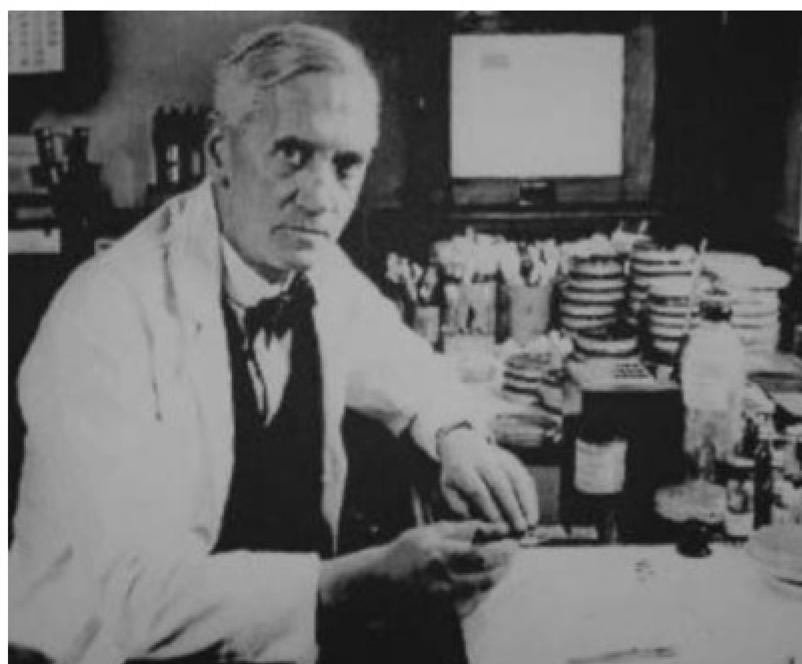
Chapter 20

The use of microorganisms in the manufacture of medicines



Alexander Fleming – discoverer of penicillin

KEY FACTS

- Microorganisms are used to produce a wide range of complex molecules, which cannot be made synthetically.
- Examples include enzymes, proteins, peptides, steroids and most antibiotics.
- Primary metabolites are produced by microorganisms during their period of active growth.
- Secondary metabolites are produced after the cell has stopped growing.
- The amount of material produced naturally is very small and microorganisms must be mutated to make them overproduce the product of interest.

The majority of chemicals used as medicines are manufactured synthetically with resultant high yield and purity. However, there are a number of instances where it might not be possible to produce the chemical in the laboratory and this is most commonly seen where the molecule is highly complex such as with steroids or proteins and peptides. Under these circumstances it might be more advantageous to use the manufacturing facilities within a microbial cell to carry out the complex synthetic processes. The molecule may then be extracted and, if necessary, modified further in the laboratory.

In fact there are a number of advantages to using microbes as synthetic factories:

- They have a high metabolic rate.
  - Microorganisms are small.
  - They have a large surface-to-volume ratio.
  - Rapid transport of nutrients into cell.
  - Cells grow very fast.
- They possess a wide range of enzymatic capability.
  - Fungi in particular are saprophytes and so live on dead and decaying matter.
  - They can produce many different enzymes.

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  - They produce an extensive range of metabolic end products.
- Microbial reactions are carried out under mild environmental conditions.
  - Many industrial chemical reactions require high temperatures, high pressures or the use of organic solvents – this makes the reactions expensive to carry out.
  - Microbial systems require low temperatures and pressures and usually employ aqueous solvents.
  - This results in lower energy costs.
- Microorganisms can grow on plentiful supplies of cheap nutrients such as the end waste products of other manufacturing processes.
  - Waste paper pulp.
  - Petroleum products.
  - Molasses.
  - Cornsteep liquor.

The summary box gives examples of a range of different products obtained from microorganisms and these will be discussed in more detail below.

Summary box showing typical products of microbial synthesis

- The cells themselves.
  - Baker's yeast/brewer's yeast used industrially and in domestic kitchens.
  - Quorn (mycoprotein from *Fusarium graminearum*) and edible mushrooms.
  - Marmite (yeast extract).
  - Probiotics.
  - Single cell protein for animal feed.
- Large molecules such as enzymes, polysaccharides and proteins (both natural and bioengineered). Tables 20.1 and 20.2 illustrate the range of pharmaceutical products that are able to be produced.
- Microbial biotransformations.
  - The main example here is the biotransformation of steroids.
- Primary metabolic products arise during active growth of the microbial culture and high cell concentrations and high growth rates give rise to high yields. The microorganisms used industrially are usually mutated to greatly overproduce the products of interest which include:
  - Alcohol.
  - Vitamins.
  - Amino acids.
  - Nucleotides.
  - Organic acids (including vinegar).
- Secondary metabolic products are produced after the cells have finished actively dividing and are in stationary phase. Conditions giving high growth rates give poor secondary metabolite yields, hence there is a need for slow growth rates but high cell concentrations. Examples of secondary metabolites include:
  - Antibiotics.
  - Toxins.
  - Alkaloids.

20.1 The cells themselves

Microbial cells are mainly used in the food industry and as feedstuffs for farm animals. Examples are given above and with the exception of probiotics they have little relevance in the pharmaceutical field and so we will not talk about them further.

20.2 Enzymes, proteins and polysaccharides

This group represents a wide range of products used in the pharmaceutical and cosmetic industries. It also includes a large number used in the food and chemical industries which do not have direct relevance here. Many of them are multibillion dollar markets including biological washing powders which contain microbially derived protease enzymes. Table 20.1 gives some examples of the different types of products which are of pharmaceutical interest. Recombinant proteins will also be briefly considered here.

20.3 Recombinant proteins

A number of pharmaceutically useful compounds of biological origin are not produced naturally by microorganisms but by human cells, and examples are given in Table 20.2. It is very difficult to culture human cells for the industrial production of these compounds and so other strategies have to be adopted. One such strategy is to identify the gene which produces the compound of interest, then to splice that into the genome of an easily grown microorganism such as *E. coli* and grow that organism in culture. Provided we have the mechanism to switch on the gene the bacterium will produce large quantities of the final product.

It is not the purpose of this book to give further details of this complex subject but the summary box on gene cloning outlines the basic process and the reader is referred to the website for further information.

Table 20.1 Examples of enzymes, proteins and polysaccharides produced by microorganisms for commercial purposes.

