78	13.17	11.69
2.	Bagasse	0.5 M H_2SO_4	2.20	16.14	25.30	23.66
3.	Bagasse	Amylase Enzyme	2.15	19.14	21.16	20.54

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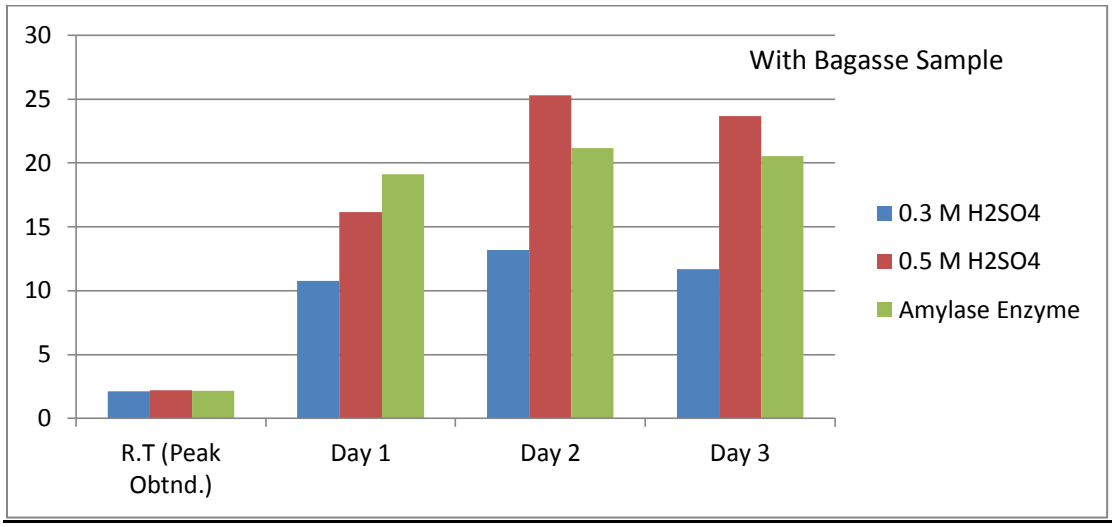

