BE401 Industrial Processing

Penicillin Recovery Strategies

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Abstract:

Alexander Fleming discovered penicillin in 1926. Penicillin has become the most demanded antibiotic for treatment of bacterial infections worldwide. In the extraction of Penicillin G there are numerous stages: The downstream process are essentially Broth Filtration, Filtrate Cooling, Further Filtration, Extraction of Penicillin with Solvent, Carbon Treatment, Transfer back to Aqueous Phase, Solvent Recovery, Crystallisation, Crystal Washing and Drying of Crystals. There are numerous technologies that can be applied to downstream processing. It is important to revise the downstream process when newer technologies become available. The main penicillin downstream processes should be analyzed in detail and any other alternatives should be identified. This requires the main downstream processes to be described in detail, and a search for alternatives through research carried out. In our investigation we discovered many alternatives to the main downstream processes. Other alternatives were also discussed including the use of alcohols to further improve efficienty, the use of SLM and ELM membranes to reduce the number of stages, and surfactants to stabilise the membranes. Alternatives to these systems have been suggested. These include the use of an extraction decanter invented by Westfalia Separator Industries to remove the broth-filtration stage of the downstream process, and the use of newly invented polymers (Skelland and Meng 2004) to remove the instability of membranes.

Introduction

1. What is penicillin?

Penicillin is a fungal secondary metabolite, which is used as an antibiotic. The most popular mechanism for Penicillin is suggested by blocking the synthesis of the membrane peptidoglycan in gram-positive type bacteria, resulting in cell lysis. The penicillin family can be used to reduce any kind of bacterial infections. The main problem associated with penicillins is that 1-5% of US adults show an allergic response to the drug, and some bacteria are showing strong resistance to penicillin using an enzyme called penicillinase, which degrades the B-Lactam ring.

Naturally occurring penicillin was first isolated by Alexander Fleming from the fungi *P. notatum* in 1926, and has also been found naturally occurring in the fungi *P. chrysogenum*. The first generation penicillin products from these fungi were benzyl-penicillin (penicillin G) and penicillins V, X, F and K. The penicillin base unit is 6-aminopenicillanic acid (6-APA; a 5-membered thiazolidine ring system fused to the square rigid B-Lactam ring). The functional group was identified as the B-Lactam ring.

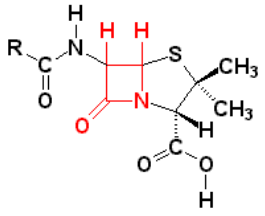


Figure 1. 6-aminopenicillanic acid highlighting the B-Lactam ring (red)

The primary objective of modifying penicillin was to produce more acid-stable penicillin, counteract both gram positive and gram-negative bacteria, and resist penicillinase. Variations were achieved by adding esters, carboxylic acids amongst others to the fermentation process, and over 20 R-group variations have been identified. The natural penicillins provided resistance to gram-positive bacteria but not gram-negative bacteria.

In the 1940's penicillin V, the most acid resistant, became a widespread therapeutic agent since it could survive digestion in the stomach and be taken orally. It was especially useful to resist staphylococcus (gram positive) during WWII. Penicillins were developed later to reduce gram-negative infections. Other penicillins were synthesized to resist penicillinase.

2. The penicillin industry

Semi-synthetic penicillin was developed in the late 50's with the commercial production of benzyl-penicillin, in which the fermentation process is induced in the above *Penicillium* mould by adding phenylacetic acid ($C_6H_5.CH_2.COOH$). Since then large-scale production of penicillin has developed. The yield of penicillin was monitored and many changes to the fermentation and extraction stages have occurred in the last 50 years to optimise penicillin production. The total world market has consistently grown over the years and has been estimated to be as much as €5 billion (2003).

