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THE LATEST STATE OF TECHNOLOGY IN THE PRODUCTION OF NATURAL SUBSTANCES BY FERMENTATION *

by

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Introduction

Despite the progress that has been made in the field of chemistry recently, microbiological processes have become increasingly important over the years. The search for new active ingredients and the improvement of production strains goes on. Even for active ingredients of a simple structure human endeavours do not match the natural ability of micro-organisms to bring about chemical synthesis. A high degree of selectivity despite the use of impure starting materials, the ability to adapt to the raw materials locally available and the possible rise in yield due to the further culture of the organism isolated are all factors that contribute to the superiority of the microbial production of complicated molecules. The efficiency of the natural organisms is best demonstrated by the fact that even now new substances are still being discovered by biological methods and brought to production stage, even before the various stages of synthesis are known. It is not surprising that the importance of fermentation methods is constantly on the increase and that this gathering is interested in the techniques necessary for manufacture.

General principles of aerobic fermentation

The starting point of large-scale fermentation is the biological cell (Fig. 1). It is the actual reactor. This seemingly huge production unit serves only to create physical conditions that will promote cell growth and are favourable for the production of the active ingredient. The most important of these are the provision of substrates in the form of a gas or liquid, the removal of waste products and the maintenance of temperature and pH at the most suitable level. As the practice is to use only aerobic methods for recovery of the active ingredient, I shall restrict myself here to fermentations that require the use of substantial amounts of oxygen.

The basic processes are carried out as batch operations and comprise two stages. Fig. 2 illustrates one of these processes with the build-up of the necessary microorganism mass and, when growth has been restricted, the subsequent formation of the required active ingredient. Continuous or fed-batch processes are being studied at research institutes but have not yet become established for use in commercial practice.

Gas-liquid contact reactors

The fermentor used for aerobic fermentation is in essence a gas-liquid contact machine. Figure 3 lists various widely used machines of this type. They differ basically in the type of drive used and in the phase representing the continuum. The first aerobic fermentors consisted of surface systems which either enabled the micro-organisms to grow on agar or, as in the case of citric acid production, enabled them to grow on packing material, through which nutrient solution has been allowed to trickle. These surface fermentors probably simulate most closely the original conditions of the organism in its natural habitat. However because of their inevitably large size, they have been overtaken by the second main method, submerged fermentation. The use of surface methods should not be ruled out wholesale, however, because they may be the answer for certain locations and where certain cost factors have to be taken into consideration. Their main advantage is that energy costs are kept to a minimum.

stirred tank fermentors

The aerated stirred tank (Fig.4) is the mainstay of antibiotic and active ingredient fermentation. It was the introduction of the first submerged aerobic fermentations that paved the way for the production of antibiotics in the quantities possible today. We must not lose sight of the fact that the process of suspending under water the normally earthbound fungi, yeast, bacteria and streptomycetes is a momentous step from the physiological viewpoint. It is not surprising that a number of organisms do not survive this measure, so it is also possible

that some useful syntheses that have been found in the laboratory could'nt technically be realized. As far as its appearance is concerned, the aerated stir-tank fermentor has changed very little over the last 30 years. Admittedly, the reactors have increased in size from about 40 to 250 m^3 and over, the heat removal is 10 times greater than it was in those days owing to the development of the biological cultures, and the power required is 5 kW/m^3, in some cases even as much as 8 kW/m^3. The table in Fig. 5 shows the power requirements for special fermentations. If we attempt to classify these data in a manner that will illustrate the technological level of chemical processing, the following factors become apparent:

1. The very efficient agitating action which is otherwise mainly found with dissolvers or mixers. This has come about in the course of development as a result of the constant increase in the viscosity of culture broths due to a higher nutrient content and higher cell density and the considerable capacity for material transport required of the cultures.

2. The quantities of heat to be removed are very high when one considers that the fermentation temperatures are only slightly above the ambient temperature. This has meant that as a rule the cooling water used for fermentation cooling has to be conditioned in special watercooling plants.

3. The aeration rates of 30 to 50 $m^3/m^3 \cdot h$ considerably increase production costs.

4. Because of the long fermentation periods required for each batch culture the costs of stirring, cooling and aeration, which on an hourly basis are not particularly high, amount in all to over 10 % of the total running costs.

Construction and design

As we have seen, the appearance of the reactor has remained unchanged for many years. The modern planning procedure would be to spread the production of an active ingredient over a sensible number of reactors to minimize the risk of losses due to failure. The level of the liquid and consequently the height of the reactor depends on the pressure stages of the air supply; for instance one can try to make do with one-stage air

compressors corresponding to an upper pressure of 3 bar. The diameter of the reactor is usually limited by the size of the approach roads, shipping route and railway bridges along which they are transported. As a rule the fermentor is equipped with a cooling jacket, and, depending on the temperature of the cooling medium on site, is fitted with an internal cooling unit. The disadvantage of the latter is that it takes up valuable production space and impairs the mixing action of the stirrer. Mounting a cooling coil on the outer wall of the vessel increases its stiffness and reduces the strength of the wall (Fig. 6). In the past the cooling jacket was made of ordinary steel, with only the actual vessel wall being made of stainless steel. This resulted in the corrosion and pollution of the cooling water, which in turn can pollute the recooling plant. Nowadays stainless steel is used for both.