Material	Producing microorganism	Applications
Alginate	*Pseudomonas* sp; *Azotobacter vinelandii*	Used in a wide range of pharmaceutical formulations
Asparaginase	*Erwinia chrysanthemi*	Treatment of acute lymphoblastic leukaemia
Dextran	*Leuconostoc mesenteroides*	Blood plasma substitute
Hyaluronic acid	*Streptococcus zooepidemicus; Bacillus subtilis*	Wide range of pharmaceutical and cosmetic applications
Levan	*Zymomonas* sp.	Cosmetic applications
Streptokinase	*Streptococcus* sp.	Treatment of thrombosis in myocardial infarction and pulmonary embolism

Table 20.2 Recombinant proteins that are used clinically.

Recombinant protein	Therapeutic use
α-1 antitrypsin	Treatment of emphysema
Erythropoietin	Treatment of anaemia
Factor VIII	Prevention of bleeding in haemophiliacs
Granulocyte colony stimulating factor	Stimulates white blood cells, aids recovery of cancer patients from neutropenia and chemotherapy
Human β-glucocerebroside	Treatment of Gaucher's disease
Human growth hormone	Growth promotion
Human tissue plasminogen activator	Dissolves blood clots (acute myocardial infarction)
Insulin	Treatment of diabetes
Insulin-like growth factor	Growth promotion
Pegylated interferon 2a	Antiviral, antitumour
Tumour necrosis factor	Antitumour

Summary information on gene cloning

Human genes responsible for producing complex biological molecules such as hormones, enzymes and cytokines can be inserted into bacterial cells. These cells are easily grown to high cell densities in large volumes and the desired therapeutic materials produced on a large scale. There are three elements to the process:

- The gene of interest – derived from human DNA.
- A small piece of bacterial DNA, which can act as a carrier for the gene of interest (a plasmid vector).
- A host bacterium (typically *Escherichia coli*) into which the plasmid vector will be inserted to produce the biological product.

The human DNA

The human DNA is extracted and purified and then cut into small pieces using a restriction enzyme such as EcoR1. These enzymes recognize specific base sequences on the DNA and result in an uneven cut at the ends of the pieces of double-stranded DNA (called sticky ends).

(*continued*)

Plasmid vector

These are small circular pieces of double stranded DNA which have the capacity to replicate autonomously within the bacterial cell. They contain a small number of genes including an antibiotic resistance gene (often ampicillin) and a gene to allow screening for cells containing foreign DNA (this may be a gene for β-galactosidase). The plasmid is treated with the same restriction enzymes to generate sticky ends at the sites of cleavage.

The fragments of human DNA and the plasmid vector are then added together in the presence of an enzyme called DNA ligase. This enzyme joins the complementary sticky ends together thus inserting the pieces of human DNA into the plasmid vector.

Host bacterium

The plasmid vector is added to a suspension of the host bacterium and various mechanisms may be employed to transport the DNA into the cell. These may include transduction (Chapter 13) or electroporation. At the end of the process a small number of cells will have acquired the plasmid vector. Of those, only a small number will contain foreign DNA, and of those only a very small number will contain the gene of interest.

Selection

The cells can be plated onto agar containing ampicillin and only those cells that have acquired the plasmid vector (containing the resistance gene) will be able to grow.

Screening

The vector is often designed such that the restriction site is located within the β-galactosidase gene. Sometimes the vector may be modified so that cells containing plasmid with an intact β-galactosidase gene produce blue colonies on appropriate media while those that have a defective β-galactosidase gene due to insertion of the foreign DNA will produce white colonies on the same medium. The white colonies can therefore be picked off and grown up individually so that they can be further screened for the production of the gene of interest.

20.4 Microbial biotransformation of steroids

Steroids occur naturally in the body and possess a wide array of pharmacological properties. Examples include:

- adrenal corticosteroids (cortisone, corticosterone);
- progestational hormones (progesterone);
- androgens (testosterone);
- oestrogens (oestradiol, oestrone).

They are all derived from same basic ring structure (see Figure 20.1), and variations arise by changing the substituents on this ring. Over the last 60 years hundreds of synthetic variants have been produced giving rise to a variety of molecules with the potential to be:

- anti-inflammatory drugs;
- sedatives;
- antitumour drugs;
- cardiovascular drugs;
- oral contraceptives;
- dermatological agents.

20.4.1 Historical context

In 1949 it was found that cortisone had remarkable anti-inflammatory properties when injected for the treatment of rheumatoid arthritis. This news led to a tremendous demand for the drug as there was no alternative satisfactory treatment for this condition at that time, but since the cortisone had to be extracted from natural sources the demand could not be met. There was no chemical method for the large scale manufacture of cortisone in those days.

A chemical method was designed later which used deoxycholic acid as a starting material and this could be extracted from cattle bile. However, 31 separate chemical steps were required to produce cortisone; the yield was poor and the process economically unsound. For

