Project report

on

PRODUCTION OF PENICILLIN BY *PENICILLIUM NOTATUM* USING DIFFERENT CARBON SOURCES

Submitted in Partial Fulfillment of the Requirement for the Degree of M.Sc. Biochemistry.

Submitted by

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M.Sc. Biochemistry (Semester IV)

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Uttar Pradesh

(Greater Noida)

May 2020

GALGOTIAS UNIVERSITY

(Established under Galgotias University Uttar Pradesh Act No. 14 of 2011)

School of Basic and Applied Science

CERTIFICATE

This is to Certify that **Mr.Rahul Palariya** has carried out his project work entitled "PRODUCTION OF PENICILLIN BY *Penicillium notatum* USING DIFFERENT CARBON SOURCES" under my supervision. This work is fit for submission for the award of Master Degree in Forensic Science.

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CERTIFICATE

This is to certify that the Project entitled "**PRODUCTION OF PENICILLIN BY *PENICILLIUM NOTATUM* USING DIFFERENT CARBON SOURCES**" submitted in partial fulfillment for the award of the Degree of **M.Sc. (BIOCHEMISTRY)** from Galgotias University, Greater Noida, is a record of research work carried out by **MR. RAHUL PALARIYA** from 31st January 2020 to 16th March 2020 under my guidance and supervision.

All the help and assistance received during the course of this investigation have been duly acknowledged.

Tripti Bhatnagar

Dated : 20/05/2020
Place : Noida
No : CBPL2020/108

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CANDIDATE DECLARATION

I hereby declare that the dissertation entitled "PRODUCTION OF PENICILLIN BY *Penicillium notatum* USING DIFFERENT CARBON SOURCES" submitted by me in partial fulfillment for the degree of M.Sc. in Biochemistry to the Division of Biochemistry, School of Basic and Applied Science, Galgotias University, Greater Noida, Uttar Pradesh, India is my original work. It has not been submitted in part or full to this University of any other Universities for the award of diploma or degree.

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LIST OF ABBREVIATIONS

- ACV d- (L-a-Amino adipyl)-L-cysteine-D-valine
- AA- Amino acids
- AAA-Aminoadipic acid
- Cys - Cysteine
- Val-Valine
- AMP -Adenosine monophosphate
- ATP -Adenosine triphosphate
- β -GAL- β -Galactosidase
- β -GLU- β -Glucuronidase
- bp -Base-pairs
- A-Adenine
- Å -Angstrom
- mRNA-Messenger ribonucleic acid
- nt- Nucleotide
- PDA-Potato Dextrose Agar.
- TBC- Total Bacterial Count.
- TFC- Total Fungal Count.

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In a work like this, it is impossible to accomplish the task without encouragement and valuable help of much gratitude; one feels from heart and cannot be discussed exactly. I express my immense sense of gratitude to Almighty, who blessed me in all ways in completion of this project.

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INTRODUCTION

The word Antibiotic means ‘against life’ they are low molecular weight antimicrobial agents produced as secondary metabolites by microorganism.

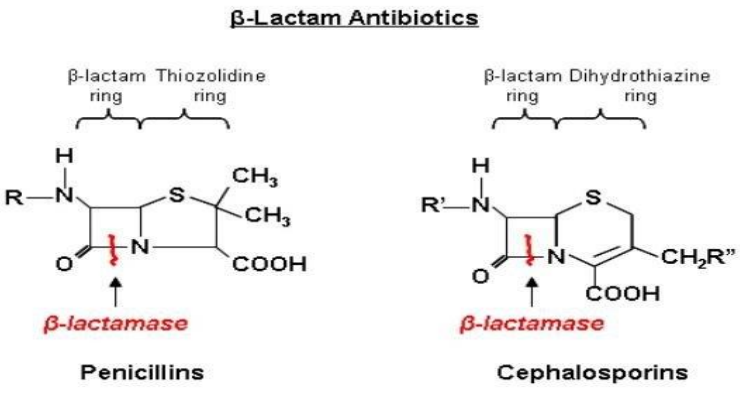
Antibiotics either kill or inhibit the growth of bacteria. Penicillin was the first Antibiotic and it was discovered by Alexander Fleming in 1928. Whilst some antibiotics are able to completely kill other bacteria, some are only able to inhibit their growth. Those that kill bacteria are termed bactericidal while those that inhibit bacterial growth are termed bacteriostatic **(Walsh, 2003).**

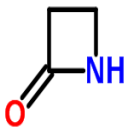
Antibiotics do not work against infections that are caused by viruses - for example, the common cold or flu. because bacteria and viruses have different mechanisms and machinery to survive and replicate. The antibiotic has no “target” to attack in a virus.

Penicillin was the first antibiotic discovered in September 1928 by an English Bacteriologist, late Sir Alexander Fleming who accidentally got the antibiotic from a soil inhabiting fungus Penicillium notatum but this discovery was reported in 1929 **(Aminov, 2010)**, and first clinical trials were conducted on humans in 1940 **(Russell, 2004; Schlegel, 2003)**.

Examples of some commonly used Antibiotics-:

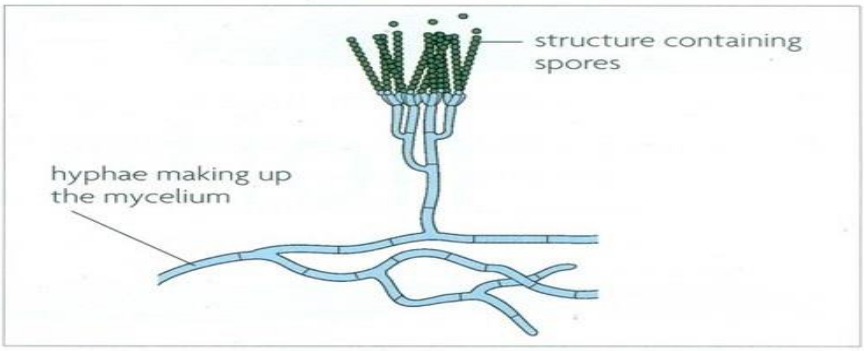
Beta-lactam Antibiotics- Members of this class of antibiotics contain a 3-carbon and 1-nitrogen ring that is highly reactive. Penicillin and Cephalosporins both contain β-lactam ring.