3. The production process of penicillin

Penicillin is produced from fungi. It is only produced when the fungi are under stress. The mycelial density tends to be high when fungi tend to cluster together, a common feature of fungi. This high mycelial density produces great stress on the fungal population. To ensure the fungi are in a stressed environment they are previously inoculated to occupy about 10% of the total volume container and cultured for 30-40 hours. This culture preparation is known in industry as seed preparation and takes place in a smaller fermenter, which is churned with an impeller, aerated, cooled and supplied with water and nutrients. After the seed preparation mass production of penicillin is begun. This takes place in a larger fermenter. Batch-fed fermentation dominated over continuous fermentation since continuous fermentation showed many problems with contamination and process control. The *P. notatum* was first used for penicillin mass-production but fermentation yields were poor. Yields were found to be increased with *P. chrysogenum* by the addition of precursors. The main precursors are phenylacetic acid ($Ph\text{-}CH_2\text{-}COOH$) in the production of Penicillin G and phenoxyacetic acid ($Ph\text{-}O\text{-}CH_2\text{-}COOH$) in the production of penicillin V. Semi-synthetic penicillins can be produced from the 6-APA penicillin base unit. This is produced from the cleavage of penicillin G. The enzyme penicillin acylase cleaves the penicillin G into its two main precursors; phenyl acetic acid and 6-APA. (figure 1-2). This base unit may then be chemically modified to form a second order penicillin. Penicillin is excreted from the cells during the process and can easily be extracted. Extraction is carried out followed by crystallization producing penicillin crystals.

Penicillin Recovery

There are ten steps in the recovery of Penicillin:

1. Broth Filtration
2. Filtrate Cooled
3. Further Filtration
4. Extraction of Penicillin with Solvent
5. Carbon Treatment
6. Transfer back to Aqueous Phase
7. Solvent Recovery
8. Crystallisation
9. Crystal Washing
10. Drying of Crystals

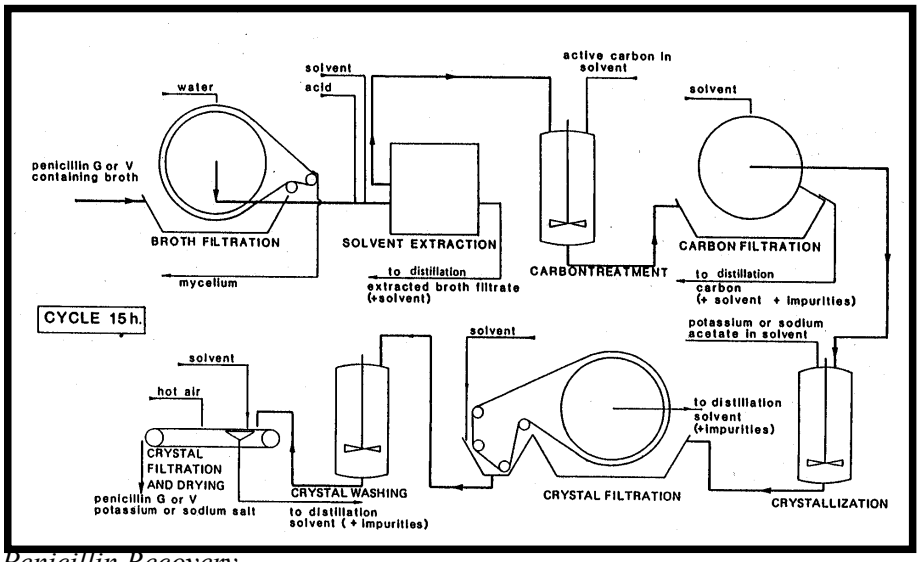


Fig 2: Penicillin Recovery

1. Broth Filtration

By analysing a fermentation broth at the time of harvesting it will be discovered that the specific product may be present at a low concentration in an aqueous solution that contains intact micro-organisms, cell fragments, soluble and insoluble medium components and other metabolic products. In the first stage, the main objective is to remove large solid particles and microbial cells by either centrifugation or filtration. Filtration is the most versatile and most established method for removing insoluble from our broth. In filtration, the micro-organisms are captured in a concentrated cake, which looks like sand, sludge or paste.

Many factors influence which type of filtration will take place; viscosity and density of filtrate, solid:liquid ratio, size and shape of particles, scale of operation, need for aseptic conditions, need for batch or continuous operation and the need for pressure or vacuum suction to ensure an sufficient for rate for liquid.

The Rotary Vacuum Filter is the most common piece of equipment used for the extraction of penicillin, and is used in continuous processing. Rotary Vacuum Filter designs vary, but usually outline as follows:

- The Filter Drum: Cylindrical, hollow drum which carries the filter cloth. On the inside it is segmented into rows to which a vacuum can be applied or shut off in sequence as the drum slowly revolves.
- Trough: Filter is partially immersed in through which contains the penicillin broth. The trough is sometimes fitted with an agitator to maintain solids in suspension.
- Discharge Nodes: Filter cakes are produced from the filtration of to penicillin broth. Because of this a node is devised to scrap off the cake after filtration. When this happens the vacuum is broken.