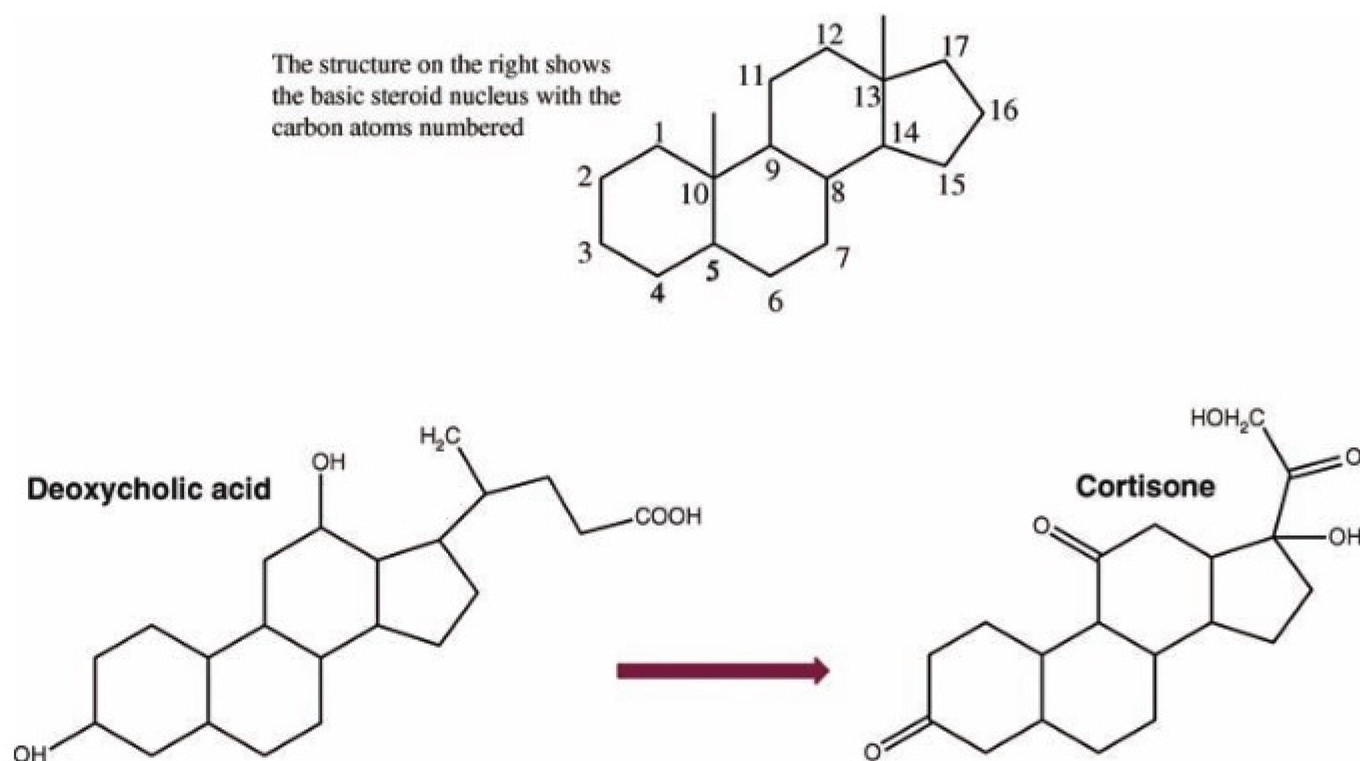

Figure 20.1 The basic steroid nucleus and the first example of the chemical synthesis of cortisone.

example, over 600 kg of deoxycholic acid were needed to produce 1 kg of cortisone. Figure 20.1 shows the oxygen shift from C12 to C11, which is essential for activity, but this move alone required nine separate chemical steps.

A more efficient production process was therefore required, which used cheaper and more plentiful supplies of starting materials. It was also necessary to reduce reliance on chemical synthetic pathways for the more complex reactions. In the first instance it was found that yams were a source of diosgenin and soybeans were a source of stigmasterol (both natural plant products containing the steroid nucleus). Independently it was observed that bacteria and fungi could carry out oxidation, isomerization and hydroxylation reactions on the steroid nucleus and hence these two discoveries led to an alternative approach to the production of therapeutic steroids.

20.5 Microbial modifications of the steroid nucleus

Rhizopus arrhizus (*nigricans*) was found to carry out 11α hydroxylation of the steroid nucleus at C11 and this had a greater than 85% yield. In addition, *Curvularia lunata* carried out 11β hydroxylation; *Streptomyces argenteolus* carried out the 16α hydroxylation and *Streptomyces lavendulae* introduced a 1,2 double bond. Figure 20.2 shows that from the more plentiful starting materials described above a combination of microbial and chemical processes can give rise to a range of steroidal agents with high yield and purity.

20.6 Primary metabolic products

Primary metabolites are typically small molecules, which arise as a result of the normal growth and metabolism of the cell. Figure 20.3 illustrates the production of primary metabolites during the stage of active growth of the culture. Rapid growth and high cell concentrations give rise to high yields of primary metabolite. In most cases the cells in the wild produce very little of the product we are interested in and the amounts are regulated by various feedback mechanisms.

An example is shown in Figure 20.4 where the desired product B is obtained by the action of enzyme 1 on compound A. The production of enzyme 1 is controlled via a negative feedback loop where, as the concentration of B increases, the level of enzyme 1 decreases. In the example shown here the picture is further complicated because our desired product is further metabolized in the cell to compound C via the action of enzyme 2. The levels of enzyme 1 may also be controlled by the concentration of compound C. If we wish to maximize the production of compound B then we must select strains of cultures in which these control mechanisms have been removed or greatly reduced. It would be necessary to block the negative feedback via compounds B and C and also to

Stigmasterol
Chemical steps
Progesterone
Microbial steps
11α hydroxy progesterone
Chemical and Microbial steps
Prednisolone
Phytosterol starting materials
Chemical steps
Microbial steps
Diosgenin
Chemical steps
Compound S
Microbial steps
Hydrocortisone
Cortisone
Prednisone

From naturally occurring plant sterols a combination of chemical and microbial processes are used to produce the range of steroids used clinically today.

Figure 20.2 Examples of the use of microbial biotransformations in steroid synthesis.

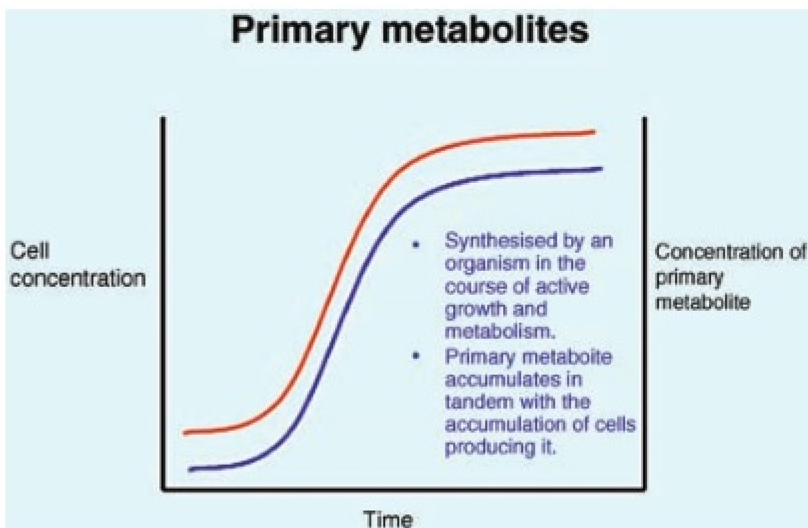

Figure 20.3 Primary metabolites (blue line) are produced during active growth of microorganisms (shown by the red line).