Some new answers have been found connection with the stirring mechanism. As the growth phase and the active ingredient formation phase often require different degrees of agitation, a device has now been installed to enable the rotational speed to be varied for the two phases. The following alternatives are now available:

a) Thyristor-controlled three-phase motors.
   Advantage: purely electrical operation, easy to use, degree of efficacy: over 95 %.
   Drawback: the torque falls with the speed; a high proportion of wattless current results, and the motor can stall as the resistance is increased.

b) Hydrostatic torque converter
   Advantage: the torque increases as the speed is reduced.
   Degree of efficacy: over 93 %. The machine is very small, a very small motor can be used.
   Disadvantage: Noisy operation.

c) Hydrodynamic torque converter
   Advantage: sturdy, simple design, torque increases as the rotational speed is reduced.

Disadvantage: Rotational speed inconstant, degree of efficacy depends on rotational speed and decreases from about 93 % at the nominal speed.

d) Hydrodynamic clutch
Advantage: the torque is constant with the rotational speed, design is very simple.
Disadvantage: Degree of efficacy decreases with the rotational speed. Oil cooling necessary.

e) Three-phase motor, commutable poles, with two or three asynchronous rotational speeds.
Advantage: low capital expenditure, slight rise in torque at the low rotational speed.
Disadvantage: No continuous control; as the culture changes, the rotational speed can only be adjusted by means of belt pulleys or a change in spur wheels.

In all there is a tendency to increase the output of the motor to a considerably higher level than was required by original lay-out-data. because lower power requirements on the part of the culture cause only a slight loss in effectiveness, but so far trials have shown that, as the cultures have improved, the reserves of power have subsequently been fully utilized in every case. If the market for a certain fermentation product is good enough to ensure that a unit will be fully utilized, it may be advantageous to invest in a three-phase motor with commutable poles.

As it is essential for the procedure to be carried out under aseptic conditions, the shaft seals are especially important. For a long time it was considered preferable for the entire drive system to be situated at the base of the vessel. The seals used were double-action mechanical seals with a sterile sealing liquid (Fig. 8). These seals are expensive, particularly in the case of the large vessels which require shafts of a diameter of 200 mm and over, and are sensitive to jarring and vibration. Owing to the use of better, self-lubricating packing materials the faithful old stuffing box has now made a come-back, although admittedly it is best used in a gas-filled

space (Fig. 9). In other words, for large fermentors it is advisable to use an overhead drive with a double-action stuffing-box seal.

The stirrer blades have undergone surprisingly little refinement. The disc stirrers (Fig. 10) of the early 1950s are still used. Fig. 10 shows several different types such as the self-suction stirrer, Frings system, developed for the production of acetic acid and yeast, a multistage counter-current stirrer, Ekato system, and an axial propeller with a flow guide. There have recently been attempts to consider the stirrers as pumps and design them accordingly in terms of pressure and throughput. Machinery manufacturers with initiative can establish which is the best stirrer for a certain system by carrying out trials in their own workshops.

Other reactors

The aerated stir-tank fermentor still leads the field as the best reactor for most forms of production. This is mainly because of its flexibility, which enables it to ferment even highly viscous culture broths (up to 4 Pas).

For special fermentation processes, such as steroid recovery, yeast production and single-cell protein (SCP) recovery, the treatment of waste water and in particular the treatment of sulphite waste liquors, the fermentors shown in Fig. 11 were developed. The only one of these that is suitable for nutrient solutions of very high viscosity is the paddle-wheel fermentor. Most of the machines were designed for the fermentation of low-viscosity solutions. The very widely used principle of the loop current is particularly worthy of special mention. It postulates - instead of the entire reactor contents being homogeneously mixed with uniform distribution of all the substrates - a requirement that can never be met in all respects, namely the purpose-linked circulation of the entire contents with a very narrow spectrum of the circulation time through the various zones of the reactor (Fig. 12). In this way the time it takes for the substances fed in to be circulated and used up can be correlated with each other.

Active metabolite fermentation

So far the fermentor has been recorded here as a general submerged fermentation reactor. In the unit as a whole, however, it is surrounded by a comprehensive battery of back-up devices, supply and control systems. These depend largely on the type of substrate and the active ingredient formed.

Production of the inoculum culture

Fig. 13 illustrates the route, with which you are all no doubt familiar, of the inoculum culture to the fermentor. The production strain selected is transferred from the agar slant in the laboratory to culture shakers which are then propagated in several fermentation stages to production scale with the necessary number of cells. The inoculum culture is then kept under conditions which promote growth but not the production of the active ingredient. In order to obtain the best possible inoculum from the generally expensive production-scale stock culture a relatively large number of cultures are grown in the early stages of propagation and the best of these are selected for further processing. This takes up a considerable amount of space in the laboratory.

Fig. 14 shows a modern mechanical shaker designed to hold 300 shaker flasks of 300 ml capacity. As there were serious balance problems with earlier models, these designs have a counterbalance device built into the middle of the three platforms so that even tilting is prevented.

Most of the small (30 to 300 l) fermentors have the motor at the bottom to leave the lid area free to take tubes and measuring equipment.