The filter drum, partially submerged in the trough of broth, rotates slowly. Filtrate and washings are kept separate by the segments in the drum. The liquid is drawn through the filter and a cake of solids builds up on the outer surface. Inside the drum, the filtrate is moves from the end of the cylindrical drum onto a storage tank. As our penicillin cells move from the broth, the vacuum is used to remove as much moisture as possible from the cake, and to hold the cake on the drum. The section at the node/knife, which scrapes off the filtrate can get air pressure to burst out, helping contact with the node.

Rotary vacuum filters are expensive, but they are convenient and do provide a considerable degree of mechanisation.

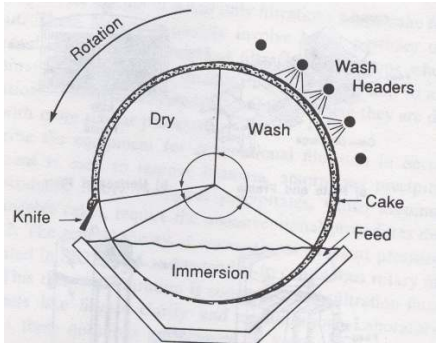


Fig 3: Rotary Vacuum Filter

2. Filtrate Cooled

From filtration, the penicillin rich solution is cooled to 5°C. As penicillin G only has a half-life 15 minutes at pH 2 at 20°C, this helps reduce enzyme and chemical degradation during the solvent extraction step (step 4).

3. Further Filtration

Further filtration again takes place using the Rotary Vacuum Filter.
In addition, we know that: *Rate of filtration = Driving force/resistance*
Resistance can be caused by the filter cloth, which also adds to the resistance of the filter cake as it accumulates. Pre-coats and filter aids can be used to assist the filtration.

The addition of a pre-coat/filter aid will increase the strength of the filter cake and minimises compaction. *Perlite,* an exploded rock, or *diatomaceous earths* are such materials. Either of these substances is built up over the conventional filter, and each time the drum completes a cycle the shave-off gear moves slightly nearer the drum. This continuous shaving away of contaminated earth prevents the filter becoming clogged, and means that there is always a clean filter starting the next cycle. The pores of their skeletons take up greasy materials also. Their addition to poor filters will increase the rate of filtration greatly.

4. Extraction of Penicillin with Solvent

For penicillin recovery, it is standard practice to use liquid-liquid countercurrent extraction processes. The basis to which liquid-liquid extraction, also called solvent extraction, works is that the extraction agent and the liquid in which the extract is dissolved are not perfectly miscible. Liquid-liquid extraction is suitable for the recovery of penicillin because of its operation at low temperatures, greater selectivity and is less expensive compared to distillation, evaporation and membrane technology.
Before starting large scale extraction, the solubility characteristics of the product must be found. “Like dissolves like”, in relation to the polarities of the molecules. Apart from being less then perfectly miscible with the carrier medium, the extract solvent has to have high capacity, ie capacity to absorb large amounts of extract, have a degree of selectivity, low levels of corrosion and toxicity, have high availability and low cost.

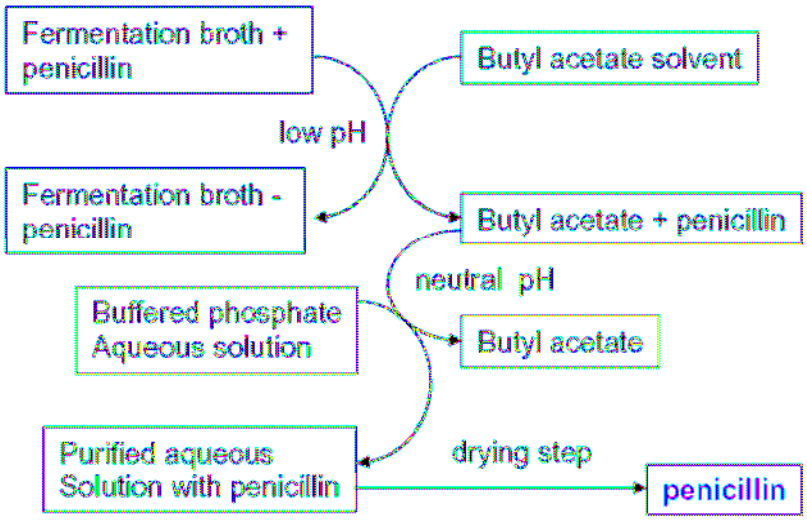


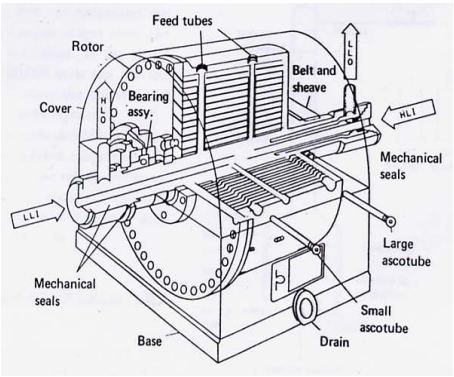
Fig 4: Extraction Process [5]

