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The Production of Industrial Enzymes [and Discussion]

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The production of industrial enzymes

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This paper reviews the essential features and current techniques used on an industrial scale in the preparation of partly purified or 'bulk' enzymes, as opposed to highly purified enzymes for analytical or diagnostic use, which are covered elsewhere in this volume.

Industrial enzymes may be derived from a wide variety of plant, animal or microbial sources, although most production processes rely on the last of these. Microbial enzymes are either extracellular, such as the proteases and carbohydrates, which account for a large proportion of total sales, or intracellular, such as glucose oxidase. Intracellular enzymes usually remain associated with the cell and therefore have to be released, unless the microorganism itself is used as the catalyst. Although specific fermentation procedures adopted by manufacturers vary to a degree, there remain only two principal methods of cultivation, i.e. solid-state and submerged fermentation. Most microbial enzymes are produced by aerobic submerged fermentation, which allows greater control of growth factors than solid-state methods.

The recovery and purification techniques discussed are typical of those used for fermentation enzymes, although they may be applied, generally, to most extraction and refining operations irrespective of whether the enzyme source is of plant, animal or microbial origin. In particular the following processes are covered; cell disruption, precipitation and solid–liquid separation including concentration by ultrafiltration. It is shown how, by using a combination of these techniques at different stages in the recovery process, it is possible to produce a complete range of product specifications.

Introduction

It is hard to imagine that at the beginning of this century enzymes were still being extracted from dogs' faeces and that there was virtually no enzyme industry of a type recognizable to today's biotechnologists. However, even before the industrial revolution began, enzymes were being used (albeit unbeknown to the users) in a variety of food fermentations to produce bread

Table 1. Worldwide distribution and sales of enzymes

enzyme	applications	distribution and sales (%)
Bacillus protease	detergents, brewing	35
amyloglucosidase	starch saccharification, brewing, distilling	14
glucose isomerase	isomerization (starch industry)	14
bacterial amylase	starch liquefaction brewing, distilling	10
pectinase	wine, fruit juice	10
microbial rennet	cheese manufacture	5
fungal amylase, fungal protease	baking, milling	4
others	—	4

alcoholic beverages, vinegar and a host of yoghurt-like drinks. Modern enzyme technology can be traced back to two pioneers working independently on opposite sides of the world. In Japan Takamine developed the idea of using an enzyme mixture for use as a digestive aid, and in 1894 takadiastase was the first microbial enzyme to be produced in a relatively purposeful and sophisticated manner. Meanwhile in Germany, Röhm was developing a process for extracting pancreatic proteases for leather bating as an alternative to the use of the dogs' faeces mentioned above. Röhm in 1913 took out one of the earliest patents on enzymes, surprisingly enough to cover the use of proteases in washing formulations.

Over the years these small beginnings have led to a thriving enzyme industry with an approximate distribution of sales and production as illustrated in table 1. It is the purpose of this paper to review the current methods used to produce enzymes on an industrial scale.

Fermentation techniques

Strain development

Most enzymes produced on an industrial scale rely on microorganisms belonging to the genera *Bacillus* or *Aspergillus*.

The *Bacillus* species, apart from the *B. cereus* group which includes *B. anthracis*, are harmless, well suited to enzyme production, and may be grown in high concentration in fairly simple growth media. All *Bacillus* species form heat-resistant spores that mark the termination of the cell growth phase. Sporulation is therefore undesirable in an enzyme production process; in addition, spores may be able to survive the recovery process and thus infect the final product. Many *Bacillus* species can produce polypeptide antibiotics, which cannot be tolerated where enzymes are to be used in food products. Fortunately strain improvement can enable the preparation of high-yielding mutants that lack the spore-forming property and antibiotic activity. Species of *Aspergillus* may be regarded as the fungal analogue of the *Bacillus* genus in their use for enzyme production. The genus is highly variable and widespread and is divided into a number of groups within which species are relatively close in appearance to one another. *Aspergillus* species are usually haploid in their vegetative phase, which means they are easily mutable. Because the inoculation of fermenters or trays in surface cultivation is dependent on conidial spores, it is important to maintain the spore-forming ability of production strains. This is a problem frequently encountered owing to the fact that strains with highly improved enzyme yields may have a reduced ability to form conidiospores.