β-lactam ring

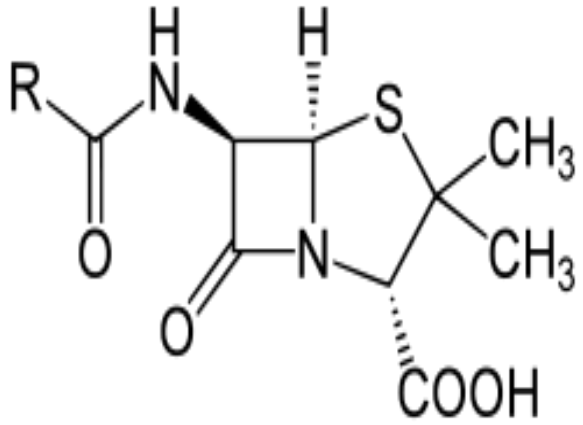
Penicillin-Penicillin was first discovered by Alexander Fleming in 1928 as a metabolic by product of the fungus *Penicillium notatum*. All penicillin shares a common bicyclic nucleus that is composed of fused β- lactam and thiazolidine rings. Penicillins are active against a variety of Gram-positive bacteria, including Staphylococci and Streptococci. They are beta lactam compounds containing a nucleus of 6animopenicillanic acid (lactam plus thiazolidine) ring and other ring side chains **(Zahner and Maas, 1972).**



Penicillium the fungus that makes

Structure of Penicillin:-

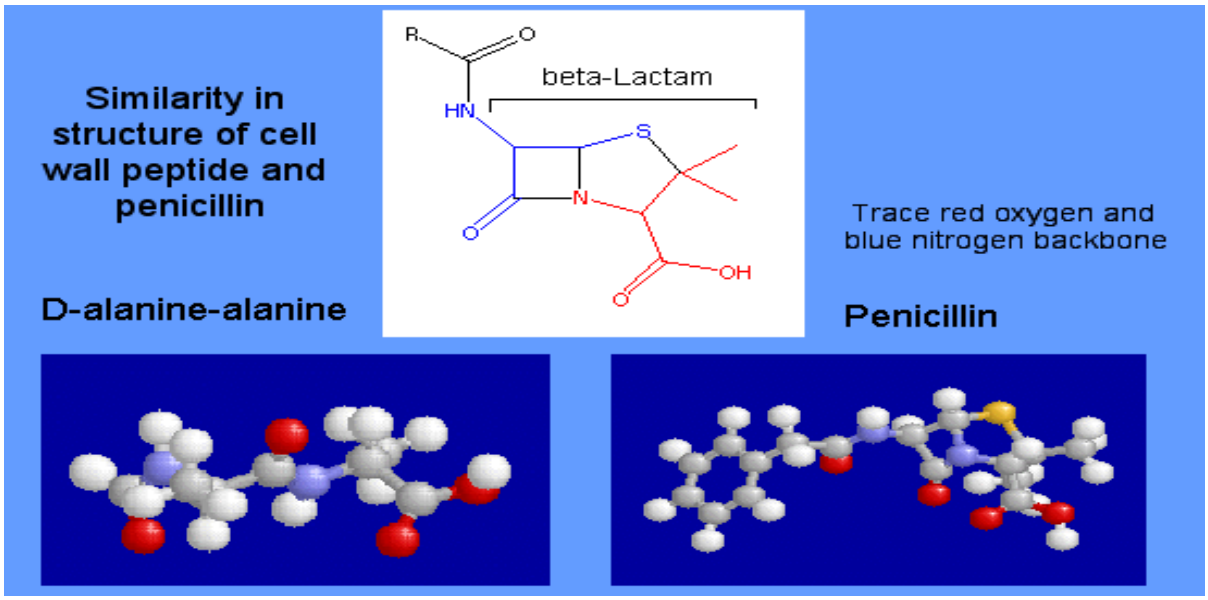
Its molecular formula is R-$C_9H_{11}N_2O$. Its main part is the β-lactam ring which is the reason for its bactericidal effects, and it is also joined to a thiazolidine ring.



The core structure of Penicillin: The "R" is the variable group.

Penicillin Mode of Action: -

The antibacterial effects of penicillin arises from its ability to prevent bacterial cell wall formation. These walls are formed by the formation of crosslinks between mucosaccharide chains.Penicillin prevents the formation of these peptide crosslinks, thus effectively preventing formation of the wall. Since the cell can no longer form and maintain its walls, water flow is unchecked and water floods in due to osmotic pressures. The uncontrolled influx of water causes the cell to swell up and then burst. The mechanism of the drug has been partially attributed to some structural similarities between the molecule and the peptides of a cell wall. Such similarities have been highlighted below.

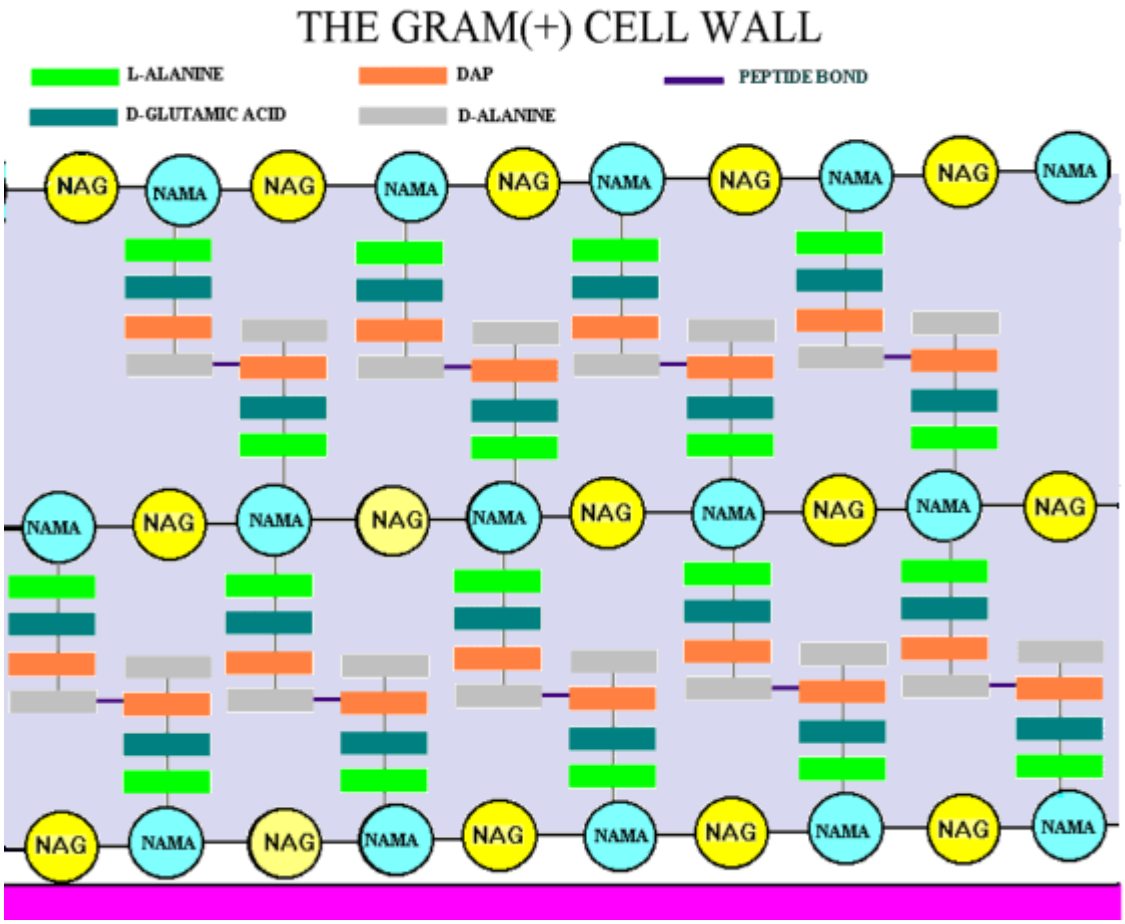


Similarity between structure of cell wall peptide and Penicillin

The cell walls of gram-positive bacteria consist of structural sugar polymers connected to peptides via peptide bonds. The peptides present may vary, but are observed to begin with L-alanine and end with a D-alanine. Between these is present a dibasic amino acid known as diaminopimelate (DAP). DAP is orange in colour and helps connect to the next peptide in the chain.