Penicillin is extracted from an aqueous phase into the solvent butyl acetate or amyl acetate. The extract phase (butyl acetate) is the one into which the extract is transferred from the raffinate (aqueous phase with penicillin). A counter current system is used when K (the partition coefficient) of the two phases is low.

K = Concentration of solute in extract
Concentration of solute in raffinate eg, the extraction of penicillin.

When working with penicillin the lower the pH, the greater the K value, thus making extraction more efficient. Sulphuric or phosphoric acid is added to created pH 2.5-3.0.

The Podbielniak Centrifugal Contractor (POD) is and example of such a countercurrent system. The Podbielniak extractor is used extensively in the commercial production of antibiotics. It is especially useful when the densities of the two liquids are very close to each other.



HLI – Heavy Liquid In
LLI – Light Liquid In
HLO – Heavy Liquid Out
LLO – Light Liquid Out

Fig 5: Podbielniak Centrifugal Contractor

The POD is made up of a horizontal cylindrical drum, which rotates at 2000-5000 rpm on its axis. The liquids are introduced into the shaft, with the heavy liquid entering the drum at the shaft while the light liquid is led by an internal route to the periphery of the drum. As the drum rotates, the liquids travel countercurrently through the cannels in the interior of the drum; the light liquid towards the centre and the heavy liquid to the periphery and then back to the shaft. The two liquid streams are then discharged via the shaft.

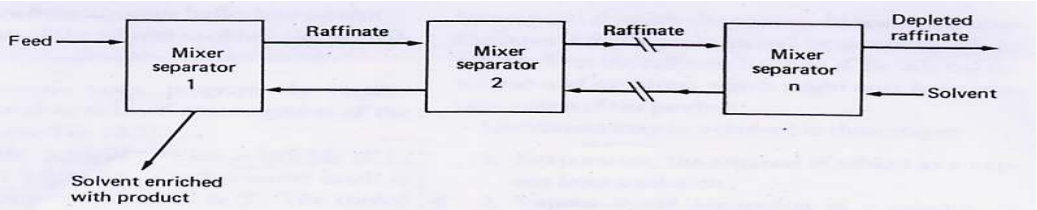


Fig 6: Countercurrent extraction summary

This centrifugal extractor offers the advantage of rapid settling, short resistance times, low hold-up volumes and flexible phase ratios, but they are finely machined and are very expensive.

5. Carbon Treatment

Our penicillin rich solution is then treated with 0.25-5% activated carbon to remove pigments and impurities. Activated carbon is an amorphous solid, and absorbs molecules from the liquid phase through is highly developed internal pore structure. It is obtained in powered, pelleted or granular form and is produced from coal, wood and coconut shells.

6. Transfer back to Aqueous Phase

Using a second Podbielniak Centrifugal Contractor, the penicillin rich solvent is passed into a fresh aqueous phase. This is done in the presence of Potassium or Sodium Hydroxide to bring the pH back to 5.0-7.5, creating the penicillin salt.

7. Solvent Recovery

The penicillin solvent is usually recovered by distillation. Distillation is carried out in three phases: Evaporation, Vapour-liquid separation in a column and condensation of the vapour.

Firstly the solvent is vaporised from the solution, then the low boiling volatile components are separated from the less volatile components in a column, and finally condensation is used to recover the volatile solvent fraction.

Solvent recovery is an important process, as solvent is a major expense in the penicillin extraction process.

8. Crystallisation

Crystals are highly organised inert matters. If grown without external interference, they grow in polyhedral shapes and exhibit many degrees of symmetry. Penicillin G is an odourless, colourless or white crystal, or crystalline powder. Crystallisation is essentially a polishing step that yields a highly pure product. It is done through phase separation from a liquid to a solid.

To begin crystallisation, we must first have a supersaturated solution. Supersaturation refers to a state in which there are more dissolved solids in the solvent than can ordinarily be accommodated at that temperature at equilibrium. Supersaturation can be achieved usually by cooling, drowning, solvent evaporation, or by chemical reaction.