Since the vast majority of wild-type microorganisms are incapable of producing commercially acceptable enzyme yields, strain improvement is very important and occupies a central role in large-scale production processes. The selection of high-yielding mutants by industrial geneticists and microbiologists still relies, in the main, on mutagenesis followed by evaluation in shake flask or surface culture.

Because the enzyme industry is highly competitive, reliable estimates of the extent to which different fermentation techniques are used are not easy to establish. Although specific procedures adopted by different manufacturers will vary to a degree, there remain only two principal methods of cultivation, i.e. solid-state and submerged fermentation. The bulk of microbial enzymes are produced in aerobic submerged culture, which allows greater environmental control of growth factors than in solid-state methods. However, solid-state fermentation continues to find use in the production of certain mould enzymes, e.g. proteases and pectinases

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from respective genera such as *Aspergillus* and *Rhizopus*, because for reasons yet unexplained the unique environment enhances formation and high yields of these products.

Solid-state fermentation

According to Cannel & Moo-Young (1980), the correct use of the term 'solid-state fermentation', sometimes misleadingly referred to under the Japanese name of 'Koji' fermentation, refers to the growth of microorganisms on solid materials with moisture existing within the solid matrix in an adsorbed form but without the presence of a free liquid phase. Three well established solid-state fermentation processes are still in use, which include Oriental food preparation, mould ripening of cheese and composting. The fungi represent the most common class of microorganism used because of their ability to tolerate the low amounts of water available as exemplified by their growth on solid materials in Nature, e.g. pieces of wood, stems and leaves.

The Koji fermentation has been used for millennia in the production of oriental foods. Koji is essentially an enzyme preparation obtained by growing a mould, e.g. *Aspergillus oryzae* or *A. soyae*, on steamed rice to produce a 'seed-koji' inoculum, which is then added to the main substrate, e.g. soya beans, for soy sauce production. Because of the laborious and complex nature of the process, various types of machine have been devised to save on labour (Numokawa 1972). The tray system allows aeration either by passing sterilized and moistened air through wire mesh tray bottoms or over the tray surfaces. The rotating drum system accomplishes both mixing and temperature control by rotating the drum while blowing in conditioned air. It is claimed (Hesseltine 1972, 1977*a*, *b*) that Koji incubators have been devised that allow automated inoculation, handling and harvesting under controlled conditions, although there appears to be some disagreement as to the extent of automation used in the industry (Cannel & Moo-Young 1980).

Because the Koji fermentation necessarily produces certain key enzymes such as proteases, which break down proteins, and amylases, which convert starches into sugars, it comes as no surprise to find that this well established process and technology is also used in the manufacture of certain enzymes. Table 2 lists enzymes that are known to be produced commercially via solid-state fermentation. Representative Koji-making apparatus used in Japan for the production of cellulase from *Trichoderma viride* has been given by Toyama (1976).

From an engineering viewpoint, solid-state fermentation offers a number of advantages over submerged fermentation; they may be summarized as follows.

1. Aeration is achieved easily owing to the air spaces existing in the medium; in addition, mixing may be done intermittently rather than continuously.

2. The absence of a free liquid phase and low moisture content per unit mass of substrate gives rise to the additional benefits:

(*a*) For a given productivity the space occupied by fermentation plant is much less than in conventional submerged culture.

(*b*) Liquid effluent treatment requirements, often neglected and potentially costly processes, are expected to be low.

(*c*) Should precipitation be necessary, much less reagent is required.

(*d*) The low moisture content inhibits the growth of most bacteria, making usual sterilization requirements unnecessary.

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3. Very simple media, e.g. steamed cereal, with few inorganic salt additions are employed.

Solid-state fermentation is claimed to be adaptable to either batch or continuous processes (Cannel & Moo-Young 1980), requiring no greater complexity than conventional stirred-tank reactors. There are, however, a number of disadvantages to solid-state methods.

1. The process is limited to moulds that are able to tolerate the low moisture conditions.
2. Metabolic heat production in large-scale operation can be difficult to remove. Moreover, since both oxygen transfer and heat removal are governed by the aeration rate, a compromise between the two effects is often necessary.