Fig; 7: Graph showing ethanol production in bagasse by Gas chromatography

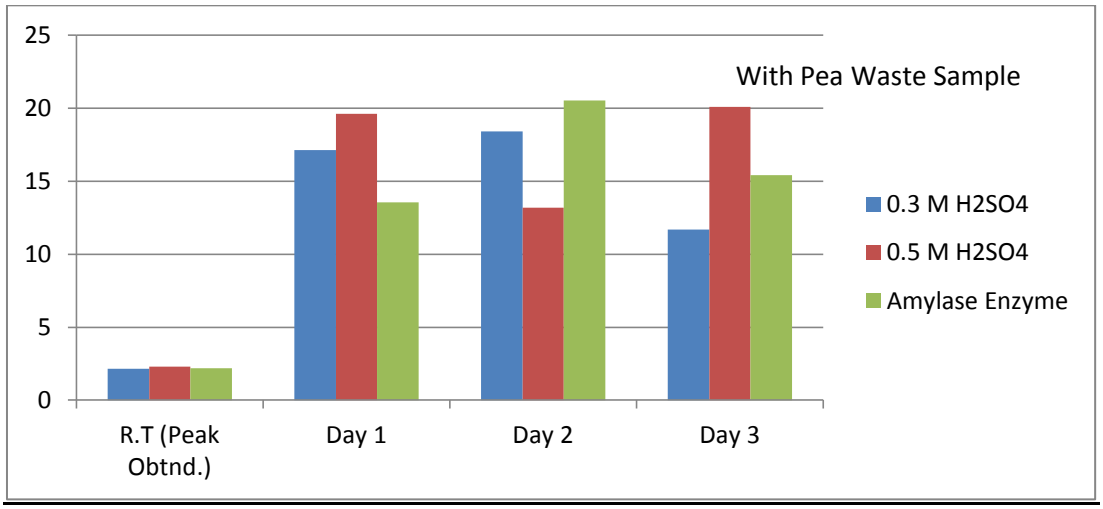

Fig:8: Graph showing ethanol production in pea waste by gas chromatography

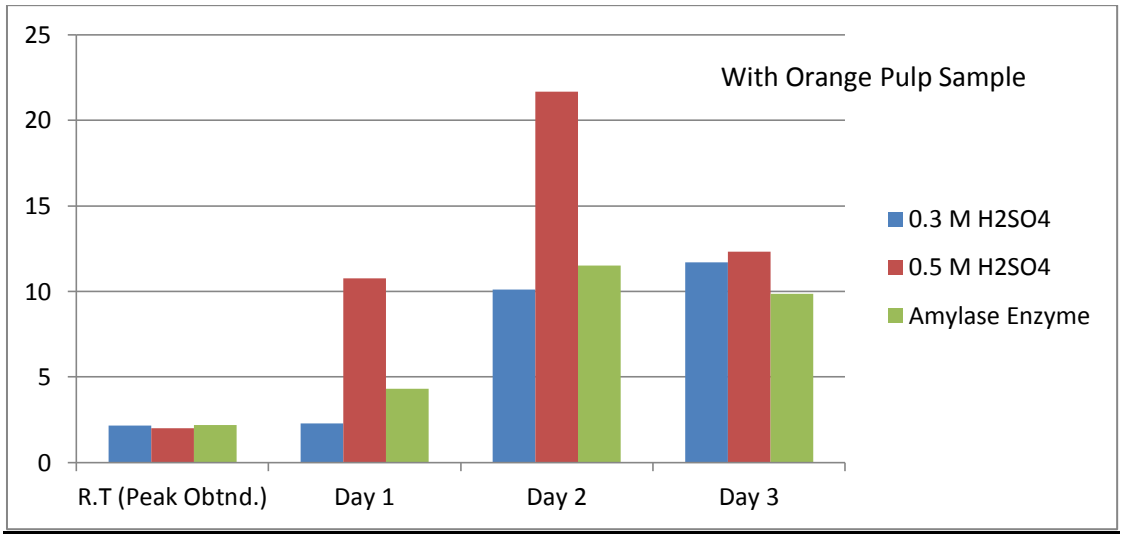

Fig: 9: Graph showing ethanol production in orange pulp by gas chromatography

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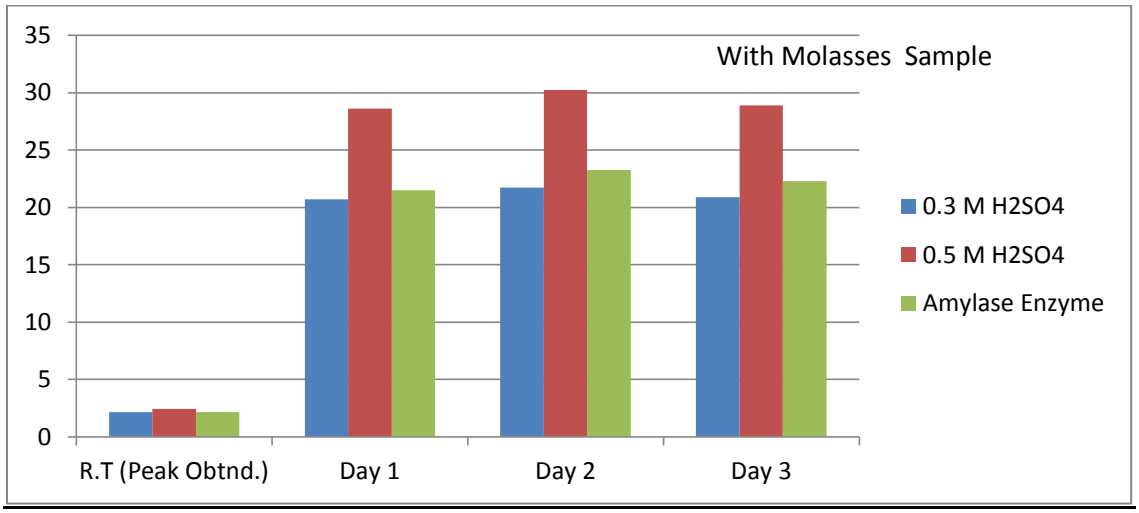

Figure: 10: Graph showing ethanol production in molasses by gas chromatography

Table: 15: Estimation of the amount of ethanol produced by gas chromatograph in Pea.

S. No.	SAMPLE	HYDROLYSIS	R.T. (PEAK OBTAINED)	DAY 1 ETHANOL COCN (gm/l)	DAY 2 ETHANOL COCN (gm/l)	DAY 3 ETHANOL COCN (gm/l)
1.	Pea waste	0.3 M H_2SO_4	2.15	17.13	18.42	14.69
2.	Pea waste	0.5 M H_2SO_4	2.31	19.6	13.17	20.10
3.	Pea waste	Amylase Enzyme	2.20	13.56	20.54	15.43

Table : 16: Estimation of the amount of ethanol produced by gas chromatograph in orange Pulp.