In order for cell wall synthesis to occur, a peptide bond must be formed between the two units. The formed chains are supported by a polysaccharide backbone. First the terminal alanine from

a peptide chain is hydrolyzed. Following that the alanine is joined to an adjacent lysine residue through an amide bond.



Cell wall of Gram -Positive bacteria

Penicillin works by binding at the active site of the enzyme responsible for cross-linking the strands. The attachment occurs because it can mimic the D-alanyl-D-alanine residues that would normally bind to this site. After binding, penicillin modifies a serine residue in its structure. The modification is permanent and disables the enzyme from forming more cell walls. Growth inhibition does not occur in mammalian cells since their cell walls are made of different components.

Cephalosporins- Cephalosporins originally isolated from the fungus *Cephalosporium*, and their lactam structure is very similar to that of the Members of the cephalosporin family of

antibiotics are effective in preventing the synthesis of bacterial cell wall and mostly useful against Gram-positive bacteria.

Tetracyclines-The tetracyclines are the family of antibiotics with a common four membered ring to which a variety of side chain attached. Tetracyclines are a class of broad-spectrum antibiotics used in the management and treatment of a variety of infectious diseases.

Tetracycline was discovered in 1945 from a soil bacterium of the genus Streptomyces by Benjamin Duggar **(Sanchez et al., 2004).** They include naturally occurring Chlorotetracycline and Oxytetracycline, Isolated from species of Streptomyces. They are used in Gram negative infections as Brucellosis, Plague, Cholera, Gonorrhea and Pneumonia; and therapy of some Protozoan infections as amoebiasis. **(Sánchez AR et al., 2004).**

Most of the tetracyclines are administrated by oral route. However, topical, intramuscular (IM) and intravenous (IV) forms of the medication do exist. Only oxytetracycline and tetracycline are administrated by IM injection. Oral tetracycline absorption occurs primarily in the stomach, duodenum, and small intestine. They contribute well in tissues, ascitic fluid, synovial fluid, pleural fluid, and bronchial secretions. However, Tetracyclines have poor penetration into the cerebral spinal fluid. The absorption of all tetracyclines decreases when administered with multivalent cations such as aluminum, calcium, iron, or magnesium. Cations cause chelation of the tetracyclines, hence impairing their absorption in the gut, leading to excretion of the drug in the urine and feces. **(Bernier C.et al., 2001; Dougherty JA., et al., 2019).**

Tetracyclines can commonly cause GI distress, including abdominal discomfort, epigastric pain, nausea, vomiting, and anorexia. While taking tetracyclines, discoloration of teeth and inhibition of bone growth in children may occur.

Aminoglycoside antibiotics- Aminoglycoside antibiotics are inhibitors of Protein synthesis in bacteria. The aminoglycosides include gentamicin, amikacin, neomycin, and streptomycin. The first drug which was discovered among members of this class of antibiotics was streptomycin, first isolated in 1943 **(Mahajan and Balachandran, 2012).** The aminoglycosides are broad-spectrum, bactericidal antibiotics. Aminoglycosides are polar drugs, with poor gastrointestinal absorption, that is why intravenous or intramuscular administration is needed. The Aminoglycoside antibiotics active against Gram-negative bacteria. The aminoglycosides consist of two or more amino sugars joined in glycosidic linkage to an hexose nucleus. Aminoglycoside antibiotics have a broad spectrum of antibacterial activity. They are able to inhibit the protein synthesis in bacteria by binding to one of the ribosomal subunits (Peterson, 2008), and are effective against aerobic Gram-negative rods and certain Gram-positive bacteria.

- Sulphnamide-Sulphonamide or sulfa drugs are structurally related to sulfanilamide,

- an analogue of p-aminobenzoic acid. Sulphonamides inhibit both Gram-positive and Gram-negative bacteria such as Nocardia, E. coli, Klebsiella, Salmonella, Shigella and Enterobacter, Chlamydia trachomatis and some Protozoa, and are widely used in the treatment of various infections including tonsillitis, septicemia, meningococcal meningitis, bacillary dysentery and some urinary tract infections **(Eyssen et al., 1971).**When sulphnamide or other suphamide

enters a bacterial cell, it competes with p-aminobenzoic acid(PABA). It results in decrease in folate concentration. Side effect includes Skin rash Itching Headache, Dizziness, Diarrhea, Tiredness.

- **Classification of Antibiotics: -**

Antibiotics are classified on the basis of two criteria, in addition to their chemical structure:

- Microbial source
- Mode of action

Classifying antibiotics based on their source is not effective, since this method is too broad. Multiple antibiotics can be isolated from the same organism. Classifying them on their mode of action can also be ineffective, since the same antibiotic may have multiple modes of action, placing them in several different categories.

On the basis of their chemical structure antibiotics have following main classes:

- Amino glycosides
- Macrolides
- Penicillins
- Tetracyclines
- Peptide antibiotics
- Chloramphenicol
- Antifungal antibiotics

Mode of action of antibiotics: -

1. **Cell wall synthesis inhibitors-**

   Penicillin and Cephalosporins both contain β-lactam ring. Penicillins and other antibiotics in the beta-lactam family contain a characteristic four-membered beta-lactam ring. Penicillin functions during the synthesis of bacterial cell wall. The molecules block the cross linking of hexose in the peptidoglycan layer during cell wall formation, causing bursting of cell.

   Gram-positive bacteria have thick cell walls containing high levels of peptidoglycan, while gram-negative bacteria are characterized by thinner cell walls with low levels of peptidoglycan. The cell walls of gram-negative bacteria are surrounded by a lipopolysaccharide (LPS) layer than prevents antibiotic entry into the cell.

Therefore, penicillin is most effective against gram-positive bacteria where DD-transpeptidase activity is highest.

2. **Protein synthesis inhibitors-:**

Such Antibiotics are Streptomycin, Neomycin, Kanamycin.

They bind to the 30S ribosomal subunit of the 70s Prokaryotic Ribosomes. Apart from this such antibiotics erythromycin, clindamycin, lincomycin, chloramphenicol, linezolid etc

There after Translation process will not occur as well as Protein will not form.

Antibiotics such as erythromycin, clindamycin, lincomycin, chloramphenicol, linezolid binds with 50S Ribosome. In generic terms, antibiotics which inhibit 50S ribosome do so by physically blocking either the starting phase of protein translation or the elongation phase of protein synthesis where the incoming amino acid is linked up with the growing nascent peptide chain **(Patel et al., 2001; Vannuffel and Cocito, 1996; Menninger and Otto, 1982).**

The 30S ribosome-inhibitors predominantly work by obstructing the access of aminoacyl-tRNAs to the ribosome. Examples of antibiotics that function in this manner include the tetracycline, streptomycin, spectinomycin, etc. **(Hong et al., 2014; Chopra and Roberts, 2001).**

3. **D.N.A Inhibitors-:**

D.N.A Inhibitors Antibiotics are Ciprofloxacin, Nalidixic Acid, Novobiocin, Mitomycin-C.

