INDUSTRIAL PRODUCTION PENICILLIN

Dear students welcome for the lecture series on Industrial Microbiology. Today we are going to discuss another fascinating topic in industrial microbiology, i.e., INDUSTRIAL PRODUCTION PENICILLIN

The topic can be divided into following five modules,

- **Introduction to Penicillin**
- **Bio-synthesis of penicillin**
- **Selection of culture**
- **MEDIA FORMULATION**
- **FERMENTATION**

I. Introduction to Penicillin

Penicillin was the first naturally occurring antibiotic discovered. It is obtained in a number of forms from Penicilliummoulds. 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.

Penicillin are the best known and probably the most important antibiotic. They have been approved for human use and they account for over most of the antibiotics produced worldwide. Penicillin was discovered by Fleming in 1929. Natural penicillin is effective against numerous gram positive bacteria. They are produced by many fungi, particularly Penicillin and Aspergillus species. They present the most favorable characteristics of being almost nontoxic to mammals.

Penicillin, being β-lactum antibiotics, are specific inhibitors of bacterial cell wall synthesis. They target the synthesis of peptidoglycan by specifically binding to penicillin binding protein (PBP) of bacterial cell wall. This binding inhibits the enzyme activity of this protein. The penicillin have a common chemical nucleus and differ principally in the chemical structure of side chain attached to this nucleus. The various penicillin fermentation also are unusual in that various compounds resembling side chain can be added as precursors to the fermentation medium, and these compounds through microbial action, are directly incorporated in the penicillin molecule. Also, the side chain can be enzymatically removed liberating the penicillin nucleus, so that unnatural side chains can be chemically added to the nucleus in order to create new penicillin.

Penicillin is a secondary metabolite of certain species of Penicillium and is produced when growth of the fungus is inhibited by stress. It is not produced during active growth. Production is also limited by feedback in the synthesis pathway of penicillin. The by-product, L-lysine, inhibits

the production of homocitrate, so the presence of exogenous lysine should be avoided in penicillin production.

The Penicillium cells are grown using a technique called fed-batch culture, in which the cells are constantly subject to stress, which is required for induction of penicillin production. The available carbon sources are also important: Glucose inhibits penicillin production, whereas lactose does not. The pH and the levels of nitrogen, lysine, phosphate, and oxygen of the batches must also be carefully controlled. The biotechnological method of directed evolution has been applied to produce by mutation a large number of Penicillium strains. These techniques include error-prone PCR, DNA shuffling, ITCHY, and strand-overlap PCR.

Chemical Structure of Penicillin

The basic structure of penicillin is 6-aminopenicillanic acid (6-APA). it consists of a thiazolidine ring with a condensed β-lactum ring. The 6-APA ring carries valuable acyl moiety in position 6. If the penicillin fermentation is carried out without addition of side chain precursors, the natural penicillin are produced. From this mixture, only benzylpenicillin is therapeutically useful. About 38% of the penicillin produced commercially are used in human medicine, 12% in veterinary medicine and 48% are used as starting material for the production of semi synthetic penicillin.

The fermentation of penicillin can be better controlled by adding a side chain precursors, so that only one desired penicillin is produced. Addition of corn steep liquor to the medium increases the total yield of penicillin. Corn steep liquor contains phenylalanine and its breakdown products phenylethylamine and phenyl acetic acid, which when added as precursors directs the mold synthesis towards required penicillin. It also minimises the recovery problem of separating the unwanted penicillin.

II. Bio-synthesis of penicillin

The first step is the condensation of three amino acids—L-α-aminoadipic acid, L-cysteine, L-valine into a tripeptide. Before condensing into the tripeptide, the amino acid L-valine must undergo epimerization to become D-valine. The condensed tripeptide is named δ-(L-α-aminoadipyl)-L-cysteine-D-valine (ACV). The condensation reaction and epimerization are both catalyzed by the enzyme δ-(L-α-aminoadipyl)-L-cysteine-D-valinesynthetase (ACVS), a nonribosomal peptide synthetase or NRPS.

- The second step in the biosynthesis of penicillin G is the oxidative conversion of linear ACV into the bicyclic intermediate isopenicillin N by isopenicillin N synthase (IPNS), which is encoded by the gene pcbC. Isopenicillin N is a very weak intermediate, because it does not show strong antibiotic activity.

- The final step is a transamidation by isopenicillin N N-acyltransferase, in which the α-aminoadipyl side-chain of isopenicillin N is removed and exchanged for a phenylacetyl side-

chain. This reaction is encoded by the gene penDE, which is unique in the process of obtaining penicillins.

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.

Industrial production of pecicillin-steps

- ☐ Selection of strain
- ☐ Culture maintenance
- ☐ Inoculum preparation
- ☐ Medium preparation
- ☐ Fermentation process
- ☐ Extraction and purification

III. Selection of culture:

1) Many strains of Penicilliumnotatum and Penicilliumchrysogenum were tested in an effort to find which one would produce good yields in submerged culture process. One of these Penicilliumchrysogenum NRRL 1951 was found to be a superior producer.

2) The strain was adopted by most of the penicillin manufacturers and the monospores isolated from it are the parent cultures of those now in use.

3) Descendants of these cultures have many properties, they produce 10 times as much penicillin.