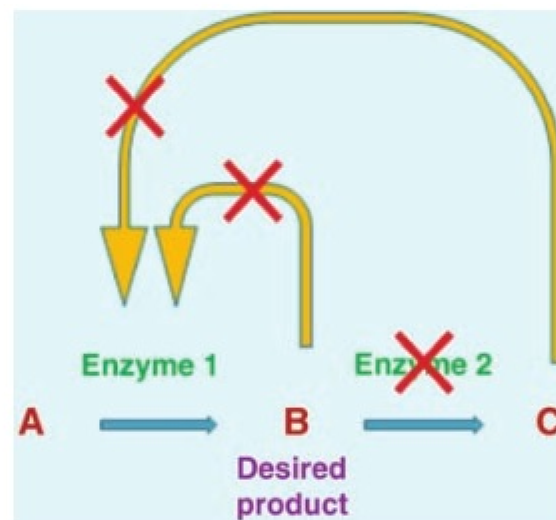

Figure 20.4 An example of how the production of a useful compound must be manipulated to maximize yields (details given in the text).

Table 20.3 Pharmaceutical uses of amino acids produced by microorganisms.

L-Amino acid	Uses
Arginine	Infusions, cosmetics
Glutamine	Assists recovery from trauma; TPN
Histidine	TPN
Isoleucine	TPN
Leucine	Dietary supplement; TPN
Lysine	TPN
Methionine	TPN
Phenylalanine	Nutritional supplement (supposed analgesic & antidepressant); TPN
Proline	Osmoprotectant in pharmaceutical formulations
Serine	Cosmetics
Tryptophan	Dietary supplement (antidepressant activity); TPN
Tyrosine	Mood modifier? L-DOPA synthesis
Valine	TPN

TPN = total parenteral nutrition.

inhibit the production of enzyme 2. Such cultures are derived through a process of mutation and selection and in this way organisms which hugely overproduce compounds of commercial interest are obtained. Examples of these highly mutated organisms include:

- *Ashbya gossypii* produces 20 000 times more riboflavin (B_2) than the natural wild type.
- *Propionibacterium shermanii* and *Ps. denitrificans* produce 50 000 times more cobalamin (B_{12}) than their naturally occurring counterparts.

We have already seen that primary metabolites include alcohols, amino acids, organic acids, nucleotides and vitamins. Table 20.3 gives examples of various amino acids produced by microorganisms and their uses in the pharmaceutical industry.

20.7 Secondary metabolic products

Secondary metabolites are also typically small molecules and, most importantly, include antibiotics. Figure 20.5 illustrates the production of secondary metabolites in relation to growth of the culture. As can be seen, the compound of interest is not produced until the cells enter stationary phase, and unlike primary metabolites high growth rates inhibit yield. In order to maximize yields we need to have slow growth but high cell concentrations. Achieving this combination is not straightforward because high cell concentrations require nutritious media which in turn give high rates of growth.

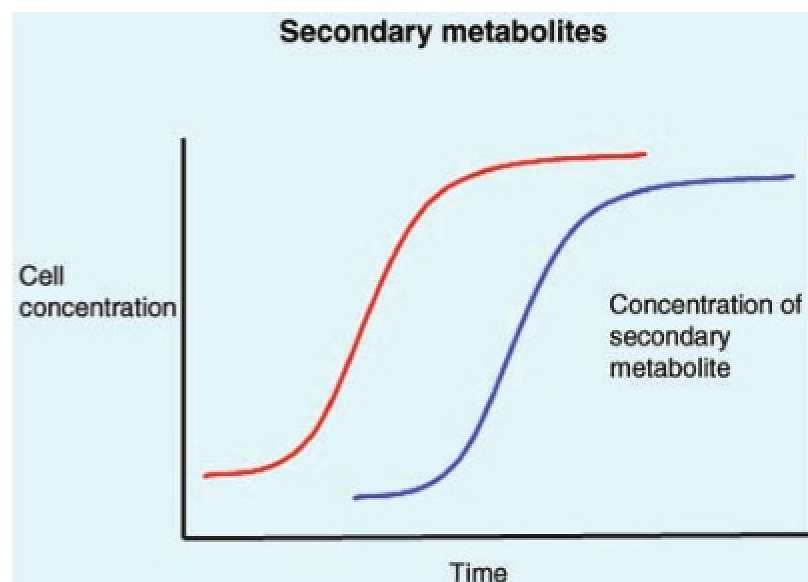

Figure 20.5 Secondary metabolites (blue line) do not accumulate in the medium until after the cells have stopped growing (shown by the red line).

Let us take a simple medium which has glucose as its sole source of carbohydrate and this is the ingredient which runs out first during growth. If this is inoculated with a small number of cells then those cells will grow at their maximum rate until the glucose is exhausted and the culture will enter stationary phase. If we now reduce the glucose concentration somewhat and repeat the process the rate of growth will be the same but the final cell concentration will be less because there is less carbon to manufacture cells. We can repeat this by gradually reducing the concentration of glucose and each time the growth rate remains at a maximum but the final cell concentration gets gradually less (see Figure 20.6). Eventually, the glucose concentration is so low that the cells cannot grow at their maximum rate and the culture grows more slowly, however, the final cell yield is now very poor.

If we require low glucose concentrations to achieve slow growth but high glucose concentrations to achieve high cell numbers then the answer may be to formulate the medium with a high glucose concentration but to feed the glucose into the culture very slowly throughout growth. This is known as an open system of culture compared to a closed (batch) culture where all the ingredients are present at the start. Figure 20.7 illustrates this process which is used to maximize yields.