They block DNA Replication (Inhibit bacterial DNA gyrase).

Hydroxyurea is a well-established inhibitor of Ribonucleotide reductase.

Quinolones that inhibit bacterial nucleic acid synthesis in this way do not interact with mammalian RNA polymerase, making them specifically antagonistic to Gram-positive bacteria and some Gram-negative bacteria.

4. **Antibiotics that block RNA synthesis-:**

Actinomycin D- Binds DNA. Actinomycin D (dactinomycin) is a natural chromopeptide composed of a heterocyclic chromophore and two cyclic pentapeptide lactone rings. The heterocyclic fragment is a phenoxazine derivative, containing a

quinonimine portion, and is responsible for the color of the compound and its intercalative ability. Actinomycin D, one of the oldest anticancer drugs.

Streptomycin- Streptomycin is an aminoglycoside antibiotic produced by the soil actinomycete Streptomyces griseus. It acts by binding to the 30S ribosomal subunit of susceptible organisms and disrupting the initiation and elongation steps in protein synthesis.

Rifampin- β-subunit of RNA Polymerase. The enzyme liable for DNA transcription Streptolydigin (Stl) is a potent inhibitor of bacterial RNA polymerases (RNAPs). (β-subunit of RNA polymerase). Cellular RNA synthesis is prohibited by Bleomycin and the inhibition is nonspecific. **(M. Tien Kuo et al 1977).**

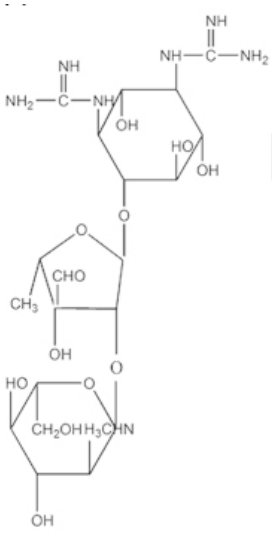
5. **Inhibitors of metabolic processes-**

Sulfonamides or sulfa drug when enters a bacterial cell, it competes with (PABA).

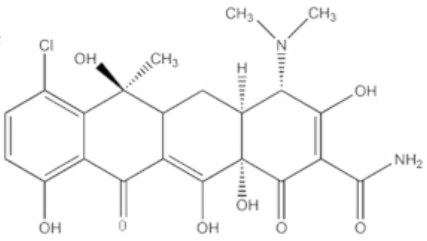
After that it inhibits folic acid synthesis by completion with p-aminobenzoic acid. In particular sulphonamides act like tetrahydrofolate which is needed for the synthesis of folic acid in bacterial cells **(Talaro and Chess, 2008).**

Trimethoprim inhibit dihydrofolate reductase synthesis.

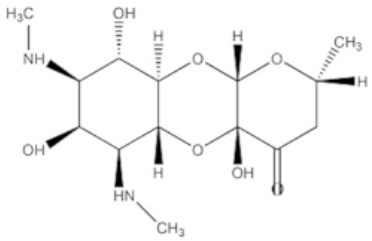
Structure of some common antibiotics-:



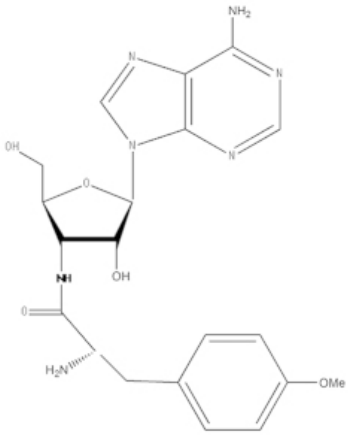
Streptomycin



Tetracycline



Spectinomycin



Puromycin

OBJECTIVES

1. Isolation of Penicillium strains from decaying bread.

2. Morphological analysis of the isolated Penicillium strains.

3. Isolation of pathogenic bacterial test cultures from soil.

4. Production of Penicillin using submerged fermentation

5. Study of different carbon sources for optimal Penicillin production.

REVIEW OF LITERATURE

Antibiotics do not work against infections that are caused by viruses - for example, the common cold or flu. because bacteria and viruses have different mechanisms and machinery to survive and replicate. The antibiotic has no “target” to attack in a virus.

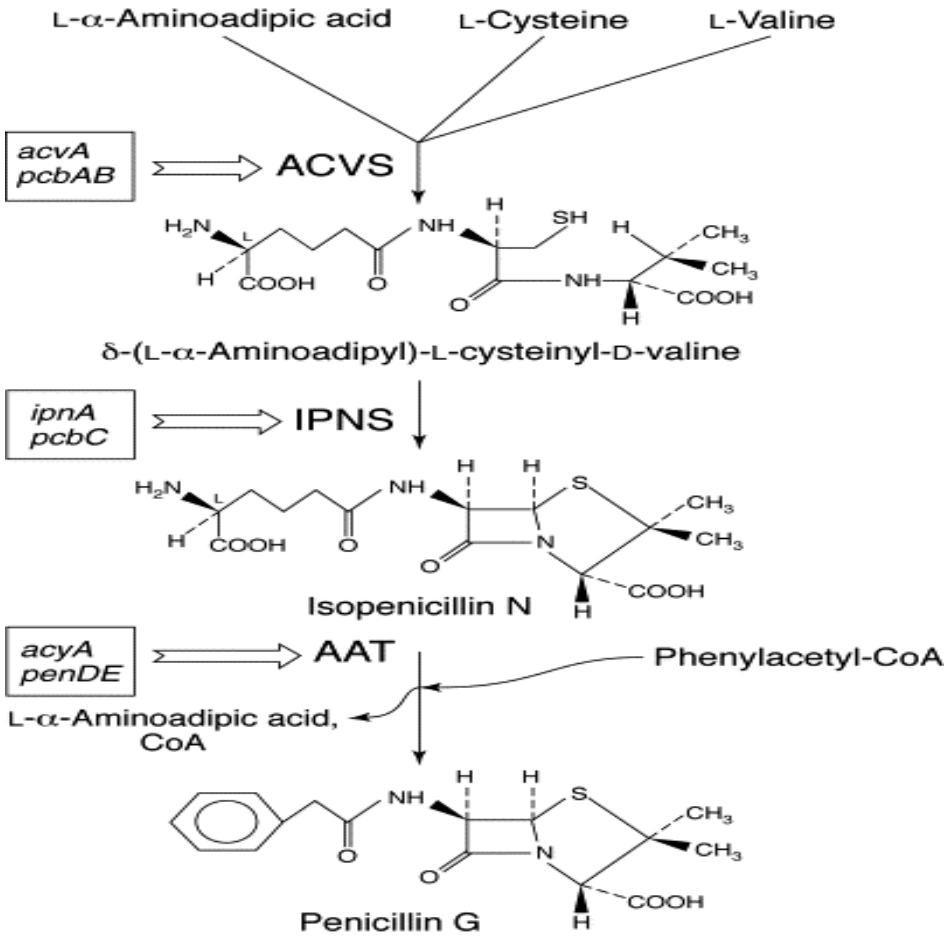
Penicillin was the first antibiotic discovered in September 1928 by an English Bacteriologist, late Sir Alexander Fleming who accidentally obtained the antibiotic from a soil inhabiting fungus Penicillium notatum but its discovery was first reported in 1929 **(Aminov, 2010)**, and clinical trials first conducted on humans in 1940 **(Russell, 2004; Schlegel, 2003)**.