4) The penicillin molds are characterized by unusual variability, the greater the productivity of strain, the less stable it is.

5) Stock cultures can be maintained on agar slants, in dry soil, in lyophilized form or as spore or as cell suspension stored in liquid nitrogen.

6) Stocks carried on agar most liable to variation. Frequent transfer tend to propagate selectively those portions of the culture population that sporulate more readily.

3.1 Culture medium:

The medium of a typical feed batch culture may vary depending on strain and usually consists of corn steep liquor, an additional nitrogen source such as soy meal, yeast extract, or whey, a carbon source such as lactose and various buffers. Phenyl acetic acid are used as precursors. Inoculum medium is similar to production medium except lactose and precursors are not included. The medium constituents have profound effect on penicillin yield. The corn-steep liquor provides peptides, amino acids and amines which are deaminated to provide the ammonia required in the early stages of fermentation. The glucose is rapidly utilized to provide mycelial growth but allows very little penicillin production. About 65% of the metabolizes carbon source is utilized for maintaining energy, 25% for growth and only 10% for penicillin production.

The lactose is only slowly degraded to glucose and it is this slow glucose availability for lactose that allows starvation conditions required for penicillin production. Lipid nutrients are also utilized by fungus during penicillin production and fatty acids and fatty oils are also effective. Some oils are added as anti-foam reagent. These nutrients increases both the amounts of mycelium and yields.

IV. 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

3.3 Production of penicillin:

1) Commercial production of penicillin is usually via a fed batch process carried out aseptically in stirred tank reactor.

2) The fermentation involves an initial vegetative growth phase followed by antibiotic production phase.

3) For inoculum production, spores from heavily sporulated working stocks are suspended in water.

4) These spores are then added to flasks of wheat bran plus nutrient solution and these are incubated for 5-7 days at 24°c, so as to provide heavy sporulation. The resulting spores are then used directly to inoculate inoculum tanks. These tanks are equipped with air spargers, agitators, cooling coils for temperature control and anti-foam addition devices.

5) The inoculum tanks are incubated for 24-48 hrs with aeration and agitation in order to obtain heavy mycelial growth.

6) After several stages of growth, the production culture is ready. In a typical penicillin fermentation, there is growth phase of about 40 hrs of duration with a doubling time of 6 hrs. During this period, the greatest part of the cell mass is formed. During the first 20-30 hrs, the fungal growth becomes very thick and heavy. The O2 supply in the growing culture is critical, since the increasing viscosity hinders O2 transfer.

7) This resulting inoculum is then inoculated in the production tank. These tanks are equipped with devices for continuous addition of sterile glucose syrups, pH control, foam sensing devices to activate automatic addition of anti-foams and metering pumps for continuous addition of sterile phenyl acetic acid.

8) The production tanks are inoculated by employing air pressure to force inoculum in the tank.

9) During production, periodic samples are removed for determination of penicillin yields and for contamination checks. This contamination checks are important, because penicillin fermentation are quite sensitive to contamination by penicillinase producing organisms.

10) The pH remains constant at the start of fermentation. But as soon as the carbon compounds becomes depleted and some of the lactic acid of corn steep liquor is utilized, ammonia is liberated and pH rises. The mold uses lactose to produce penicillin and a very little further growth occurs.

11) At the end of fermentation, the pH rises to a more higher level because depletion of lactose causes autolysis of the mycelium. the penicillin fermentation is harvested at this time.

Purification and recovery:

Penicillin in the acid form is solvent extractable, and the antibiotic dissolved in an organic solvent can be back extracted as a salt in aqueous solution. these considerations in general are made use of for the recovery and purification of penicillin from harvested broth.

V. 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 (Penicilliumchrysogenum 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.

Steps of purification and recovery:

1) The penicillin broth is harvested from the fermenter and chilled at 5-10°c.

2) The Penicilliumchrysogenum mycelium is then filtered on a rotary vacuum filter to remove the mycelium and the other solids.

3) Phosphoric acid are added to lower the pH, in order to convert the penicillin to the anionic form.

4) The broth is immediately extracted in counter current extract with an organic solvent such as butyl acetate.

5) The penicillin is then back extracted into water from the organic solvent by adding enough potassium or sodium hydroxide to form a salt of the penicillin.

6) the resulting aqueous solution is again acidified and re extracted with butyl acetate. this shift between water and solvent aid in purification of penicillin.

7) The solvent extract finally is back extracted with aqueous potassium to crystallize penicillin as potassium penicillin salt.

The penicillin thus crystals thus obtained are mixed with volatile solvents to remove further impurities. the crystals are collected by filtration and air dried. At this stage the penicillin obtained is 99.5% pure.

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.

Penicilliumchrysogenum 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 (Penicilliumchrysogenum) 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 Penicilliumchrysogenum. In this case the metabolite is being utilized as a defense mechanism against other microorganisms in the environment. In essence Penicilliumchrysogenum 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.

5. Conclusion

The discovery of penicillin and its medicinal uses are arguably the most important scientific discovery of the 20th century. The process of production of penicillin has changed dramatically since its beginning. Now, industrial productions were found to ensure that more penicillins are being produced and purification process is as effective as possible, the production could increase.