TABLE 2. FUNGAL ENZYMES PRODUCED IN SOLID-STATE FERMENTATION

enzyme	source	reference
α-amylase	*Aspergillus oryzae*	Cannel & Moo-Young (1980)
cellulase	*Trichoderma viride*	Toyama (1976)
lactase	*Aspergillus oryzae*	Kiuchi & Tanaka (1975)
pectinase	*Aspergillus niger*	Beckhorn *et al.* (1965)
protease	*Aspergillus oryzae*	Yoshida & Ichishima (1964)

3. Measurement and control of moisture levels, pH, temperature, oxygen and carbon dioxide levels, biomass concentration and product yields are difficult because of the inhomogeneity of the medium.
4. Very little design information for solid-state fermentation plant is available. This situation is aggravated by the fact that most technological advances are written in Japanese.

Submerged fermentations

The equipment used in submerged enzyme fermentation is similar, generally, to that used in the preparation of antibiotics. Deep-culture methods, according to Sakaguchi *et al.* (1971), may involve volumes in the range 4.5–135 m^3; however, the scale of operation at our company for the production of mould enzymes extends up to 220 m^3 working volume. The fermenters are usually stainless steel pressure vessels, oftrn designed to withstand extremes ranging from full vacuum to 2 bar (200 kPa) gauge steam pressures, equipped with single or multiple Rushton turbine-type agitators, aeration devices such as simple pipes or ring spargers for introducing sterile air, and jackets or coils for temperature control. The vessels are cleaned with caustic alkali, detergent solutions or water, and sterilized with live steam between batches.

The fermentation media can be sterilized in batches in the fermenter or, more economically, on a continuous basis by using plate (and frame) or spiral plate heat exchangers, with energy conservation sections, such as those available from The A.P.V. Co. Ltd, Crawley, West Sussex, or Alfa-Laval Co. Ltd, Brentford, Middlesex. The sterile medium in the fermenter is cooled to the desired operating temperature, typically 25–40 °C by passing aseptic air through the vessel. Air depth-filters employed comprise glass microfibre pad systems (electrically sterilized) or hollow cartridge hydrophobic systems: borosilicate glass bonded with silicone resin (steam sterilized). The pH of the medium may be adjusted if required and a sterility check made by standard bacteriological techniques. The medium is inoculated with either a spore suspension or a vegetative inoculum. Suitable agitation and aeration are supplied to the growing culture, and the fermentation exotherm (combined agitation, aeration and metabolic heat outputs) removed, if necessary, by circulating water over the cooling surfaces of the fermenter. Con-

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tinuous monitoring of hydraulic agitator power draw, oxygen and carbon dioxide levels in the exit gas stream, as well as control of temperature, pH and dissolved oxygen concentration may be exercised. Culture samples are taken periodically throughout the course of the fermentation for testing and analysis of certain medium constituents, cell dry mass and enzyme concentration. The fermentation may extend from 20 to 160 h, depending on the enzyme system being produced.

The formulation of balanced fermentation media is critical to good growth and enzyme yields. The use of chemically defined media tends to be expensive and usually leads to slow growth and low yields. Thus most media for large-scale production are made up of a number of complex undefined materials. Mixtures of carbohydrates (molasses, corn syrup, starch, starch hydrolysates, milled cereal products, lactose, etc.), nitrogenous materials (ammonium salts, corn steep liquor, distillers' solubles, yeast extract, gelatin, casein, etc.) and minerals are often used. The choice of carbohydrate is very important because the synthesis of many enzymes is governed by catabolite repression. For example, glucose is known to repress the formation of α-amylase in *Aspergillus oryzae* (Nikolov *et al.* 1970) and cellulase production by *Trichoderma virida* (Mandels 1975). To keep carbohydrate concentrations low during fermentation, it is therefore often enecssary to add the carbohydrate during the fermentation, or to use a slowly decomposable type such as starch or lactose. For high yields of certain enzymes such as lactase and pectinase, inducers are normally required in the form of tne enzyme substrate.

Optimization of medium composition, to maintain yield and productivity, takes place over a number of years, especially in the face of changing raw material specifications. However, the composition of the medium is based not only on requirements of the fermentation but also on the subsequent purification steps. Special attention is often paid to medium formulation such that final fermentation broth is low in viscosity, contains easily separable cell mass, minimal free residual carbohydrate and low trace metal concentrations, which might otherwise affect the final product specification.