The β-lactam antibiotic penicillin is one of the mainly used antibiotics for the therapy of infectious diseases. **(A.A.Brakhage et al).** The discovery of penicillinsin1929 by Fleming is probably the most important observation in the history of therapeutic medicine development. Penicillins belong to the group of β -lactam antibiotics and are produced as secondary metabolites by several filamentous fungi **(Brakhage, A. A., et al. 2004)** For industrial production the filamentous fungus *Penicillium chrysogenum* is used.

The discovery of antibiotics is perhaps the most important discovery in the history of therapeutic medicine. It may conceivably have saved more lives than any other medical therapy. In the first reaction of penicillin biosynthesis, the three precursor amino acids L-α-aminoadipic acid, L-cysteine, and L-valine are condensed to the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This step is catalyzed by ACV synthetase, a single multifunctional enzyme with non-ribosomal peptide synthetase activity that is coded by the pcbAB gene (synonym, acvA). **(Ziemons et al).**

The second step is characterized by the oxidative ring closure of the linear ACV tripeptide, leading to the formation of a bicyclic ring comprising the β-lactam and thiazolidine ring. This reaction is catalyzed by the isopenicillin N synthase, encoded by the pcbC gene (synonym, ipnA).The resulting compound, isopenicillin N, is the first bioactive intermediate of the penicillin biosynthesis pathway. **(Ziemons et al).** In the third reaction of penicillin biosynthesis, the hydrophilic L-α-aminoadipate side chain of isopenicillin N is exchanged for a hydrophobic phenylacetyl or phenoxyacetyl group, resulting in the formation of penicillin G and penicillin V, respectively. This final step is catalyzed by the acyl-coenzyme A: isopenicillin N acyltransferase, and the corresponding gene is penDE (synonym, aatA). **(Ziemons et al).**

Biosynthesis of Penicillin: -



A literature survey covering more than 23,000 microbial products possessing some biological activity, i.e. antifungal, antibacterial, antiviral, cytotoxic and immunosuppressive. The discovery of cephalosporin C generated a whole new group of clinically significant b-lactams. The success of b-lactams in the treatment of infectious disease is due to their high specificity and their low toxicity. Despite a growing number of antibiotics and the incidence of penicillin-resistant isolates,b-lactams are still by far the most frequently used antibiotic.

Penicillin, which remains an important part of our antimicrobial armamentarium, had a significant impact on the second half of the twentieth century. Deep-fermentation methods, which were primarily developed for the production of penicillin during the war, gave rise to the development of antibiotics and contributed to the nascent biotechnology industry which appeared in the 1970s.

Akin to other antimicrobials, penicillin is a secondary metabolite, thus it is only produced in the stationary phase. The industrial production of penicillin was generally classified into two processes – upstream processing and downstream processing.

Upstream processing encompasses any technology that leads to the synthesis of a product and includes the exploration, development and production. The extraction and purification of a biotechnological product from fermentation is referred to as downstream processing.

Pursuit for a Better Yield: -

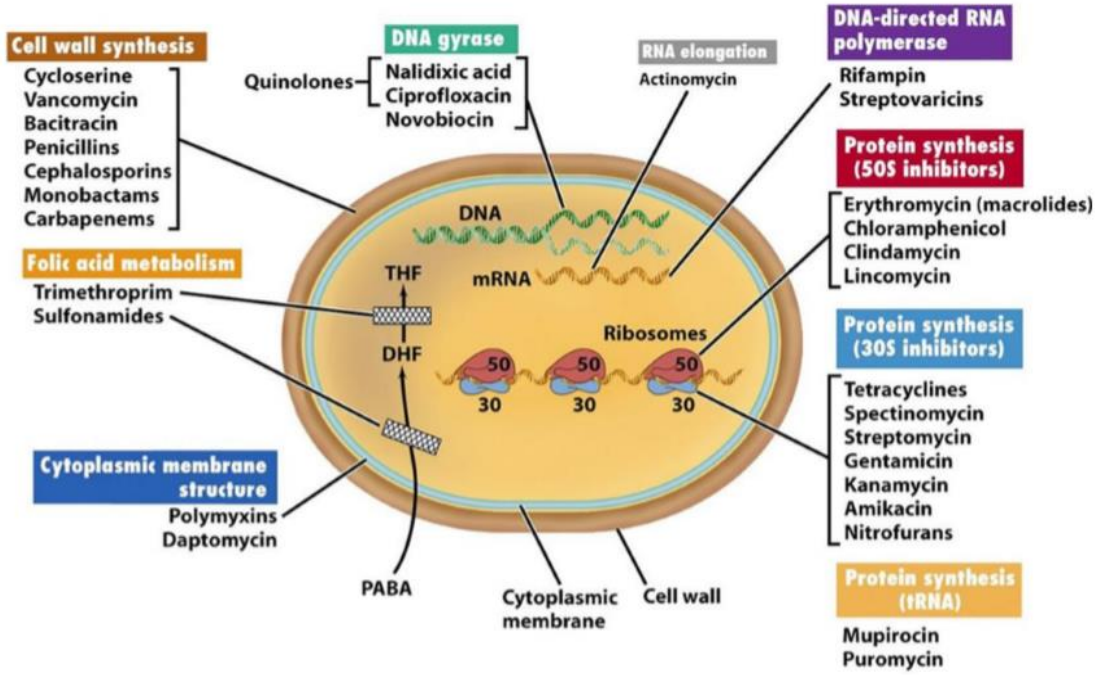
When penicillin was initially made at the end of the Second World War using the fungus Penicillium notatum, the process yielded one milligram per cubic decimeter. Today, with a use of a different species (Penicillium chrysogenum) and improved extraction procedures the yield is 50 grams per cubic decimeter.

The yield of penicillin can be further increased by improving the composition of the medium, isolating aforementioned Penicillium chrysogenum which grows better in huge deep fermentation tank, but also via the development of submerged culture technique for cultivation of mold in large volume of liquid medium through which sterile air is forced.

Modern Production Methods: -

Significant improvements in modern production methods have increased production and decreased cost. Today, commercial producing strains of Penicillium chrysogenum are grown using submerged culture in constantly agitating and aerated 50,000-gallon stainless steel tanks. These industrial strains can now produce 40-50 grams of penicillin per liter of culture with a 90% recovery yield. This is an overwhelming improvement from the earliest Peoria farmer's market strain that only produced 0.15 grams per liter with very low recovery rates. In order to achieve these production rates, modern Penicillium strains display a host of genetic and cellular modifications that result in increased production, including amplification of the penicillin biosynthesis gene cluster, an increased number of peroxisomes, and elevated levels of transporter proteins that secrete newly produced penicillin out of the peroxisomes and the cell.