Nowadays the ring-main system for the inoculation piping is dispensed with wherever possible; instead the fermentor to be inoculated is connected to the pre-fermentor (Fig. 15) by means of rigid pipe bends or flexible hose to prevent cross-infection. The piping itself should be positioned at an angle and suspended in such a way that no pools of nutrient solution can be

left behind. As can be seen from Fig. 16, these could in time dry up to form solid patches, which cannot be adequately sterilized at the time and temperature conditions prevailing and may therefore eventually cause an infection.

Method of making additions

Depending on the active ingredient being processed, a number of additions have to be made to the fermentor and must be incorporated into the system under sterile conditions. Typical examples are: sugar solution for penicillin, sugar solution with ammonium sulphate for tetracycline, acid or caustic soda lye to control the pH of all fermentations, antifoaming agents in the form of oils and of course the volumes of air required for metabolism. Sterilization is achieved by means of liquid sterile filtration, which is still problematic from the point of view of long-term sterility, and by heat sterilization. The latter is still the most common form of sterilization, whereby in the batch processes the media are pumped under sterile conditions or passed through the machine by gas under pressure, or in continuous sterilization are passed from a non-sterile vessel first over a heating section and then, through an insulated pipe system (heat retention) followed by a cooling section. The continuous system is the safer method for temperature-sensitive substrates but it requires a steady throughput so that the heat transfer remains uniform. The incubation air introduced (the amounts needed for several products are given in Fig. 5) has usually undergone sterile filtration. There is no doubt that the sterile filters with a pore width of 0.1 to 0.2 u that are now available are highly suitable for small units, but for large plants these filters consist of several cartridges connected in parallel. This means that the risk of failure increases commensurate to the current of air. If the fermentor becomes contaminated, all the cartridges must be examined or exchanged. There is considerable controversy as to whether for large-scale fermentation it would not be better to stick to traditional, large glass wool filters which would also prove more economical in the long run.

Measurement and control systems

Fig. 17 is a diagram showing the control systems normally used today for batch fermentation. It necessitates a means of regulating the internal temperature, which is best linked with cascade control of the temperature of the cooling medium. Otherwise the fermentation temperature tends to fluctuate. The flow of air, cooling water and additions - in the case of the cooling water also the entry and exit temperatures - should be measured in order to balance out the amounts used up. The flow of additions is controlled by means of a level-or weight-measuring gauge which is also to be provided for the fermentor. Wherever possible the measurements are made on the non-sterile side in front of the filters or heat exchangers. I consider it vital to record the amounts of oxygen and CO_2 present in the exhaust air, and if necessary also the concentration of volatile substrate. The pH value, the redox potential and the oxygen content of the broth in the fermentor itself are determined by inserting probes into the fermentor. The output of the agitator motor and its rotational speed offer valuable clues to the viscosity and therefore also the density of the culture. Unfortunately the majority of biochemically important data concerning continuous fermentation still has to be elaborated in lengthy laboratory work. We are still a long way from developing a real time control of the cell density, enzyme content and amount of active ingredient contained in a culture.

Constant progress is being made in the development and marketing of systems to eliminate, by mechanical means, the foam that forms under unfavourable conditions (Fig. 18). But so far there is no effective means of dealing with really stubborn foam. Consequently we still fall back on the antifoaming oils that are particularly unpopular with the harvesting experts. It should be noted here that the disadvantage of the mechanical means of combating foam is that they only treat the symptom and have little effect on the underlying cause. Further research into the basic principles of foam formation and improvement of the nutrient media may go some way towards solving this problem.

Sterilization of the fermentor

At the start of fermentation all micro-organisms and spores must be removed from the container that has already been cleansed. There are two ways of doing this: the tank liner is sterilized either empty or 10 % full of water, or when completely full after all the nutrients have been introduced. In the first case the nutrients are sterilized separately and then introduced into the fermentor that has been previously sterilized. In both cases it is important to ensure that all the pipes leading in to the fermentor are flushed into the tank with steam and raised to sterilization temperature, during which the tank and the water it contains act as a large spray condenser. The most reliable procedure is for the piping to and from the fermentor to be equipped with temperature probes at the hazard points so that the effect of sterilization can be monitored. It is also advisable to measure the pressure at the end of the piping system. The first phase of sterilization consists in completely ventilating all the systems as sterilization has to be carried out in a current of steam. Narrow flanges in which gas could accumulate must therefore be avoided and if necessary separately ventilated. Branched steam connections (Fig. 19) are also unsuitable because it is impossible to ensure that there is a proper flow through both pipes.

Connections and flanges that are not used should be protected from infection by means of a "steam seal" (Fig. 20) - a heat zone which maintains the connections in a sterile environment. Instruments must be arranged so that the fermentor is sealed off by the seat of the valve but the easily contaminated stuffing-box packing material does not come in contact with the medium. In our experience the ballcocks shown in the Fig. constitute the least problematic shut-off device.