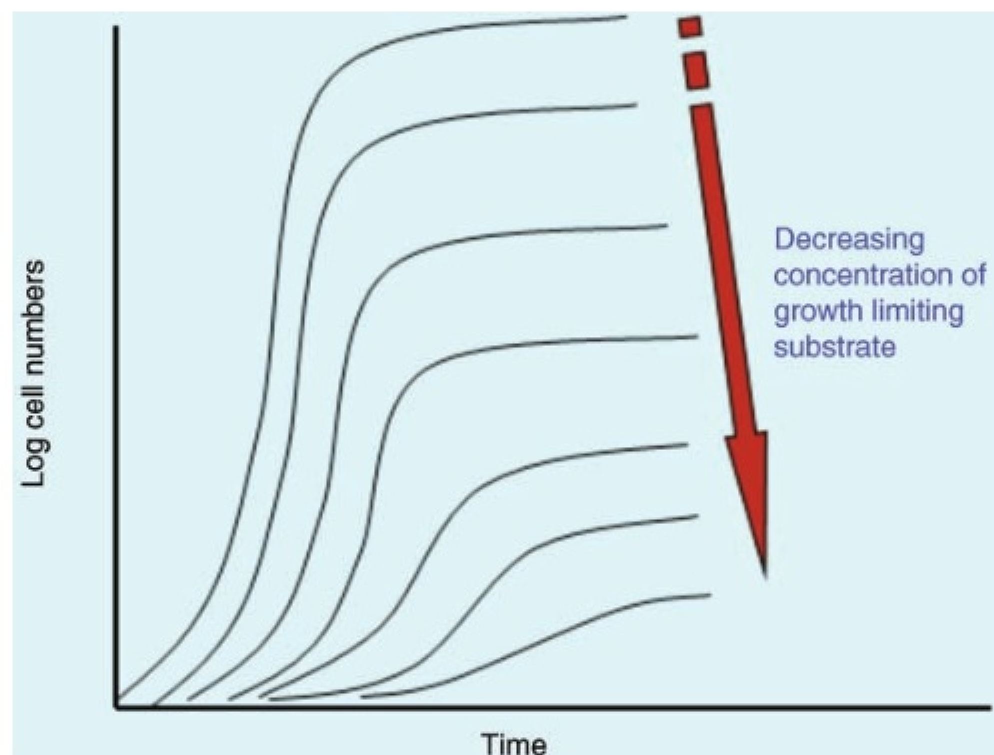

Figure 20.6 Decreasing the concentration of growth limiting substrate initially does not affect growth rate – only the final cell yield is reduced. As the concentration is reduced further both rate of growth and yield are reduced.

20.8 Commercial production of antibiotics

Antibiotics are secondary metabolites but it is important to realize that they are not waste products.

They are elaborate molecules constructed in the cell via a number of complex steps. In nature they are produced in very low amounts but industrially the cells are mutated such that they vastly overproduce the antibiotics. The production of antibiotics is now a multibillion dollar industry.

20.8.1 Penicillin

Penicillin was discovered by Alexander Fleming in 1928 at St. Mary's hospital, Paddington. He was studying the bacterium *Staphylococcus aureus* which required regular examination of his agar plates over several days but his agar plate became contaminated with a mould culture from the air. The important observation made by Fleming was *not* that there was a zone of inhibition, as that is quite a common occurrence, but that the colonies of bacteria had become established and were then subsequently lysed by the mould. Fleming tried without

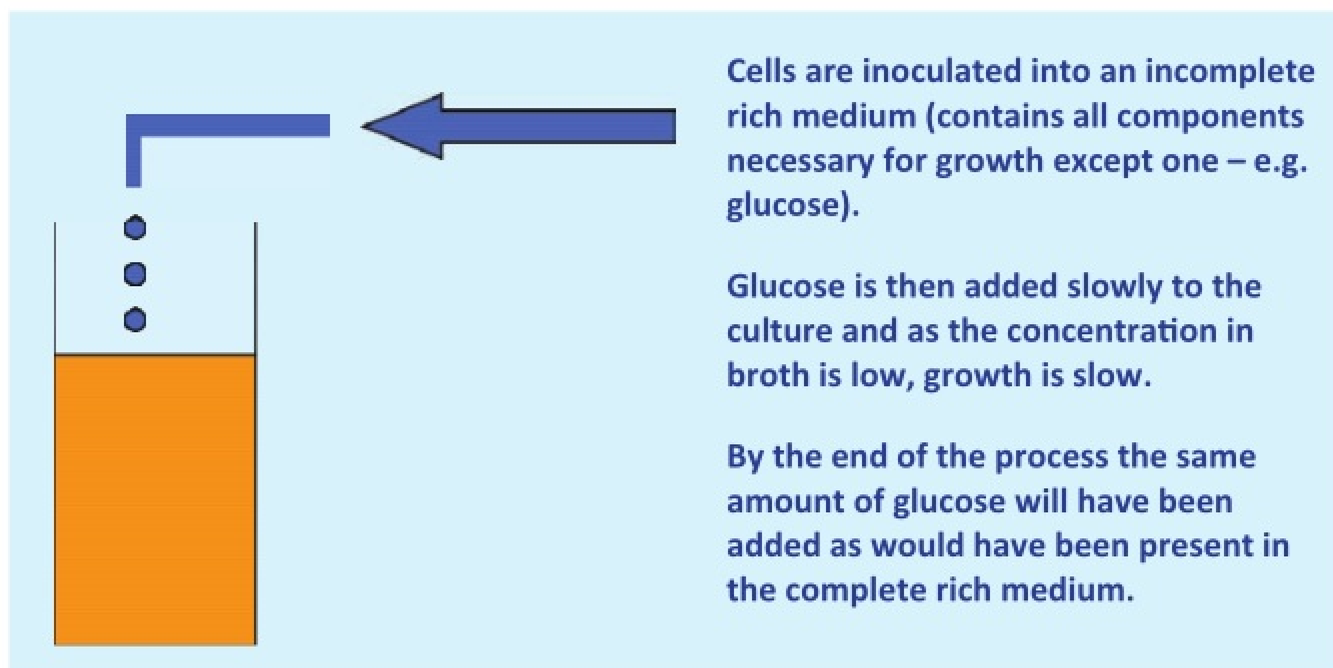

Figure 20.7 Glucose is added slowly to a growing culture to force the microorganisms to grow slowly.

success to isolate the active ingredient from mould cultures and this was eventually achieved by Florey and Chain in 1939. Norman Heatley developed a suitable production process in 1941.

Fleming described penicillin production using *Penicillium notatum* and this was used for the initial development work. However, yields were poor and the mould only produced antibiotic when growing on the surface of agar. Subsequently, *P. chrysogenum* was tested and found to give good yields in liquid culture.

Early clinical use of penicillin

The first clinical application of penicillin was in 1930 when Cecil Paine, a pathologist at the Royal Infirmary in Sheffield, attempted to use crude culture filtrates topically to treat patients with sycosis barbae (infection of the hair follicles on the face) but this was not successful due to lack of skin penetration. However, he did successfully treat an infant with ophthalmia neonatorum (gonococcal infection of the eyes) and this was the first recorded cure for penicillin.

The first human patient to receive purified intravenous penicillin was Albert Alexander, in 1941. He had developed bacteraemia from a cut hand whilst gardening and responded well to the novel therapy. However, stocks of the new drug were limited and there wasn't sufficient to complete the course. He died shortly afterwards.