Different-different antibiotics target sites-:



Antibiotics target sites

MATERIAL AND METHOD

ISOLATION OF FUNGUS (*Penicillium* sp.) FROM BREAD-

- Taken 2-3 Pieces of bread in a tub and moist with water.
- Covered the tub from silver foil.
- Kept tub into BOD at 25°-26° C
- Observed the growth on bread and fruits
- Performed the staining to identify the growth of fungus

LACTO PHENOL COTTON BLUE MOUNTING OF FUNGI-

- **MATERIAL REQUIRED:**

  Fungal culture plates, lactophenol cotton blue solution, cover slip, needles, slide, sprit lamp, microscope.

- **THEORY:**
  - Lactophenol cotton blue stain is commonly used for microscope examination of fungi. Rapid examination of all types of fungi and their spore bearing structure can be done. The fungal structure is either hyaline or of different colours. The hyaline mycelia/spores/conidia, etc and their cytoplasm can be stained by using lactophenol and cotton blue. Cotton blue stains the cytoplasm and also result in light blue background.
  - Phenol acts as a fungicide. Lactic acid acts as a cleaning agent and separates the mycelia network of fungi. Glycerol is also added to make a semi-permanent preparation. Thus, the fungal structures are stained blush in colour with a light blue background against which the walls of hyphae can be readily seen.
  - The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which will kill any live organisms.
  - lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls. The identification of moulds is based on the shape, method of production, and arrangement of spores (conidial ontogeny).
  - Lactophenol Blue Solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal elements are stained intensely blue. Lacto phenol Cotton Blue is recommended for mounting and staining

yeast and molds. It is formulated with lactophenol, which serves as a mounting fluid and cotton blue. Organisms suspended in the stain are killed due to the presence of phenol. The high concentration of the phenol deactivates lytic cellular enzymes thus the cells do not lyse. Cotton blue is an acid dye that stains the chitin present in the cell walls of fungi.

- **COMPOSITION:**

  Ingredients In 100 ml aqueous solution

Cotton Blue (Methyl Blue)	0.075g
Phenol crystal	20g
Lactic Acid	20ml
Glycerol	40ml
Distilled water	20ml

PROCEDURE: -

- I have taken few drops of lactophenol cotton blue stain on a clean slide.
- Transferred a small tuft of fungus usually with the spores and spore bearing structure into the drop of stain using a flamed and cooled needle.
- Gently tease the material (separate the mycelia network) with the help of needles.
- And put the cover slip taking care to avoid trapping of air bubbles.

  Observed the slides under low and high-power objectives of the microscope and noted the type of hyphae and spore structures.

PREPARATION OF POTATO DEXTROSE AGAR (PDA):-

Liquid growth media containing nutrients are usually solidified by agar. Agar-agar is a complex polysaccharide obtain from marine red algae. It is composed of 3, 6 anhydro-L-galactose and d-galactose. It liquefies on heating to 96^0 C and solidifies in to a jelly on cooling to 42^0 C or less. Liquefied media can be added to test tubes and Petri plates and allowed to harden in a horizontal position. Slants can also be prepared by keeping the test tubes at an angle for solidification.

PDA is a general media for growth. The growth can be carried out either in PDA Petri plates or PDA slants. Slants are mainly made for maintenance of fungi culture. PDA contains high

amount of starch and sugar which enables fungal growth. Isolation of fungi from soil can be carried out on PDA plates.

Potato Dextrose Agar (abbreviated "PDA") is common microbiological media made from potato infusion, and dextrose (corn sugar). Potato dextrose agar is the most widely used medium for growing fungi and bacteria which attack living plants or decay dead plant matter.

MATERIAL REQUIRED: -

Potatoes, dextrose sugar, agar, peptone, beef extract, distilled water, beaker, measuring cylinder, funnel, filter paper, muslin cloth, mortar and pestle, heater, autoclave, test tubes, Petri plates, aluminum foil, cotton plug.

COMPOSITION –

Potato	200gm
Dextrose	20gm
Agar	15gm
D/W	1000ml

PROCEDURE-

- Take potatoes peel off and weigh 200gm.
- Chopped it into small pieces with a knife and boiled 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.
- Mashed the potatoes and filtered the solution through a muslin cloth.
- Take the potato extract in a beaker and added 20g of dextrose and 15g of agar.
- Make up the volume to 1 ltr by adding distilled water to it.
- Boiled 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^{o}C for 15-20 min at 15psi pressure.
- take the entire material in a laminar air flow cabinet which is previously sterilized for 30 min in U.V. light.
- When temperature cools down a little poured about 15-20 ml of medium aseptically into the bottom half of petri plates.

- Placed the plates on the horizontal base and after 20-30 minutes, it solidifies to form PDA plates.
- I used the plate for fungal cultivation or store in refrigerator for further future use.

GROWTH OF *Penicillium* ON PDA PLATE-:

MATERIAL REQUIRED: -

PDA plate, laminar air flow, match box, spirit lamp, inoculation loop, tissue paper, 70% ethanol, fungus culture containing *penicillium.*

PROCEDURE: -

- Switched on the UV light of laminar air flow for 30 minutes prior to start the experiment for sterilization
- Switched off the UV light, on the main light and blower. Now open the door of LAF and cleaned the surface by using 70% ethanol.
- Now spirit lamp is lighted and inoculation loop is sterilized by using 70% ethanol and by taking it to nearby flame.
- Now I have taken some penicillin culture with help of sterilized inoculated loop and transferred to the PDA plate.
- Place the plate in incubator for 48 hr. for the growth of desired fungus.

PRODUCTION OF PENICILLIN USING *Penicillium Chrysogenum* IN GLUCOSE: -

REQUIREMENTS: -

250 ml flask, shaker, incubator, autoclave, nutrient agar media, *Penicillium chrysogenum.*

COMPOSITION: -

Sodium nitrate	3 gm
Potassium Dihydrogen phosphate	1 gm
Potassium chloride	5gm
Magnesium sulphate	5 gm
Ferrous sulphate	5gm
Glucose	20 gm
Yeast malt agar (10:10:5)	25 gm
Distilled water	1000 ml

PROCEDURE:-

- Media preparation: this media was prepared by adding given composition in 1-liter flask and make up the volume with distilled water up to 1000ml.
- Media sterilization: flask is sterilized in auto clave for 30 min at 121 ^{o}C.
- Inoculums preparation: spores from a 48 hr slant culture of *P. chrysogenum* were transferred to sterile inoculum medium consisting of the above composition.
- Fermentation was carried out on a rotary shaker for 10 days. Samples were drawn after 10 days of incubation and antimicrobial activity was performed. The potency of each sample was tested using well diffusion method against isolated oral pathogen.