Special fermentation conditions

Fig. 21 illustrates the fermentation data relating to the active ingredients penicillin G, penicillin V, streptomycin, erythromycin, tetracycline, vitamin B 12 and amylase. As it

would take too long to explain the differences between the various cultures. I shall restrict myself merely to the main problems. For instance the use of soybean meal, starch granules, yeast extract and peanut hull meal make sterilization difficult because the temperature has to penetrate to the core of the solid particles and, as can be seen from Fig. 22, lags behind the temperature of the whole solution. The sugar that has to be added in the case of penicillin must be sterilized very quickly otherwise there is a tendency for caramelling to occur. Consequently, this process is carried out using heat exchangers of the type shown in Fig. 23 because, in addition to a good heat transfer, they also ensure a short dwell time and low resistance to viscous broths. Moreover the system can be cleaned very easily after removing the lid which is not the case with a plate or tubular heat exchanger.

The structure of the mycelium formed by the microorganisms constitutes a completely different problem. Some strains such as the Streptomycetes form filamentous mycelia which rapidly become highly viscous whereas others, like Penicillium notatum, tend to form pellets which have lower viscosity. As there is a relationship between the structure and extent of antibiotic formation, it is important to ensure, when operating the stirrer motor, that optimum mycelium formation is not impaired, for instance by excessively high shear stresses.

The variation in culture media presents another problem. The term 'cornsteep liquor' used for the culture medium shown in Fig. 24 indicates a substance which is admittedly satisfactorily defined for the purpose of a raw materials list but, to those involved in fermentation, it contains a constituent which tends to vary in composition depending on the year of recovery, the quality of the soil, country of origin and method of cultivation. The very term 'tap water' covers such a wide range of analytical possibilities that, depending on the location, the water will contain either large or small amounts of trace elements or conceal the fact that it is short of them.

Contract problems

It can be concluded from all that has been said above that even detailed written and verbal operating instructions, together with all the important data and submission of the strain do not automatically guarantee a viable process. The most important of all the basic criteria is to have well-trained laboratory staff and operators who have experience of micro-biology and the patience to adapt the processing data to the prevailing local and technical conditions.

The following table (Fig. 25) contains a number of details which are of importance for the active metabolites to be treated. Obviously the amounts of active metabolites given can only apply to the optimum culture of a certain strain. In some cases it is better to use a lower amount of active metabolite. for fermentation if this makes processing easier. The risk of contamination can be reduced in every case by maintaining absolutely sterile conditions.

A broad-spectrum antibiotic like tetracycline is of course much less at risk than penicillin for instance. On the other hand with a continuous production process, such as is used for SCP, a certain amount of the foreign micro-organisms that have penetrated are washed out. All in all, however, it is not possible to design and operate a plant under anything other than absolutely aseptic conditions because 'slightly sterile' is just as difficult to adhere to as 'slightly pregnant'.

With regard to the origin of the culture it should be noted that in the case of those working on fermentations licenses can be obtained and that a number of firms have specialized directly in the mutation of strains to higher levels of synthesis by means of extensive automatic processes.

In the field of actual active-metabolite fermentation continuous production processes have not gained a foothold and prolonged fermentations (fed batch) are not used on a large scale. This is due firstly to the risk of uncontrolled mutation and the degeneration of organisms that have fermented for too long,

and secondly to the fact that with prolonged culture broths the recovery concentration is lower. This is because culture solutions that have fermented out can no longer grow prolifically and in some cases are inhibited by their own waste products; consequently with fed batch processes only low concentrations of the product are harvested.

Trends in biotechnological development

The high energy costs incurred in stirred-tank fermentation have made the search for improved and more economical reactors a matter of pressing urgency. The models that have been found so far however are not as flexible as the aerated stirred-tank It is more or less certain though that a stirred-tank of similar flexibility and with loop flow will eventually be developed. With the nutrient solutions employed, there is an increasing tendency to use liquid substrates to a greater extent, thereby reducing the viscosity of the culture broths and in some cases, despite the lower energy consumption, achieving a higher degree of synthesis. This should also be a contributing factor in the development of more economical reactors for fermentation.

Basic principles of recovery

The ability to isolate active metabolites amounts of 0.1 to 3 % by weight from the culture broth is restricted by the conditions which dictate the composition of the broths recovered. The layman proceeds under the misapprehension that he is dealing with an aqueous suspension or solution. Fig. 26 however shows two fermentor samples immediately after sampling. The culture is of such high viscosity that, on tilting, the 'liquid level' remains vertical. The composition reflects the entire fermentation history, the type of inoculum culture and nutrient solution, the type and quantity of the additions and the growth properties of the mycelium. It is important to realize right from the outset that most of the properties of the broth will vary from batch to batch and processing must make allowance for this.

Processing procedures

The block diagram (Fig. 27) of the recovery plant may give

an idea of the tasks involved. Firstly, an attempt is made to concentrate the active ingredient in one phase and separate off the mycelium. If this stage, which we shall call isolation, proves practicable, cells are filtered off. Access to the cultures which contain a large amount of the active metabolite stored in the cells must be obtained, if necessary by opening up the cell walls.

The second stage, which we shall call purification, characterizes the procedures carried out to remove impurities from the carrier phase and the third stage concentrates the active ingredient in this phase until the dry product, in a suitable form for storage, can be separated off. All the auxiliaries used must be regenerated. The subsequent formation of chemical derivatives and manufacture of the forms of issue would not normally be included in the subject of this lecture

Separating procedures - methods of disintegration

There are many different ways of destroying the cell walls (Fig. 28). As it is best to manage without adding foreign substances if possible, intensive research into mechanical methods is being carried out at present. In the future a combined system of mechanical/thermal decomposition will probably be used.