Since the solubility of penicillin in its aqueous solution decreases with decreasing temperature, as the solution cools, its saturation increases until it reaches supersaturation and crystallization begins.

Drowning is also common of recovery of penicillin G. It is the addition of a non-solvent to the solution to decreases the solubility of the solid. A chemical reaction can be used to alter the dissolved solid to decrease its solubility in the solvent, thus working toward supersaturation.

From here, crystallisation is a two phase process:

PHASE 1: Primary Nucleation

Primary nucleation is quite simply the growth of new crystals. A large supersaturation driving force is required to start this primary step. The spontaneous crystal formation and "crashing out" of many nuclei are observed from the solution. This

step is not fully understood. After primary nucleation begins, it will continue until the remaining solution concentration is at equilibrium.

PHASE 2: Secondary Nucleation

Again, this step is not fully understood. Crystal production is initiated by "seeding", and occurs at a lower supersaturation. Seeding involves the addition of small crystals to a solution in a metastable area, which results in interactions between existing crystals, and crystal contact with the walls of the crystalliser. The crystals will grow on those crystals until the concentration of the solution reaches solubility equilibrium.

Batch crystallisation is the most the most used method for polishing antibiotics, including penicillin G. Batch crystallisers simply consist of tanks with stirrers and are sometimes baffled. They are slowly cooled to produce supersaturation. Seeding causes nucleation and growth is encouraged by further cooling until the desired crystals are obtained.

While the crystallisation procedures product of very high purity, improves appearance and has a low energy input, the process can be time consuming due to the high concentration of the solutions during crystallisation. It can also be profoundly affected by trace impurities and batch crystallisation can often give poor quality, non-uniform product.

9. Crystal Washing

While the penicillin G crystals we have formed are essentially pure in nature but adsorption and capillary attraction cause impurities from its mother liquor on their surfaces and within the voids of the particulate mass. Because of this the crystals must be washed and pre-dried in a liquid in which they are relatively insoluble. This solvent should be miscible with the mother solvent. For this purpose we use anhydrous l-propanol, n-butanol or another volatile solvent.

10. Drying of Crystals

Drying can stabilise many heat sensitive products like penicillin. The drying of penicillin must be carried out with extreme care to maintain its chemical and biochemical activity, and ensure that it retains a high level of activity after drying. There are many methods for drying penicillin:

- Lyophilization: Another name for freeze-drying. The wet penicillin is frozen to solidify it. Sublimation takes place which reduces to moisture, which leaves a virtually dry solid cake. Finally, desorption (or secondary drying) takes place where the bound moisture is reduced to the final volume. These three stages do overlap somewhat.

- Spray Dryers: the precise atomization of solutions in seeded in a controlled drying environment for spray drying to take place. Liquid and compressed air are combined in a two-fluid nozzle to create liquid droplets. Warm air streams dry the droplets and a dry powder is created. This is a continuous process and the transition from liquid to powder is almost instantaneous.
- Vacuum Band Dryers: A thin wet layer of penicillin crystals are fed onto a slow rotating heated drum. Radiant heat dries the layer and scalpels remove the product from the end.

Variant Strategies

Aliphatic Alcohols

Further studies by WANG, B and collegues [10] have shown the use of aliphatic alcohols in the organic phase to improve the efficiency of penicillin transfer. The major contribution of the aliphatic alcohols to the butyl acetate organic phase is the functional hydroxyl group. This group is most effective in a branched aliphatic form. The optimum conditions are the addition of aliphatic alcohols at pH 4 to increase the efficiency of solvent extraction. The main problem is again associated with this low pH. The penicillin G is stable at pH 5-8 and has a half life [9] of 15 mins at pH 2.0 at 20^{o}C. The problem with low pH can be overcome by reducing the extraction time at low pH to seconds under continuous extraction. This allows a fractional penicillin decomposition and high recovery.

Membrane Transfer Principle

The most popular alternatives in the past have been membrane derivatives which potentially select the penicillin. Liquid membranes [8] are considered as an organic liquid phase separating two aqueous phases. This can be applied to penicillin extraction where the penicillin is transferred using a carrier into the membrane/organic phase and then further into a second aqueous phase, see Figure 7 below. The advantages of using a membrane are twofold; the purification of the penicillin in one step and the use of a carrier molecule to catalyse the transfer across the membrane – resulting in a higher transfer efficiency. The major difficulties using membranes have always been emulsion swelling and membrane breakage [4]. The emulsion swelling can be prevented by add more stabilising surfactant to the sample and increase viscosity by adding more viscous liquid, but this reduces permeability of the sample through the membrane defeating its purpose. The most common surfactant used is Amberlite LA-2.