S. No.	SAMPLE	HYDROLYSIS	R.T. (PEAK OBTAINED)	DAY 1 ETHANOL COCN (gm/l)	DAY 2 ETHANOL COCN (gm/l)	DAY 3 ETHANOL COCN (gm/l)
1.	Orange pulp	0.3 M H_2SO_4	2.16	2.29	10.12	11.69
2.	Orange pulp	0.5 M H_2SO_4	2.01	10.78	21.68	12.33
3.	Orange pulp	Amylase Enzyme	2.20	4.30	11.50	9.85

Table: 17: stimation of the amount of ethanol produced by gas chromatograph in Molasses

S. No.	SAMPLE	HYDROLYSIS	R.T. (PEAK OBTAINED)	DAY 1 ETHANOL COCN (gm/l)	DAY 2 ETHANOL COCN (gm/l)	DAY 3 ETHANOL COCN (gm/l)
1.	Molasses	0.3 M H_2SO_4	2.16	20.73	21.71	20.89
2.	Molasses	0.5 M H_2SO_4	2.42	28.59	30.25	28.90
3.	Molasses	Amylase Enzyme	2.15	21.50	23.25	22.31

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Table: 18 Estimation of the amount of ethanol produced in Small scale Fermenter. (Mixture of all lingo cellulosic and household waste) by using a gas chromatograph in Agricultural Wastes

S. No.	Sample	Hydrolysis	R.T (Peak Obtained)	Area obtained on 2nd day peak	Day 1 Ethanol Conc. (gm/l)	Day 2 Ethanol Conc. (gm/l)	Day 3 Ethanol Conc. (gm/l)
1	Agricultural Wastes	0.5 M H_2SO_4	2.40	6681463	19.49	21.16	18.99

Table: 19: Estimation of amount of ethanol in agricultural wastes after distillation

S. No.	Sample	R.T (Peak Obtained)	Area obtained on 2nd day peak	Day 1 Ethanol Conc. (gm/l)
1	Agricultural Wastes	2.32	17612669	30.49

IV. DISCUSSION

The chemical and enzymatic hydrolysis of different ligno-cellulosic waste to convert cellulose into reducing sugars had shown positive results. The maximum amount of sugar was produced from hydrolyzed bagasse which is a waste product of sugar cane industry. Agriculture waste also produced sufficient amount of sugar. The released sugars were fermented for 2 days to produce ethanol and estimation of ethanol was done after every 24 hours. Ethanol estimation was done from preliminary potassium dichromate method and by using a gas chromatograph after bioethanol was successfully produced from different ligno-cellulosic wastes.

Gas chromatography has advantages over potassium dichromate method as it is rapid, sensitive and accurate. Potassium dichromate method is preliminary method for quantification of ethanol. More amount of ethanol was estimated by potassium dichromate method than by using a gas chromatograph as the chances of error are more in potassium dichromate method. Thus, amount of ethanol estimated by using gas chromatograph was considered more appropriate.

The maximum amount of ethanol estimated using a gas chromatograph was 30.25 grams/litre which was produced from Molasses on second day which was used as standard. Bagasse produced 25.30 grams/liter of ethanol on second day when hydrolyzed using 0.5 M of sulphuric acid Pea waste, Orange pulp which was household waste produced ethanol was about 13.17 gm/L, 21.68 gm/L respectively when hydrolyzed by 0.5 M H2SO4 And Agriculture waste (mixture of all household & lignocellulosic waste) in small scale 3 liter fermentor fermentation was done and produced ethanol of amount 21.16 gm/L on second day and after purification(distillation) production was 30.49 gm/L.

This recovery of ethanol is comparable to other studies done by different scientist on different raw materials. Thus, agricultural wastes can be economically used for ethanol production.

V. CONCLUSION

It can be concluded that ligno-cellulosic waste materials such as agriculture waste, sugarcane waste, fruit waste and biodiesel waste have the capability to undergo acid and enzymatic hydrolysis and fermentation to produce bioethanol. Ligno-cellulosic wastes are rich in cellulose thus can be used to produce cellulosic ethanol. Ligno-cellulosic wastes consist of high amount of glucose which can be converted to bioethanol. Use of ethanol as a fuel can reduce greenhouse gas emission thus reduces air pollution. Fuel ethanol reduces the dependency on the fossil fuels by reducing the use of petroleum for automobile transporation.

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