The list of separation techniques given in Fig. 29 attempts to distinguish between them by means of the selection principle. The entire processing procedure hinges on filtration which aims to separate the cells as completely as possible from the residual water. Compartment filters and rotary drum filters are both used in pressure or vacuum processing (Fig. 30) which can be carried out under readily controllable conditions. The belt discharge is a continuous operation. For the separation of tetracycline a precoat layer of silica gel is applied to improve filtration. Difficulties are usually encountered with the filtration of mycelia because of the severe branching and in many cases because of the presence of slimy substances such as polysaccharides. Some culture solutions, of which streptomycin is a typical example, prove difficult to filter. They are therefore

left unfiltered and subjected with mycelium to extraction or absorption.

Compartment filters are used wherever high pressure is required. As can be seen from Fig. 31, with these filters a good deal of work still has to be done by hand during the emptying process. They also complicate the formation of the precoat layer.

In some cases ultrafiltration plays a role in separating off bacteria cells from relatively pure culture solutions, e.g. in enzyme recovery. Membrane techniques are widely used in the processing of waste water, when salts are separated off by means of reverse osmosis and when high-molecular substances are recovered by dialysis. As far as we know, electrodialysis is not yet used for processing on an industrial scale.

Ion exchangers, electrophoresis (not yet on a large scale) and flocculation methods separate according to the particle charge.

Extraction methods were the first processing methods used in the production of most antibiotics, with sophisticated machinery being developed even in the early days. The extraction columns normally employed in classical chemistry are not used because of the long dwell times involved.

Separation of cell mass by means of the centrifugal principle is seldom used because the cells can only settle slowly due to the high viscosity of the broths, and in many cases the cells have the same density as the broths.

It can be taken as a general rule that all methods of separation which are associated with the generation of considerable amounts of heat are unsuitable for the separation of the active ingredient. Thus distillation methods are only used in vacuum processing and drying methods with a short dwell time are preferred.

Finally, mention should be made of the constantly growing importance of methods of chromatography (Fig. 32). Because of the advances

that have been made in carrier materials and active groups, selectivity, capacity and range of application are always on the increase. On the other hand, as methods of chromatography gain an increasing foothold, it must be borne in mind that the recovery units have always to be used exclusively for one specific product, whereas the classical stir-tank operations can be utilized for several products.

Evaluation of separation procedures

In many cases the same jobs can be done by a number of different methods. The final decision as to which method to use is reached on the basis of technical trials which give an indication of the quality of the product and its economic viability. Separation of the precipitated active metabolite from the carrier phase can be achieved by drying, centrifuging or filtration. From the purely economic viewpoint the overall costs of the procedure would be the critical factor. Fig. 33 however shows how each method has different selectivity as regards the residual impurities because of the different separation principles involved: the drying process removes the volatile carrier substances and leaves all the impurities in the product. Cake filtration retains only some of the impurities in the product, but the remainder are removed with the filtrate. However, during the centrifuging process the separating force of gravity acts on the active metabolite particles and extracts them preferentially from the carrier phase: it is here that maximum selectivity is achieved. Other methods must be similarly assessed - a fact that should not be overlooked in view of the increasing demands for purity in pharmaceutical products.

So that it can evaluate the many different drying processes that can be used, a company engaged in pharmaceutical development must have access to a comprehensive range of trial driers or alternatively carry out drying trials with companies specializing in this field. Fig. 34 contains a list of conventional driers which are classified according to intensity of product movement and the chief form of heat transfer.

Restrictions

Because of the product to be processed, careful consideration must be given to the choice of method. The following points are particularly important:

the decomposition rate increases as the temperature rises,

the decomposition rate increases the longer processing is carried out under unfavourable conditions,

the decomposition rate depends on the pH.

Most soluble proteins, which are eliminated in every culture solution, have a pH of minimum solubility, namely the isoelectric point at which the proteins precipitate.This can occur in a machine, e.g. in the centrifuge, causing an obstruction and an interruption in processing. In many cases all the processing procedures must be carried out under sterile conditions so that there is no contamination from external sources or, more importantly, any escape of traces of antibiotic into the surroundings. The latter factor is very carefully controlled but, according to our latest information, it is probably not as dangerous as we had previously thought.

Recovery of lipophilic active metabolites

Using the type of substances mentioned in the title, let us have a closer look at the different phases of extraction. The separation stages used are very similar to the basic procedures of recovering technology although for product-related reasons restrictions have to be imposed and special models used.

Penicillin G and V

A flow diagram demonstrates the recovery of penicillin (G or V) that is possible today:
The destruction of bacteria in the harvesting tank by adding formalin which retards the breakdown of penicillin by inhibiting respiration, followed by filtration through a vacuum belt drum filter with a simultaneous washing operation. The penicillin-rich aqueous solution is removed from a supply tank, admixed with wetting agent and acidified to pH 2.0 shortly before the

extraction process. During extraction the penicillin converts into a polar solvent, referred to here as butyl acetate. The penicillin is precipitated out of the solvent by the addition of an organic base, after which the precipitating salt is hydroextracted on a filter centrifuge, washed again, hydroextracted a second time and then dried. The dry product has good storage stability and can be further processed in chemosynthesis.