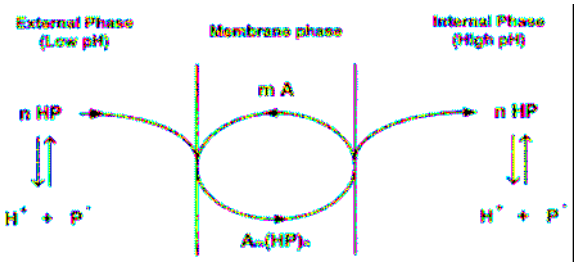


Figure 7. Liquid Membrane transfer

Supported Liquid Membrane (SLM)

Supported liquid membranes are based on ionic charge and are more suited for alkaloids, metals and inorganic ions. However, there have been some recent developments on this technology where the SLM chip [11] can be used to select penicillin from the broth. The SLM structure is two aqueous phases separated by an organic phase imbedded in pores of a micro-porous support e.g. polypropylene. This has been found to be applicable [14] to penicillin G extraction. The disadvantages include instability (loss of membrane to the aqueous phases).

Emulsion Liquid Membrane (ELM)

The most popular membrane to extract penicillin has been developed based on a surfactant membrane. Known as an emulsion liquid membrane (ELM), the system [6] uses di-*n*-octylamine and butyl/amyl-acetate as the organic phase and Amberlite LA-2 as

the membrane surfactant (abbreviated LA-2 or B). The principle is based on the formation of an emulsion membrane, which encapsulates the second aqueous phase and flows continuously with the broth aqueous phase. The encapsulation of the second phase is essentially droplets of aqueous solution surrounded by the organic phase. This allows a maximum surface exposure for efficient transfer. The emulsions are stabilised by the use of a surfactant. The penicillin is then removed from the droplets by breaking the emulsion, typically electrostatic coalescence, followed by electroplating or precipitation.
To promote membrane stability a mixture of LA-2 and n-butyl acetate is used in the ratio of 7:3 respectively [12]. The use of the membrane replaces the three step of aquous->organic->aquous into one process simultaneously using a membrane. This has significant economic advantages – reduces the complexity by reducing the number of steps. Some derivations [7] include the addition of tributyl phosphate and Shellsol TK, which is found to increase the distribution co-efficient allowing much greater extraction (up to 90%). The main advantage of this principle is the extraction can be carried out continuously. However, the LA-2 can be expensive and is often uneconomical to be used.

Future Technologies

Polymer Membranes

A breakthrough by Skelland and Meng (2004) has developed with the creation of a new polymer-based membrane. It makes use of the principle that viscuous liquids can be used to stabilize the membrane. This is a viscous liquid and stabilizes the surface. The concentration is prepared close to the critical membrane-saturated limit without any loss in permeability. This polymer-membrane also shows higher stability against breakage compared to its previous surfactant-stabilized membrane. The penicillin is transferred effectively from the external aquous phase (low pH) to the organic internal phase (high pH) using a carrier (forms a reversible complex with penicillin to allow transition across the membrane). This has a high selectivity for penicillin and with the new polymer membrane has a high stability. This has the potential to replace solvent extraction since membrane stability problems appear to have been resolved. The main disadvantage is very expensive polymers.

Extraction Decanting

Extraction Decanting [13] is an alternative to mycelial filtration. Research by the Westfalia shows an alternative downstream processing to the above. The fungal mycelium does not require to be filtered beforehand as this task is carried out by the extraction decanter. The decanter removes the solids by a mechanical motor, and the penicillin is then isolated using countercurrent or direct current extraction. The main advantage of direct extraction here is the reduction of upstream filtration from the process. This has significant economic benefits.

Conclusion

We have researched the downstream processes of penicillin recovery. These steps cover the stages of process from broth filtration to crystallization. While the basic principle of solvent extraction used by Alexander Fleming initially have always remained the same, huge advances in technology, effiency and yield have taken place. These include filtration with a rotary vacuum filter, countercurrent-based Podbielniak centrifugal contractor and crystallization techniques. The use of aliphatic alcohols and membranes alternatives or extraction decanters can all be used to optimise the recovery of penicillin.

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