Very little published information exists about the effect of inoculum size on the fermentation, although it usually ranges from 0.1 to 10% by volume. The morphology of filamentous microorganisms is important because this directly influences the power consumed by turbine-type agitator systems. As expected, the dissolved oxygen concentration has a very marked effect on growth and enzyme synthesis. All enzyme fermentations are carried out aerobically but there are cases where levels of oxygen limitation may be advantageous, for example-amyloglucosidase production in *A. niger* starts after th dissolved oxygen level has dropped to almost zero (Aunstrup 1977).

Straightforward batch or fed-batch processes are still most widely used for large-scale enzyme production, although certain enzymes, e.g. glucose isomerase from *Bacillus coagulans* (Diers 1976), are known to be produced by continuous culture. The production of proteases in continuous or semi-continuous culture is unknown in commercial practice, despite the fact that several authors have shown the feasibility of continuous high productivity for long periods of time (Jensen 1972). Fed-batch processes are reported to be used (Aunstrup 1980) to keep the concentration of carbohydrates as low as possible, and owing to economy of operation it is more of an attractive proposition than the relatively inefficient medium utilization during continuous fermentation.

Strict maintenance of asepsis is necessary if high enzyme yields are to be obtained and to ensure that potentially harmful microorganisms are excluded from the fermentation. Aseptic

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conditions are particularly difficult to maintain in many enzyme fermentations conducted at pH levels close to 7. This means that equipment used must be designed and constructed in accordance with strict cardinal rules described elsewhere, e.g. by Aiba *et al.* (1973).

General recovery schemes

Improvements in industrial enzyme recovery technology tend to be a secondary consideration compared with the traditionally more glamorous role of fermentation research and development. This situation is due, in part, to the often dramatic reduction in overall yield, which follows as a direct consequence of the number of steps involved in the recovery process. A fact of life, sadly not appreciated by many, is that simple manipulative functions in the laboratory e.g. simple Büchner-type vacuum filtration, represent discrete unit operations at plantscale requiring inevitable increases in overall processing time and, owing to the particular operation and labile nature of most enzymes, a significant reduction in final recovery yield. To underline this point, if each step in a multistage extraction process achieves an apparently credible 90% yield, simple arithmetic indicates that the maximum attainable output will decrease in direct proportion to the product of the stage efficiencies.

The term 'industrial' enzyme covers a range of products, some of which are little more than concentrated cell-free fermentation liquor, through varying degrees of purity, such as the 'bulk' enzymes, to highly purified enzyme products, as used in diagnostic reagents. Generally it may be assumed that the higher the product specification then the more numerous and sophisticated are the stages involved in its preparation. To achieve maximum yield and minimize production costs, therefore, the recovery process must be capable of attaining the desired specification by the most direct route.

Despite the fundamental differences in origin between enzymes derived from plant, animal and microbial sources, many of the subsequent downstream extraction and recovery operations are similar. In view of this, and because the majority of enzymes produced on a commercial scale are derived from microorganisms, the techniques that will be discussed here are typical of those used for fermentation enzymes. In addition, although the extraction of intracellular enzymes necessitates cell disruption and involves generally smaller volumes than for the recovery of extracellular enzymes, many of the unit operations are common to both processes. It is therefore convenient to concentrate on these similarities and discuss the various unit operations e.g. solid–liquid separation, concentration and precipitation, to avoid the duplication that would otherwise occur if the isolation of intracellular and extracellular enzymes were treated separately. However, before we give a detailed description of individual operations it is instructive to examine overall enzyme production processes.

The enzyme recovery process is considered to begin once the fermentation has achieved peak yield. At this point the enzyme-containing culture (a complex mixture of microorganisms, proteins, carbohydrates, dissolved salts, etc.), is cooled to 5 °C by using a counter-current heat-exchanger to stabilize the product and inhibit further microbial growth. Many of the subsequent recovery steps are also carried out at this temperature to minimize activity loss and risk of contamination.