The disintegration rate of the penicillin is highest at the optimum pH for extraction, i.e. acidification must not be carried out until just before extraction. In contrast the product is stable in the solvent phase.

The model designed by Podbielnak (Fig. 35), which has been in use for 30 years has proved to be an effective extractor. It corresponds to a multistage sieve plate column built into a centrifugal field. As the dissolved protein precipitates readily at the extraction pH and its density is lower than that of water and higher than that of the solvent, there may be some enrichment of protein in the separating layer of the two phases. The exchange of both phases is prevented and the processing procedure cannot continue. To prevent this happening the surface active wetting agent is added in advance so that the precipitated protein remains in suspension.

The penicillin salt that has precipitated is now separated from the solvent in a peeling centrifuge, which no longer has to be manually cleaned (Fig. 36). The solvent-moist product is automatically peeled in thin layers from the hermetically sealed centrifuge which operates in a nitrogen atmosphere, and carried to a separator via a pneumatic rising main. The transport nitrogen is heated and during transportation uses the vertical pipe as a pneumatic drier. The solvent is condensed out of the nitrogen and used again. The nitrogen is then heated again and returned to the centrifuge. This patented method conforms to the explosion prevention regulations, protects the staff from hazardous solvent vapours and entails relatively low losses. It could be used as an example to demonstrate that it is more advantageous to consider all the various stages of

a process collectively and therefore reduce the work and expense involved.

Erythromycin

In the case of erythromycin separation is carried out without prior filtration of the mycelium, using a three-phase extractor in which the mycelium is removed from the outside in a separator at pH 9, the aqueous phase is separated off via a peeling tube and the loaded solvent is separated off on the inside (Fig. 37). Recently a three-phase decanter (Fig. 38) was also developed for the purpose. With this device extraction is carried out from the solvent with dilute acid and the product precipitates at pH 7. A higher degree of purification can be achieved by recrystallization or by means of adsorption and ion exchange columns. There is every chance that the three-phase decantation method may enable the extraction of other antibiotics to be simplified.

Tetracycline

The culture solution is cooled, diluted with water and acidified. It is then passed through a vacuum filter with the aid of precoating, washed and filtered again. The cake is discarded. The filtrate is admixed with sodium sulphite and the untreated tetracycline base is precipitated by the addition of sodium hydroxide. The precipitate is separated off on a filter press. After pasting up with water and admixing with hydrochloric acid, it is dissolved in a mixture of acetone and water, and purification and decolorization are achieved by addition and absorption on to activated carbon and filtering aids. When it has been filtered clear, the pure base is precipitated by the addition of NaOH. The mother liquor is separated off with two centrifuges and the crystallisate simultaneously washed with a mixture of acetone and water. The moist product is then dried. The acetone is again recovered from the mother liquor/ wash liquor. As a result of technological progress there are now filters which empty automatically, like the centrifugal filter shown in Fig. 39.

Unfortunately the compartment filters that are also used still have to be emptied manually.

Vitamin B_{12}

After fermentation the broths are admixed with potassium cyanide and heated in the stirred-tank (Fig. 40), after which the cobalamine is adsorbed on to bentonite (previously activated carbon) and then eluted with aqueous pyridine solution. Ballast materials are removed from the eluate by adding Zn (OH_2). The next stage is countercurrent extraction with phenol, followed by countercurrent distribution with benzyl alcohol, further extraction with phenol, adsorption on to bentonite and finally separation of the corrnoid from the cyanocobalamine using cellulose exchangers. After elution with aqueous acetone solution, the substance crystallizes out in a very pure form. The machines used are the same as those employed in the processing of antibiotics.

Recovery of hydrophilic active metabolites

Although the active metabolites cannot be clearly divided into categories of polar substances that can be easily separated with nonwater-miscible solvents, and non-polar substances which become more concentrated in water, this distinction is still made. It is of course also possible to purify lipophilic metabolites by chromatography, and in fact these methods are often used. Streptomycin can be quoted as an example of the recovery of a hydrophilic antiobiotic.

Streptomycin is virtually impossible to filter because of a number of slimy constituents in the culture broth (Fig. 41). It is therefore sifted to open up and prevent the passage of large impurities and in the rising-flow operation is passed over weakly acidic cation exchange resins. The exchange resin is then washed with water and the streptomycin is eluted with hydrochloric acid so that it occurs in aqueous solution in the form of trichloride. The eluate is then purified with activated charcoal, linked again to a weakly acid ionic exchange resin and eluted with dilute nitric acid, after which it is neutralized with sodium

carbonate and filtered. It is then adsorbed on to cationic exchange resins and eluted with dilute sulphuric acid. This eluate is neutralized with an exchange resin, the salt is removed with the aid of another resin, after which the eluate is decolorized with activated charcoal and filtered. These procedures are followed by the vacuum evaporation of the water and precipitation by the addition of methanol. The precipitate is then centrifuged, washed and dried in a vacuum. The total yield obtained with this method of purification is not more than 70 %.