Further improvements were made to the production process by altering the growth medium. Originally simple, standard media such as Czapek Dox agar were used and these were supplemented randomly to try to increase yields. Two changes that had profound effects were the use of lactose instead of glucose, which greatly increased yields due to slower growth, and the addition of corn-steep liquor, a byproduct from the wet milling of corn. The latter increased yields fivefold because it is a source of phenylacetic acid, a component of the penicillin molecule.

With *P. chrysogenum* as the starting point, a programme of strain mutation and selection then followed using mutagens such as nitrogen mustard, X-rays and UV light. The process of mutation and strain selection is summarized in Figure 20.8 and shows the final industrial strains of *P. chrysogenum* producing about 30 mg per ml of culture.

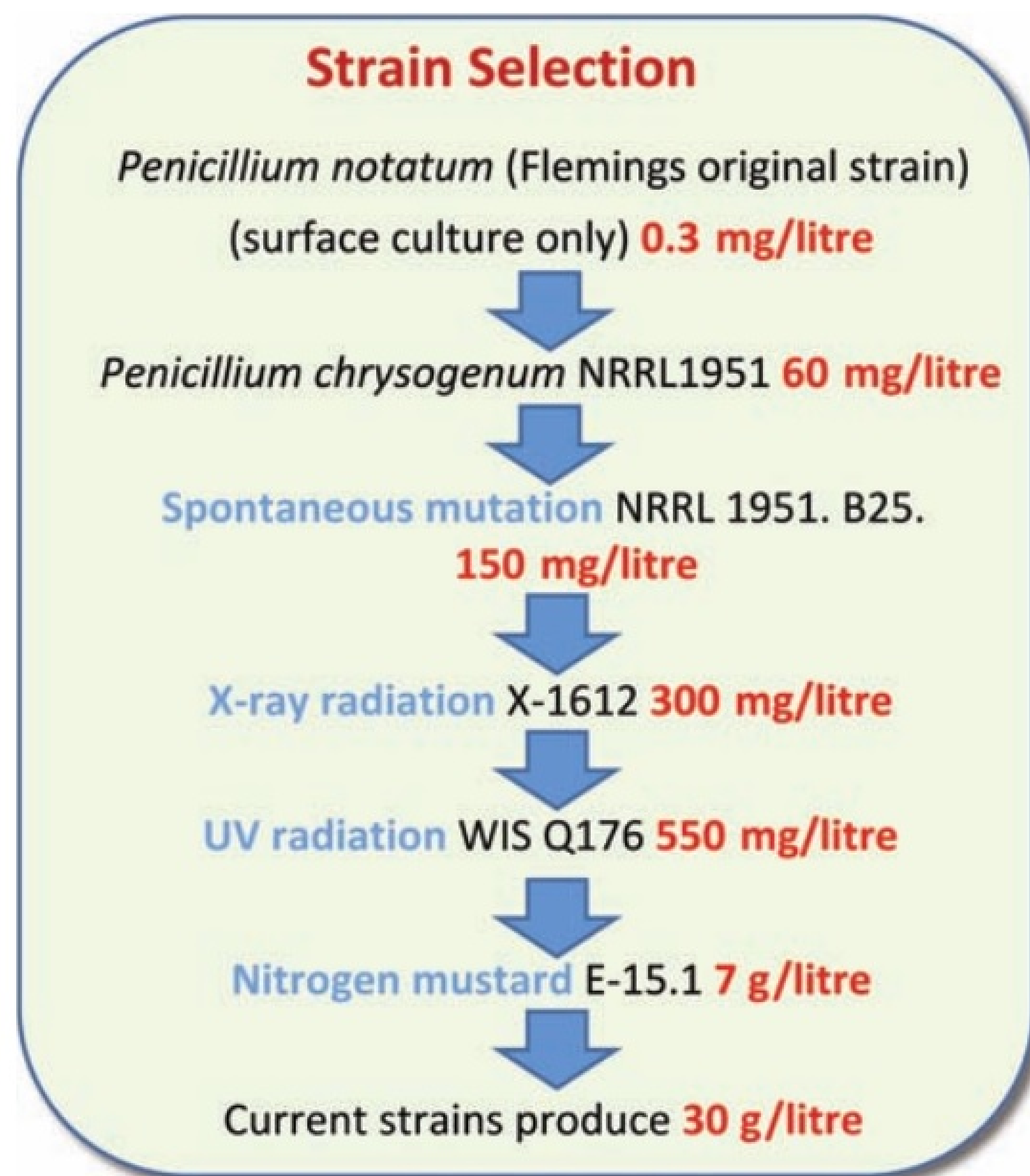

Figure 20.8 Improvement of penicillin-producing strains through mutation and selection.

20.8.1.1 The fermentation process

The industrial production of penicillin takes place in large stainless steel fermentation vessels of about 180 000 litres capacity. A diagram of a typical fermenter is shown in Figure 20.9. These vessels are stirred rapidly and aerated with forced sterile air. They are fitted with temperature, pH and foam control and pumps for the slow administration of additional nutrients.

The process flow chart (Figure 20.10) shows that the fermenter is inoculated from a seed tank of 500 litres capacity and after the fermentation is complete the penicillin is contained within the cell-free medium. This means that the culture can be filtered to remove the cells and the supernatant treated to extract the antibiotic. To extract the penicillin the solution is acidified; this allows the antibiotic to partition into a solvent such as amyl or butyl acetate. This stage must be performed rapidly and at low temperature as the penicillin is unstable in acidic solutions. Addition of phosphate buffer causes the penicillin to crystallize out where it can be