To prepare the nutrient broth and inoculate the bacteria like *E.coli* and *Staphylococcus* in broth:-

Material required: -

Two test tube, cotton plug, match box, spirit lamp, inoculation loop, swabs, a 500 ml beaker, a 50 ml conical flask, tissue paper, test tube stand.

Instrument used: -

Autoclave, incubator, laminar air flow.

Composition of nutrient broth: for 100 ml

NaCl	0.5 gm
Peptone	1 gm
Beef Extract	1 gm
Distilled water	100 ml.

Procedure:-

- Mix the entire given chemical in appropriate quantity in a conical flask.
- Shake the flask well and plugged it by a cotton plug and wrap the plugged by aluminium foil and place it along with cotton plugged wrapped aluminium foil test tube in autoclave for 15 min. at 121c for sterilization.
- Cool the broth at room temperature.
- Switch on the UV light of laminar air flow for 30 minutes prior to start the experiment for sterilization.
- Switch off the UV light, on the main light and blower. Now open the door of LAF and clean the surface by using 70% ethanol.
- Now spirit lamp is lighted and inoculation loop is sterilized by using 70% ethanol and by taking it to nearby flame.
- Transferred the broth in the test tube in equal amount. Cool the inoculated loop and take the *E.coli* and inoculated in first tube, mix it well and marked as broth containing penicillin.
- Again sterilized the loop, cool it and take the *Staphylococcus* and transferred to another test tube, mix it well and marked as broth containing *Staphylococcus*.

- Place both the test tube in incubator for 24 hr. at 37c.

To perform gram staining of bacteria: -

Material required: -

Bacterial culture, staining tray, distilled water, inoculating loop, glass slide, blotting paper, spirit lamp, microscope, immersion oil. Gram staining reagent i.e. crystal violet, iodine solution, 95% ethanol (decolouriser), saffranine (counter stain)

Procedure: -

The Gram staining procedure goes as follows:

- The smear of bacterial culture is made up a clean glass slide.
- Smear is air dried and heat fixed.
- Flood the slide with Crystal Violet (the primary stain).
- After 1 minute, rinse the slide with water.
- Flood the slide with Iodine
- After 1 minute, rinse the slide with water.
- Flood the slide with Acetone Alcohol.
- After 10 or 15 seconds, rinse the slide with water.
- Flood slide with Saffrinin (the counter-stain).
- After 1 minute, rinse the slide with water.
- Gently blot the slide dry. It is now ready to be viewed under oil immersion (1000x TM) with a bright-field compound microscope.

To prepare the Nutrient agar and culture plates: -

Material required: -

12 Petri plates, 4 conical flasks of 250 ml each, weighing machine, measuring cylinder, cotton plug, 70% ethanol.

Instrument used: -

Autoclave and laminar air flow.

Chemical composition: -

Agar	5 gm
NaCl	1.25 gm
Peptone	2.5 gm
Beef Extract	2.5 gm
Distilled water	250 ml.

Procedure: -

- Make nutrient agar each seprately in 4 conical flasks having same given composition.
- The entire flask is cotton plugged and wrapped by aluminium foil.
- Clean the Petri plate by 70% ethanol and wrapped in aluminium foil.
- Place the flask and all Petri plate in autoclave for 15 min. at 121c for sterilization.
- Cool the media at room temperature.
- Switch on the UV light of laminar air flow for 30 minutes prior to start the experiment for sterilization.
- Switch off the UV light and on the main light and blower. Now open the door of LAF and clean the surface by
- Using 70% ethanol.
- Filled the 6 sterilized plates by pouring media up to ¾ level of plate and another 6 plate up to ½ levels.
- Keep them aside and undisturbed for solidification of media.

To perform the well and disc diffusion assay by using glucose fermentation broth: -

Material required: -

Glucose fermentation broth, nutrient broth containing *E.coli* and *Staphylococcus aureus*, laminar air flow , sterile water, swab , tissue paper ,micro pipette , sterilized tips ,spirit lamp , match box., autoclaved nutrient agar

Procedure: -

- Switch on the UV light of laminar air flow for 30 minutes prior to start the experiment for sterilization.
- Switch off the UV light and on the main light and blower. Now open the door of LAF and clean the surface by using 70% ethanol.
- Now label the Petri plates and filled with nutrient agar as-1 (¾) filled plate for *E.coli* glucose well diffusion and 1 (½) filled for *E.coli* glucose disc diffusion. Another 1 (¾) filled plate for *Staphylococcus aureus* well diffusion and 1 (½) filled for *Staphylococcus aureus* disc diffusion assay.
- Kept the plate undisturbed for solidification of nutrient agar media.

Well diffusion assay for *E. coli* using Glucose as carbon source: -

- Divide the plate in four equal parts and make well in each part and labelled as control, 1, 2, 3.
- Now dip the swab in *E.coli* culture and spread on the surface of solidified media.
- Now take 50ul of glucose fermentation broth and pipette in each 1, 2, 3 well respectively.
- Take 50ul sterile water and pipette in control well.

Well diffusion assay for *Staphylococcus aureus* using Glucose as carbon source: -

- Divide the plate in four equal parts and make well in each part and labelled as control, 1, 2, 3.

- Now dip the swab in *Staphylococcus aureus* solution and spread on the surface of solidified media.
- Take 50ul of glucose fermentation broth and pipette in each 1, 2, 3 well respectively.
- Take 50 µl sterile water and pipette in control well.
- Take 20ul sterile water and pipette on control disc.
- Now take 20ul of glucose fermentation broth and pipette on each disc placed in 1, 2, 3 part respectively. Take 20ul sterile water and pipette on control disc.

Keep the four plates in an incubator for 48 hr. at 37 °C

RESULT AND DISCUSSION

PDA preparation: -

PDA plates were obtained, after solidification of broth in the plates. They can be used for cultivation of fungi.

Fig 4 - PDA- AGAR

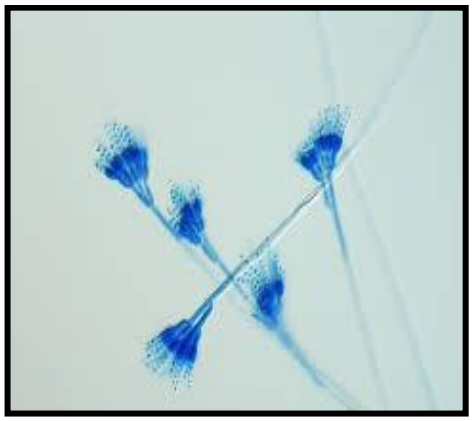
Growth of Ppenicillin on PDA plate: -

Penicillium notatum spores are observed which turns into orange coloured colonies on maturation.

Fig 5 - *Penicillium chrysogenum* spores in PDA plate

Mounting of fungi: -

When we observed the slide under microscope (10x & 40x), *Penicillium* appeared like a paint brush.



