

Microorganisms and Processes Involved in the Production of Antibiotics and Bacteriocins



HOW DID THEY MAKE PENICILLIN?



FOR MANY YEARS, scientists knew that certain molds killed some bacteria. However, researchers needed to understand how to harness this antibacterial microbe and to manufacture enough of the substance before they could make a useful medicine.

) *Penicillium* mold naturally produces the antibiotic penicillin Scientists learned to grow Penicillium mold in deep fermentation tanks by adding a kind of sugar and other ingredients. This process increased the growth of Penicillium.) Then, scientists separated the penicillin product from the mold. Finally, penicillin is purified for use as an antibiotic medicine.

microscopic view of Penicillium growth

> Initially surface culture method: A variant of original culture of *Penicillium notatum* discovered by Sir Alexander Fleming was employed.



C)

a) Penicillin fermentation:

- When shifted to submerged cultivation, strain of *Penicillium chrysogenum* (NRRL 1951) discovered in 1943, was introduced.
- *P. chrysogenum* strains: More productive than their parents.
 Produced through natural selection, and mutation using UV irradiation etc.
- d) Naturally occurring: Penicillins G, X, F, and K.
- e) Penicillin G (benzyl penicillin) was selected because more effective against pyogenic cocci.
- f) Higher yields achieved by supplementing medium with phenylacetic acid, analogues (phenylalanine and phenethylaninie) of which are present in corn steep liquor used to grow penicillin in United States.
- g) Produced from Aspergillus, Cephalosporium, Trichophyton, Epidermophyton, Actinomycete, Streptomyces.



Penicillin fermentation can be divided into three phases

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Fermentation for penicillin production

- Inoculum is usually built up from lyophilized spores or frozen culture and developed through vessels of increasing size to a final 5-10% of fermentation tank.
- Fermentors vary from 38,000 to 380,000 liters in capacity and work by computerized automation, which monitor oxygen content, Beta-lactam content, pH, etc.
- Medium for penicillin production has as carbohydrate source glucose, beet molasses or lactose.
- Nitrogen is supplied by corn steep liquor.
- Calcium carbonate/phosphates added as buffer.
- Sulfur compounds are sometimes added for additional yields since penicillin contains sulfur.
- Lactose is more slowly utilized and need not be added intermittently.
- Glucose suppresses secondary metabolism and excess limits penicillin production.



- pH is maintained at between 6.8 and 7.4 by automatic addition of H₂SO₄ or NaOH.
- Precursors of appropriate side-chain are added to fermentation. If benzyl penicillin is desired, phenylacetic acid is added.
- High yielding strains of *P. chrysogenum* resistant to precursors have been developed.
- 30-32°C is suitable for trophophase and 24°C for idiophase.
- Aeration and agitation are vigorous to keep components of medium in suspension and maintain yield in highly aerobic fungus.



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Extraction of penicillin after fermentation

- At end of fermentation, broth is transferred to settling tank.
- Penicillin is highly reactive and easily destroyed by alkali conditions (pH 7.5-8.0) or by enzymes. So, cooled rapidly to 5-10°C.
- Reduction of pH to 6 with mineral acids sometimes accompanied by cooling helps also to preserve the antibiotic.
- Since penicillins are monobasic carboxylic acids they are easily separated by solvent extraction.
- Fermentation broth is filtered with rotary vacuum filter to remove mycelia and other solids and resulting broth is adjusted to about pH 2 using mineral acid.
- It is then extracted with smaller volume of organic solvent (amyl acetate/butyl acetate) keeping it at this very low pH for as short time as possible.





Aqueous phase is separated from organic solvent usually by centrifugation.
 Organic solvent containing penicillin

Organic solvent containing penicillin is then typically passed through charcoal to remove impurities, after which it is back extracted with a 2% phosphate buffer at pH 7.5.

 Buffer solution containing penicillin is then acidified once again with mineral acid (phosphoric acid) and penicillin is again extracted into organic solvent (amyl acetate).
 Product is transferred into smaller and smaller volumes of organic solvent with each successive extraction process, penicillin becomes concentrated several times over up to 80-100 times.