A schematic diagram showing general methods used commercially in the preparation of so-called partly purified, refined or bulk enzymes is shown in figure 1. As indicated, the initial stage in any enzyme recovery process involves cell harvesting by using either filtration or centrifugation. The solids are often of a colloidal nature and difficult to remove, in which case

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polyelectrolytic flocculating agents may be added. For extracellular enzymes, such as the bacterial anylases and proteases, filtration with plate-and-frame presses tends to be employed. It may be also necessary to add a filtration aid, e.g. diatomaceous earth, before filtration. Filamentous fungal microorganisms may be separated with reasonable ease by using rotary vacuum filtration and string discharge. For intracellular enzymes high-speed stacked-disc centrifugation is the preferred route (except for mycelial cultures) because this avoids the use

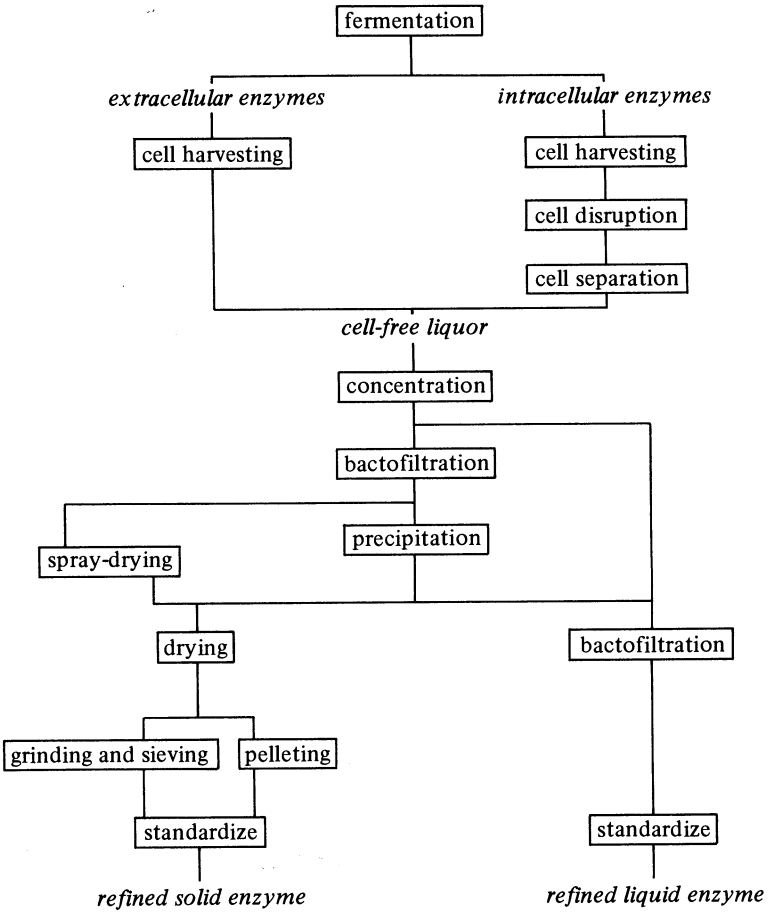

FIGURE 1. General scheme for the production of industrial 'bulk' enzymes.

of flocculating agents and filtration aid, which may otherwise interfere with the subsequent cell disruption process. After this stage a second solid–liquid separation is required to remove cell debris and produce a clarified liquor for subsequent enzyme recovery.

As shown in figure 1, the next step is to concentrate the enzyme by ultrafiltration or evaporation. Concentration is necessary to reduce process volumes. This is particularly necessary for for extracellular enzymes where the product exists, essentially, in the original fermentation volume. The concentrated enzyme solution may at this stage contain varying amounts of bacteria or other microorganisms, which may be removed by bactofiltration. In addition this removes the production strain from the product and therefore helps safeguard the manufacturer's process. Bactofiltration is achieved by depth filtration in which most bacteria are trapped within the fibre matrix of cellulose filter sheets. If a high degree of sterility is required,

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depth filtration may be followed by membrane filtration, which is based on exclusion of bacteria owing to the pore size of the membrane, typically 0.2 μm. When a fair degree of purity is desired in the final product, simple or multiple precipitation processes may be used. The various methods used by industry, i.e. salt and solvent precipitation, will be discussed later. Crude liquid enzyme preparations, which are relatively inexpensive to make and easy to use in most applications, are produced by mixing the bactofiltrate with stabilizers and preservatives, and adjusting the activity to the specified value. Stabilizers are incorporated in the final product to increase the self-life of the enzyme preparation. Agents commonly used are salts, proteins, starch hydrolysates, sugars and sugar alcohols.