This form of processing is complicated by the many stages involved and more especially by the fact that the mycelium is almost impossible to filter. Because of this so much water has to be used that the aqueous phase that has to be passed over the first exchange column is about three times the volume of the culture solution. Fig. 42 shows the shape of an exchange column used for the rising-flow operation, whose design prevents it from becoming blocked by the mycelia passing through it, but at the same time limits the flow rate.

Generally speaking, it is important to remember with exchange columns that the packing material tends to swell during saturation with the solvent which has resulted in its being designed to cope with high pressures. As a rule the exchangers can only be used for one specific product, the multiple use of this type of unit by another product being virtually impossible. As regards the theory it is not, or any rate not yet, possible to calculate certain characteristics of exchange resins. Neither the capacity nor the selectivity or re-usability of a packing material can be determined in advance. On the contrary, even when chemically identical structural materials and active groups are used it is not unusual for these parameters to vary by 1000 %. On the other hand because of the high degree of selectivity possible there is bound to be a considerable increase in the use of chromatographic methods in the future.

Enzyme production

I should like now to say a few words about the production of enzymes. Although submerged aerobic fermentation methods are gaining

an increasing foothold, there are a number of enzymes which can even now only be economically produced on solid culture media, for which pollard wheat is the most suitable substrate. The more important submerged fermentation process using complex nutrient solutions corresponds to that described with the methods for antibiotics.

The process is described in greater detail using the amylases as an example (Fig. 43). It can be achieved by adsorption methods (using α-amylase from the culture of Bacillus subtilis)

a) Adsorption on to starch (saturated with $(NH_4)_2SO_4$)
b) Elution with 1/30 M Na_2HPO_4
c) Eluate dialysed
d) Decolorization with resin (Duolite A2)
e) Salting out with $(NH_4)_2SO_4$
f) Dissolution in dilute aqueous $Ca(OAc)_2$ at pH 10
g) Dialysis
h) Addition of acetone at 0^oC, 60 % -> precipitation
i) Precipitate dissolved in aqueous $Ca(OAc)_2$ at pH 10

Adjustment to pH 6, freeze dry aq

The starch-adsorption procedure in particular must be carefully adjusted to give a water content of less than 20 % because otherwise the starch itself will be adversely affected.

The second general method used is extraction (demonstrated below by means of TAKA amylase from a culture of Aspergillus oryzae).

a) Extraction from TAKA diastase using water
b) Addition of $Ca(OAc)_2$ followed by filtration
c) Addition of water, salting out with $(NH_4)_2SO_4$
d) Centrifuging, precipitate dissolved in water
e) Dialysis
f) Addition of 1 % Rivanol solution, with subsequent filtration
g) Addition of 1 % Rivanol
h) Solution in acetate buffer at pH 6
i) Addition of acid clay, filtration
j) Addition of acetone, centrifuging of the precipitate

k) Dissolution in $Ca(OAc)_2$
l) Addition of acetone and freeze-drying

These methods are established and are used with various modifications. However the trend is towards fractionated ultrafiltration, gel chromatography or affinity chromatography, though this has not yet been used on an industrial scale. The idea is that specific inhibitors, which are linked with carriers and have extremely high selectivity, isolate the enzyme direct from the first filtrate and release it under suitable conditions.

Recovery of the cell mass

In cases where some or most of the active metabolites is to be found in the cells, as is the case with Flavomycin(R), or when the cells themselves represent the product required, as with single-cell protein, or when processing is best carried out from the anhydrous dry product, as it is with griseofulvin, the entire culture solution must be subjected to mechanical dehydration and subsequent evaporation.

Flavomycin(R)

The entire culture solution is concentrated in a thin-film evaporator and then dried in a spray drier (Fig. 45). The solution is not previously filtered because about half of the active ingredient dissolves in the aqueous phase. To eliminate the risk of dust explosion the drier, as can be seen from Fig. 45, operates on the principle of recirculation of the drying air and a condenser connected in series. The heat required by evaporation enters the circulatory system through addition of the hot fumes generated by direct combination of the air , as a result of which the oxygen content of the air is reduced to less than 8 %. In this low oxygen atmosphere there is litte risk of the dry product dust igniting.

Recovery of single-cell protein

Similar procedure to that used for Flavomycin. However, as can be seen from Fig. 46, the water is first separated off as far as possible, and returned to the fermentation. After lengthy

development work bacteria measuring 0.5 µm in size were concentrated in nozzle separators to a cell mass of about 16 %. On an industrial scale the separator processes the inflowing suspension at a rate of 40 000 l/h. By modifying the decanters it then proved possible to concentrate the bacteria mass to 30 % (Fig. 47). This was done by using a relatively high liquid level to bring out the bacterial sludge which otherwise flows back. The machine that is to be used for production will process 25,000 l/h. The additional costs of operating a decanter will be recouped as soon as the dry substance content can be increased by about 4 %.

Focal points of future development

In the last 30 years the fermentors and to a large extent recovery have undergone only minor technical changes. In the meantime several factors, including the rise in energy costs, have made it necessary to approach the problem from a different angle.