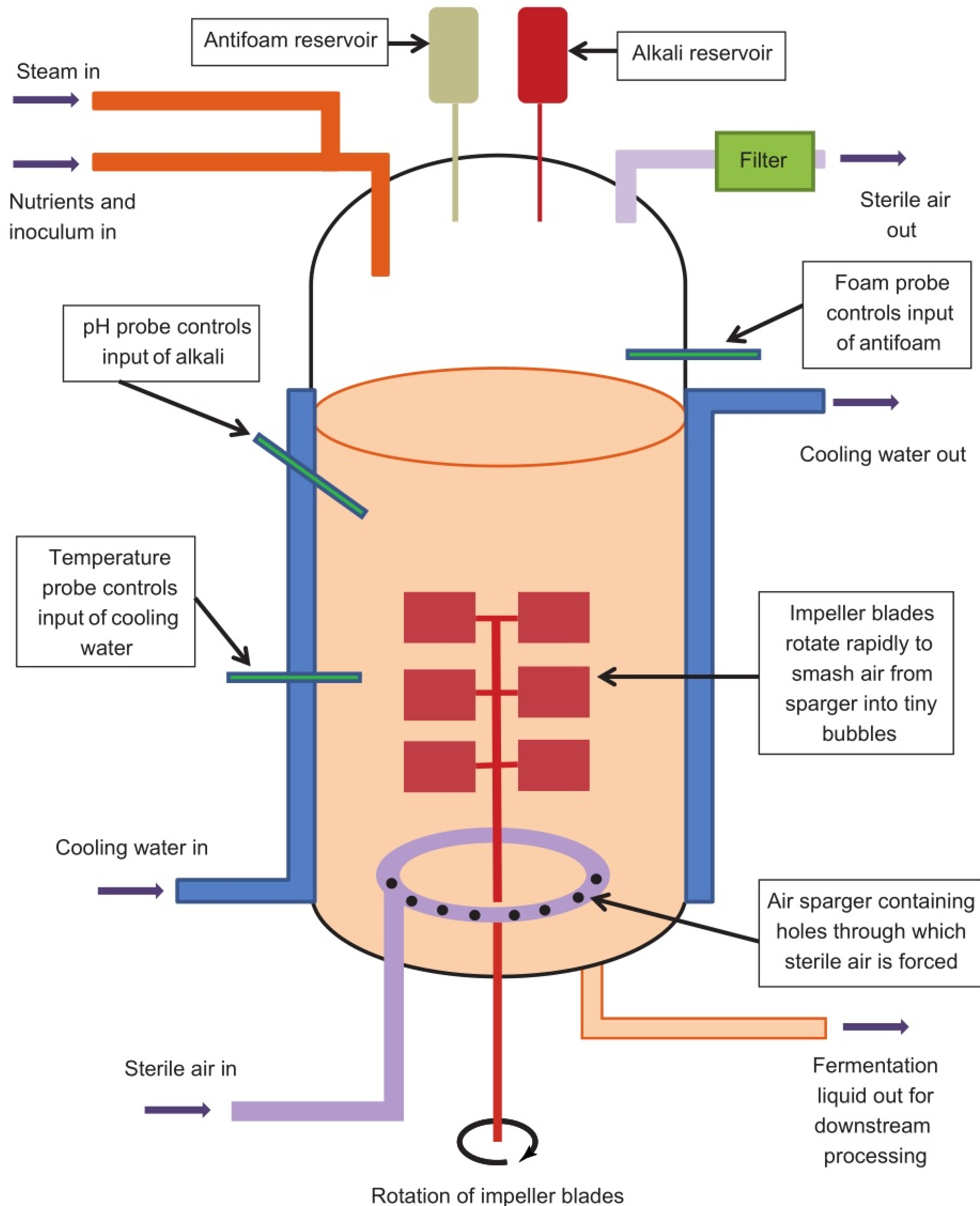

Figure 20.9 Schematic diagram of a large-scale fermentation vessel.

washed and dried. If the culture is able to produce 30 mg per ml of penicillin, a fermenter capacity of 180 000 litres will give a total yield of penicillin per batch of around 5400 kg. With a dose of 250 mg each batch therefore yields 20 million doses.

The final product of the industrial fermentation process is benzyl penicillin (penicillin G) but, although this is effective, it has a number of limitations. Its spectrum of activity is restricted to Gram-positive bacteria with little or no activity against Gram-negative bacteria. It is acid labile and is therefore destroyed by gastric acid in the stomach thus requiring it to be administered parenterally. Finally, it is readily inactivated by β-lactamases leading to the development of resistance (Chapter 10).

Chemical analysis of fermentation broths always gives higher concentrations than biological analysis. The difference is the presence of 6-amino penicillanic acid (6-APA) which is a precursor of benzyl penicillin but has no antimicrobial activity. 6-APA is benzyl penicillin without the side chain and it is the nature of the –R