Should a solid enzyme preparation be required, the simplest method is to spray-dry the clear bactofiltered concentrate. For good results, however, the concentration of low molecular mass compounds, which tend to make the product sticky, must be kept to a minimum. Therefore concentrates produced via ultrafiltration are better suited to spray-drying than those produced by evaporation. The preparations from precipitation or spray-drying are then dried in vacuum ovens or fluidized bed driers to the required moisture levels. The simplest method of preparing a commercial product is to grind the enzyme preparation to a fine powder, sieve and standardize the activity by the addition of suitable diluents. For many applications this technique is satisfactory but suffers from the disadvantage that dust formation during the handling will be high. Since exposure to enzyme dusts may cause allergic reactions it is necessary to make the enzyme preparation in a way that minimizes the dust-forming ability. A method of doing this is to mix the enzyme with an inert filler (e.g. salt), a binder (e.g. carboxymethylcellulose), and water to make a paste. The paste is then extruded and shaped into spheres. After drying, the spheres are coated with a layer of wax. In this way it is possible to obtain a uniform particle size, e.g. 0.5–2.0 mm diameter, with minimum dust formation.

Solid–liquid separation

Procedures such as harvesting cells from fermentation broth, separating crude enzyme-containing liquors from cell debris after cell disruption and collecting precipitates represent an important unit operation in large-scale enzyme production. Although the equipment used is similar to that used in the chemical industry there are a number of factors requiring special attention, e.g. enzymes may be liable to shear inactivation (Charm & Wong 1981) under adverse physical and chemical conditions, careful hygienic operation is often required to avoid growth of contaminant microorganisms, and it is necessary to prevent the creation of potential allergy-inducing substances, as with protein-rich materials.

Centrifugation

Centrifugation sedimentation techniques for separating biologically active materials have reached a high standard in the laboratory, but these methods are often difficult to realize in a production environment. The reasons for the poor performance of these devices at plant scale becomes apparent if one considers the following expression governing the throughput, Q, for complete particle removal in a cylindrical bowl centrifuge:

$$Q = \left(\frac{d^2 \Delta\rho\, g}{18\mu}\right)\left(\frac{\omega^2 r\, V}{Sg}\right),$$

where d is the partial diameter, $\Delta\rho$ the density difference between the particles and fluid, g the acceleration due to earth's gravity, μ the dynamic viscosity, ω the angular velocity, r the rotation radius, V the liquid volume in the centrifuge, and S the thickness of the liquid layer in the centrifuge. The right-hand side of the expression comprises two parts. The first corresponds to the terminal velocity and is defined by the physical properties of the fluid and particles. The second part deals with the area-equivalent of a gravity settling tank of sedimentation capacity equal to, and dictated by, the design of the centrifuge. Efficient operation is favoured by a large density difference between particles and fluid, and a low liquid viscosity. Biological particles, however, especially broken cell components, tend to be of very small size and exhibit small density difference between themselves and the liquid phase, which in addition-may be highly viscous. These biological characteristics therefore tend to make the solid–liquid separation process slow and inefficient. These disadvantages are overcome in the laboratory by employing low-capacity centrifuges with high angular velocities. On an industrial scale, capacity cannot be greatly enhanced by radius increase alone, since mechanical stresses increase with r^2, thereby jeopardizing safe limits.

With the foregoing discussion in mind, industry hasd eveloped a range of centrifuges applicable to the removal of microbial cells, cell debris and precipitates. These include the tubular bowl, multichamber, disc-stack and basket centrifuges.

The tubular bowl centrifuge represents a versatile and most useful device and is often used during enzyme purification. This comprises a cylindrical bowl with a fairly small capacity, typically up to 4 kg net mass, suspended by a flexible shaft from an overhead drive. In the Sharples no. 6 machine (Pennwalt Ltd, Surrey) liquid is pumped upwards through the bottom and solids collect on the bowl wall, which rotates at up to 15000 rev. min^{-1} generating in excess of 13000 g. This type of centrifuge is also available as a totally enclosed, pressurized unit suitable for liquids producing hazardous inflammable vapours or aerosols. A smaller laboratory-sized turbine-driven unit, the no. 1A, suitable for higher resolution techniques, operates up to 50000 rev. min^{-1}, with a maximum centrifugal force of 62000 g.