More soluble culture solutions

We still have only limited knowledge about what constitutes the optimum culture media. Many organisms can be fermented with purely synthetic culture media, the best known example being SCP. It appears however that complex culture media usually ensure a better growth and yield. Nevertheless, every effort is made to use soluble constituents to obtain cultures that are better maintained and easier to process. As we said before, the highest fermentation titre is not necessarily the best titre for the whole process.

Even for the screening of new strains, a process that will become increasingly mechanized, the use of synthetic media can be considered. Then there is the fact that all over the world progress is being made with other types of fermentory. A fermentor produced in 1990 will no doubt have a more specific flow pattern and lower energy consumption than the aerated stirred-tank fermentors of the present day.

Besides the improvements that have been made in classical processing methods, particularly mechanical separation, in which

the Federal Republic of Germany for instance is making encouraging progress, ultrafiltration and methods of chromatography have gained a foothold. In conjunction with this, problems of waste disposal and the recycling of waste products are increasing all the time.

Last but not least, the improvement in our educational standards is significant. There is no point in acquiring know-how and equipment unless there are qualified people available to use qualified techniques that will turn the good processes of today into even better processes of the future.

I was given a vast subject to talk about and I have done my best to give examples that will indicate where our latest developments are taking us. I have raised more questions than I have answered, and therefore hope that they will stimulate further discussion.

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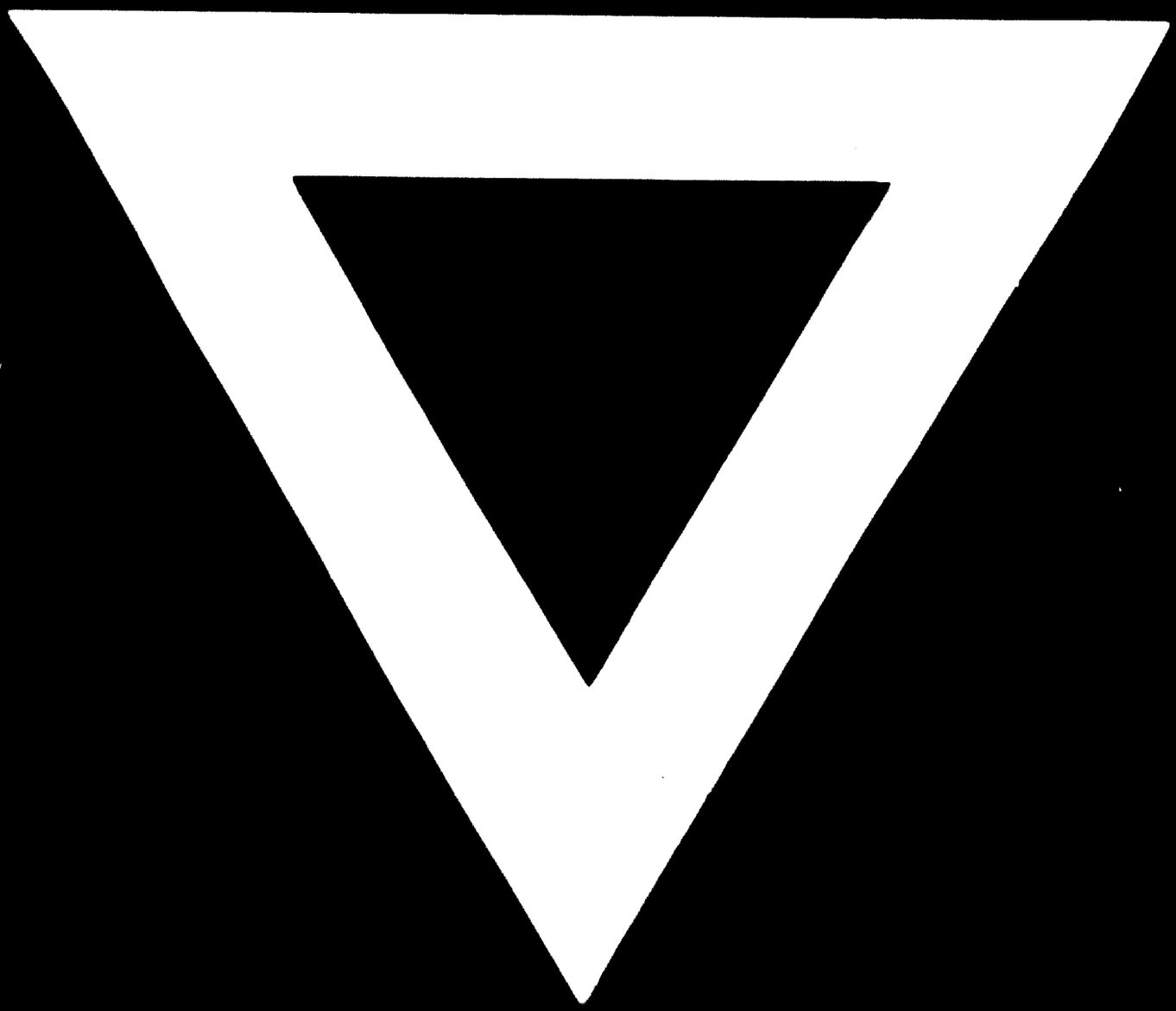

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