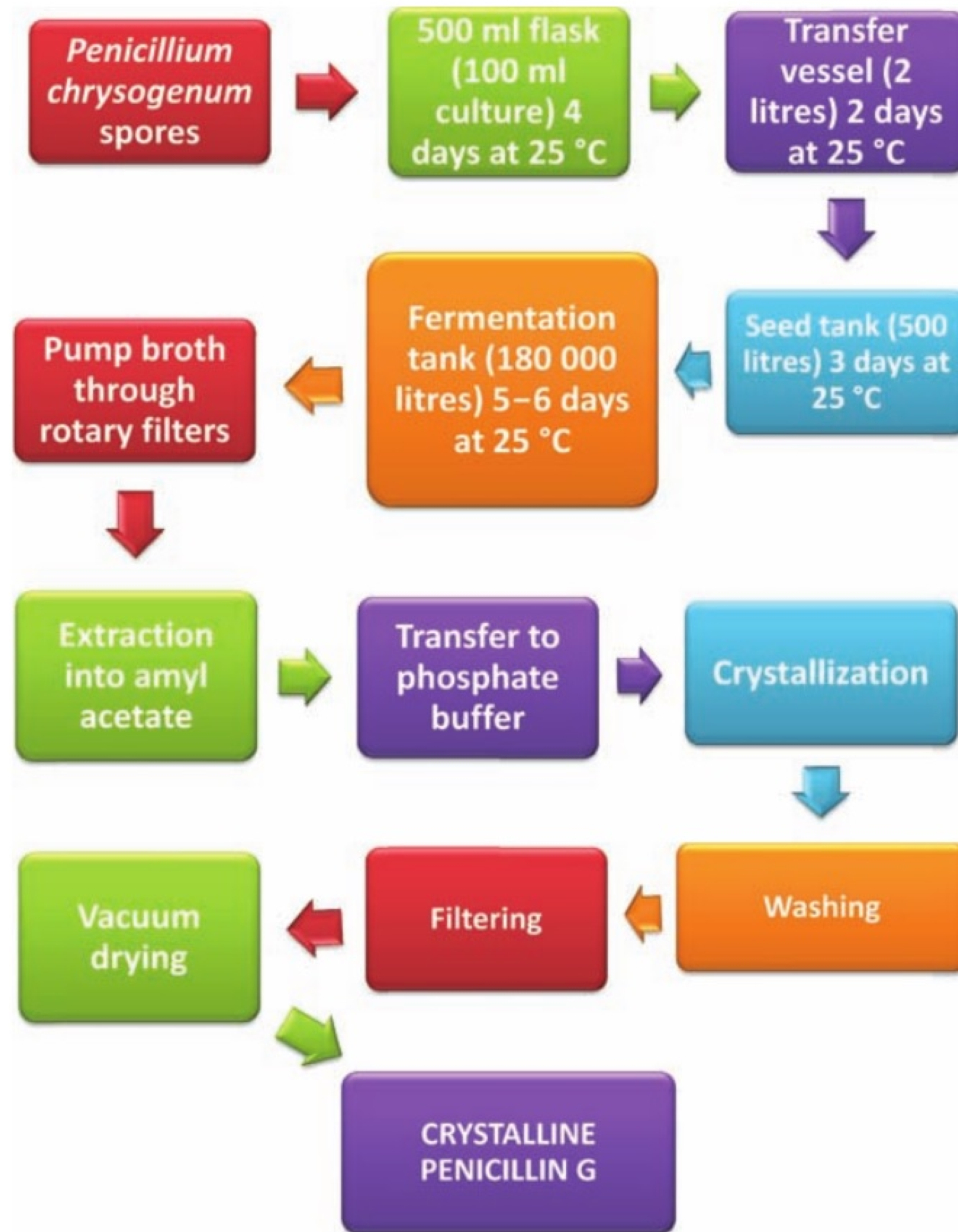

Figure 20.10 Penicillin fermentation process flow chart.

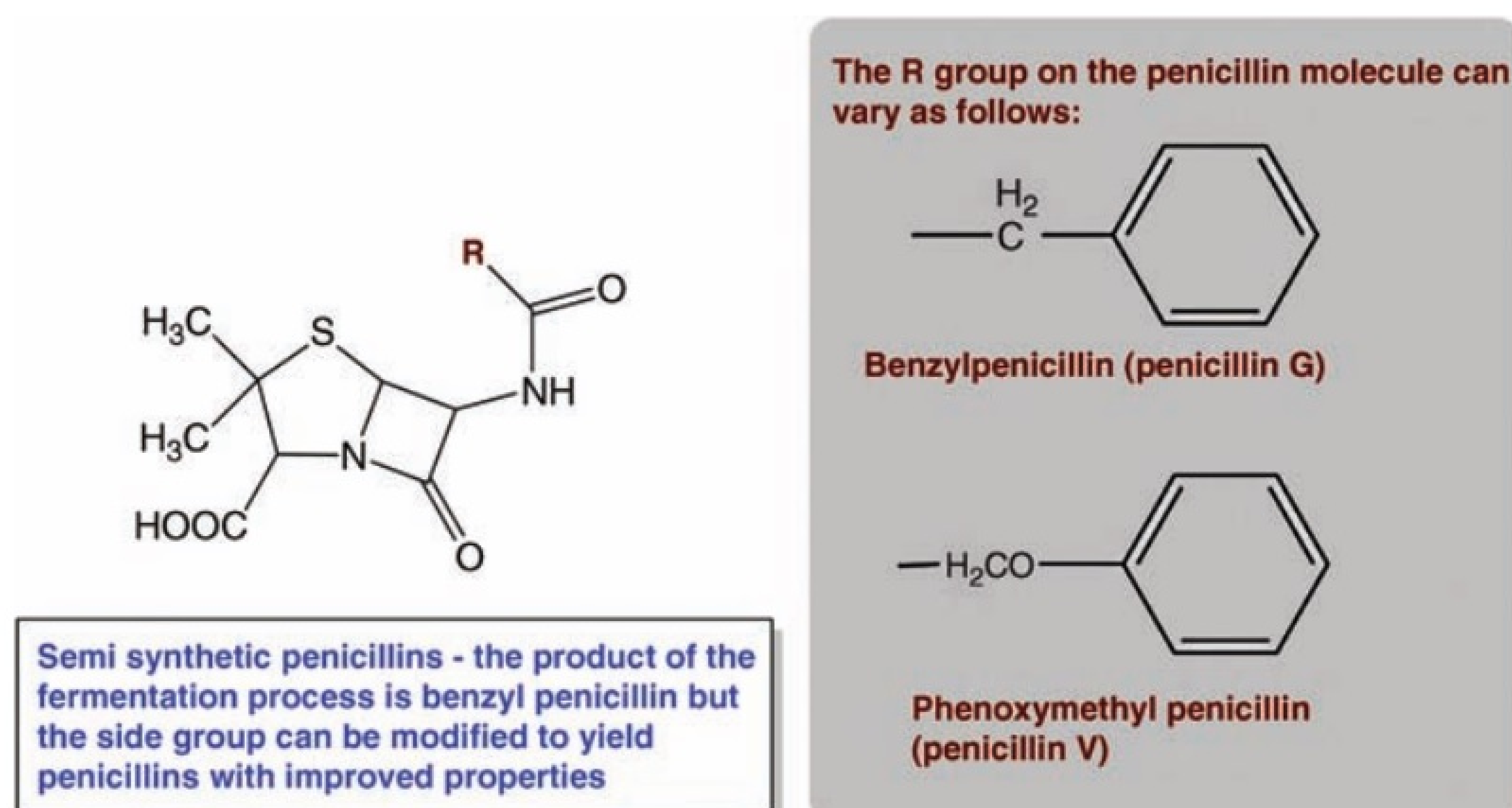

Figure 20.11 The basic penicillin nucleus illustrating how the side chain may be manipulated to produce products with different characteristics.

group, which determines the characteristics of the penicillin molecule. 6-APA is therefore a useful starting material for making penicillin molecules with different properties (see Figure 20.11).

It is not practical to manufacture 6-APA directly as it is much more difficult to extract from the fermentation broths; therefore benzyl penicillin is manufactured and converted to 6-APA using microbial enzymes. After extraction and purification it is possible to chemically add different side chains to give penicillin molecules with improved properties. Thus the production of penicillin is known as a semisynthetic process where the cells carry out part of the process and the remainder is carried out chemically.

Acknowledgement

Chapter title image: Alexander Fleming – discoverer of penicillin. http://commons.wikimedia.org/wiki/File:Alexander_Fleming.jpg