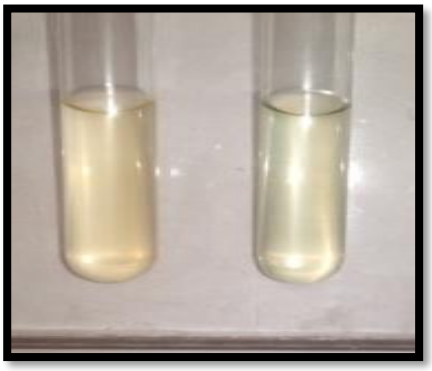
Penicillium in Lactophenol cotton blue solution

To produce penicillin using *Penicillium notatum* in glucose fermentation broth: -

We observed the growth of fungus in the fermentation media and noted the pH of the broth by using pH strips. pH of the broth was 6.

To prepare nutrient broth and inoculate *E.coli* and *Staphylococcus* in broth:-

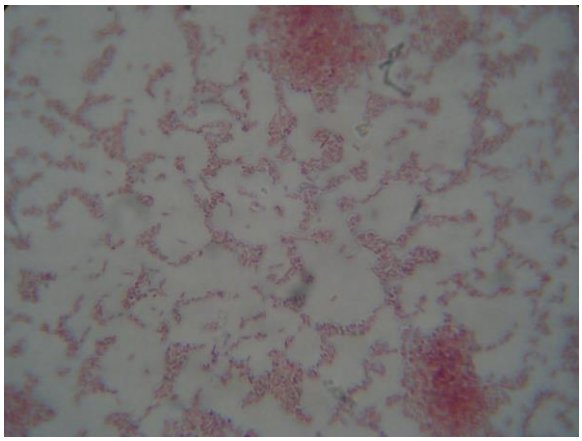
After 24 hr. turbidity in the test tubes indicate the growth of bacteria.



Nutrient broth for inoculation of *E coli* and *Staphylococcus*

To perform gram staining of bacteria: -

E.coli appears rod shaped with pink colour.



Visualization of *E.coli* under microscope

Staphylococcus appears purple retaining primary stain.

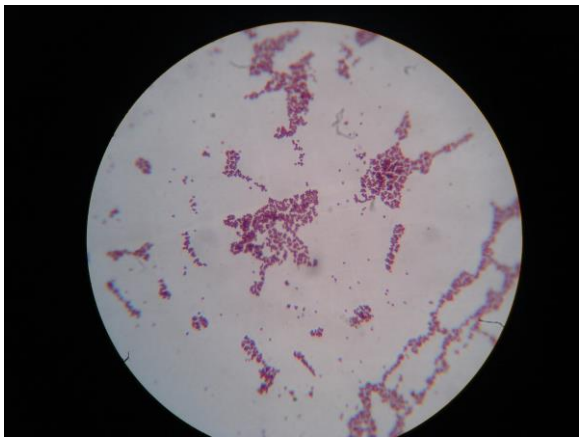


Fig 9- Visualization of *Staphylococcus* under microscope

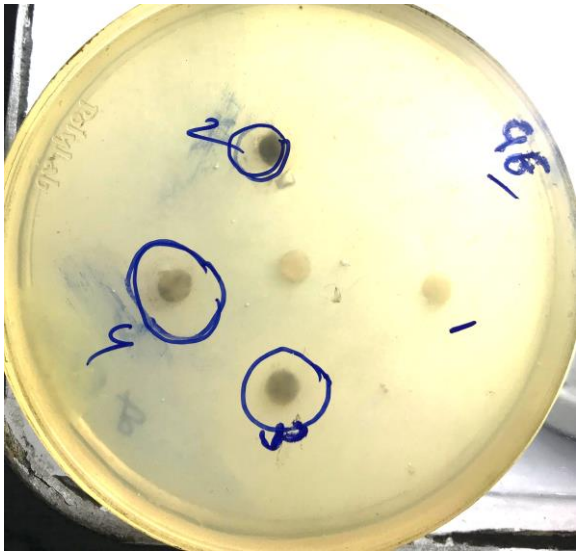
To prepare nutrient agar and media plate: -

After solidification of media we can use the plates.

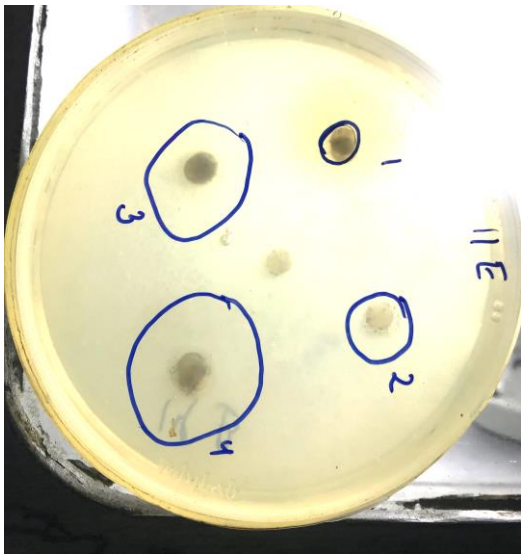
To perform the well diffusion assay by using Penicillin produced in Glucose fermentation broth: -

Well Diffusion assay for *E.coli and Staphylococcus aureus*:-

Zone of inhibition are formed which show the antimicrobial activity of antibiotic.



Zone of Inhibition against E.coli by Well diffusion (4 Days- Glucose media)



Zone of Inhibition against *Staphylococcus <u>aureus</u>* by Well diffusion (4 Days- Glucose media)

We measured the radius of the zone around the well representing zone of inhibition: And we found that the radius of zone of inhibition of *Staphylococcus* is greater than zone of inhibition of *E.coli* on the 4th day of Penicillin production. This shows that the penicillin production increases from 1st to the 4th day.

BACTERIA	**Zone of Inhibition (radius) of Penicillin (In mm) Well Diffusion Assay**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	
E.coli	**-**	**5**	**9**	**12**	
Staphylococcus aureus	**4**	**6**	**10**	**15**	

Radius of Zone of Inhibition of Penicillin by Well Diffusion Assay (Glucose Media)

Zone of inhibition denotes the antibacterial activity of penicillin. It was observed that penicillin killed gram-positive bacteria faster than gram-negative bacteria. Penicillin production was maximum in glucose fermentation media.

The present study clearly shows that glucose is the satisfactory carbon source for optimum penicillin production from *P. notatum*.

In a study by Florey on paediatric bacterial infections, he showed that bacterial resistance to antimicrobial agents is a serious problem in the treatment of paediatric bacterial infection. One of the most effective ways to control antibiotic resistance, is the development of surveillance programs. For this purpose, isolates were collected from paediatric wards of different hospitals. The result shows that isolates were highly resistant against majority of selected antibiotics with increase in the MIC's. In Penicillin group, the most effective agent is Amoxicillin and Clavulanic acid, more than 90% isolates of *Staphylococcus aureus* were susceptible while other agents. This seemed similar to our study where crude Penicillin showed better antimicrobial effect against *Staphylococcus aureus.*

CONCLUSION

Isolation of *Penicillin notatum* was done from Decaying bread and it was studied under a microscope.

Penicillin notatum was inoculated in fermentation media having glucose as the carbon sources and then studied the Penicillin antibiotic production by using well diffusion assay with sensitive bacterial strains of *Staphylococcus* and *E.coli*.

We conclude that glucose is the most satisfactory carbon source for the production of penicillin.

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