Concentrated penicillin may be converted to stable salt form in several ways which employ fact that penicillin is an acid: (a) it can be reacted with calcium carbonate slurry to give calcium salt which may be filtered, lyophilized or spray dried (b) it may be reacted with sodium or potassium buffers to give salts of these metals which can be freeze or spray dried; (c) it may be precipitated with an organic base such as triethylamine. When benzyl penicillin is administered intramuscularly it is given either as the sodium (or potassium) salt or as procaine penicillin. Former gives high blood levels but it quickly excreted. Procaine penicillin gives lower blood levels, but it lasts longer in body because it is only slowly removed from blood. Produced by dissolving sodium or penicillin in procaine hydrochloride.

MEDIA PREPARATION medium usually contain its carbon source which is found in corn steep liquor and glucose.

HEAT STERILISATION Medium is sterilised at high heat and high pressure.

FERMENTATION

Fermentation done in the fed-batch mode. Penicillin is a secondary metabolite of the fungus, so the fed batch mode is ideal as it allows high production of penicillin. **Temperature:** 20-24°C **pH:** 6.0-6.5.

SOLVENT ADDITION

To dissolve the penicillin in the filtrate organic solvent butyl acetate is added.

BIOMASS REMOVAL

Rotary vacuum filter is commonly used to do this. To maintain the Ph between 6.0-6.5 phosphoric acid is added as the Ph will be high upto 8.5.

CULTURE

The seed culture is developed by addition of Penicillium spores into a liquid medium. When it has grown up to an acceptable amount, it will be inoculated into the Fermenter.

CENTRIFUGAL EXTRACTION

This is done to separate the solid waste from the liquid solution containing Penicillin.

DRYING

Drying is necessary to completely remove the moisture content from the penicillin salt.

Nisin:

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Bacteriocins prevent growth of undesirable bacteria in a foodgrade convenient for health and accepted by the community. Classified in three different groups: lantibiotics and non-lantibiotics.

- Several strategies for isolation and purification of
 bacteriocins from complex cultivation broths to final products.
- Biotechnological procedures: salting out, solvent extraction, ultrafiltration, adsorption-desorption, ion-exchange, and size exclusion chromatography.
- Most characterized bacteriocin is nisin.
- There are 11 genes making the nisin cluster that code for nisin production, immunity etc.



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Shaping Lives... Empowering Communities... Nisin is a 34-amino acid antimicrobial polypeptide produced during fermentation of *L. lactis* subsp. *lactis*. Antimicrobial activity against *Clostridium* and *Listeria*. Approved for food applications by FDA and produced commercially.

At pH 6.80 (controlled fermentation), more than 80% of nisin synthesized bound to cells, whereas at pH below 6.0, more than 80% of nisin was in the culture fluid.

Removal of nisin during fermentation would reduce chance of the nisin being degraded or adsorbed and thus increase amount of nisin that can be recovered.





Purification steps of large-scale nisin production are commercially sensitive (foam precipitation (frothing), sodium chloride precipitation, centrifugation/ultrafiltration, spray/drum drying. Laboratory scale purification of nisin includes an ammonium sulphate precipitation step, followed by combinations of ion-exchange and hydrophobic interaction chromatography, with final reverse phase-high pressure liquid chromatography purification step. Nisin is manufactured by controlled fermentation of *L. lactis* in a milk-based medium at pH 2.0. Above pH 3.0 nisin adsorbs to producer cells, but is completely desorbed at pH 3.0 or below. **Consequently, at pH of fermentation all nisin is** released into medium, from which it can be extracted at end of fermentation.



- Solvent extraction methods have been used and one-step immunoaffinity chromatography method is highly efficient.
- In industrial-scale process, it is concentrated and separated by simple low-cost foaming process. This involves merely bubbling nitrogen or air through a column of completed fermentation medium. As nisin is a surfaceactive agent it accumulates in the foam at airaqueous interface. Foam is collected, broken mechanically and nisin recovered. It is then spray-dried before being milled into fine particles and finally standardized by addition of sodium chloride.