Disc-stack centrifuges are available from Alfa-Laval Co. Ltd, Brentford, Middlesex, and Westfalia, Separator Co. Ltd, Wolverton, Bucks. The original disc-stack centrifuge is the solids-retaining machine and is used for feeds containing low solids content of up to 1% by volume. Liquid enters the centrifuge down a central pipe and passes around the bottom of a distributor and into the disc stack. The denser solids are thrown radially outwards to the underside of the discs, then travel down along the disc surface until they are thrown off to be retained in the periphery of the bowl. The machine must, however, be shut down at intervals to remove solids manually. A variant of the latter is the cylinder bowl (multi-chamber) centrifuge in which solids collect on the walls of a series of concentric cylinders between which liquid is forced. For industrial-scale, continuous operation, centrifuges of the solids-ejecting type are used when a solids content of 1% up to 6–10% in the feed would otherwise require the solids-retaining type of machine to be emptied too frequently to be economic, or when manual handling of the solids is to be avoided. The separated solids are discharged intermittently, either manually or automatically during separation. Depending on the time for which discharge is allowed either total discharge (of the entire bowl contents) or partial discharge (of the solids only) may be obtained. A disadvantage with the smaller machines is that partial discharge is sometimes difficult, since it is often impossible to open and shut the bowl discharge system quickly enough without sustaining liquid, and hence possible product, loss. Scaled-up larger models of the same machine,

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however, allow improved operation and make it possible to discharge only the outermost edge of the accumulated solid in the bowl. Solids-ejecting centrifuges are available with solids-handling capacities of up to 1.5 $m^3 h^{-1}$ at throughputs of up to 60 $m^3 h^{-1}$; nozzle bowl centrifuges that feature continuous solids discharge are available with solids capacities of up to 60 $m^3 h^{-1}$ for feeds containing solids ranging from 6% to 25–30% by volume at throughputs of up to 200 $m^3 h^{-1}$.

Industrial-scale solids-ejecting and nozzle-type disc-stack centrifuges operate, typically, between 6000 and 8500 *g*, measured at the minimum disc stack radius, although it should be understood that, in addition to a high centrifugal force necessary for fine particle removal, these centrifuges are designed to have a short particle-settling path, which remains constant until the bowl is full. Such machines are used extensively for harvesting procedures and for the recovery of cell debris and protein precipitates produced during enzyme isolation. The use of solids-ejecting and nozzle-type separators to remove sticky mycelial cell components during production of tetracyclines has been recorded by Larsson (1974). Large-scale polishing of enzyme solutions and fine particle removal may now be accomplished with the advent of high-efficiency continuous clarifiers as afforded by the Westfalia CSA 160 and Alfa-Laval AX 213. The latter for example, is capable of developing 14 200 *g* while maintaining throughputs of up to 36 $m^3 h^{-1}$.

Basket centrifuges such as those available from Thomas Broadbent Ltd, Huddersfield, are designed to operate at much lower *g* forces than those described above, ranging typically from 500 to 600 *g* and are in essence centrifugal filters. Porous bowl filter-bag liners are often used in conjunction with lift-out discharge facilities. Because of their design, this type of centrifuge, with feeding and washing facilities, can be used to elute enzymes from ion exchange celluloses. This type of machine is also suitable for separating enzyme-containing liquors from cell debris after cell disruption (Richardson *et al.* 1971), although the addition of filtration aid is often necessary to aid centrifugation.

Filtration

According to Rushton & Khoo (1977), the rate equation governing the time taken to filter a volume V of filtrate per unit area A of filter may be written:

$$dV/dt = \Delta P/(R_m + R_c)\mu,$$

where ΔP is the pressure drop across the filter medium and filter cake, R_m the resistance of the filter medium, R_c the resistance of the filter cake and μ the fluid viscosity. R_m is a fixed characteristic of the filter medium; however, R_c, which is proportional to cV/A, where c is the concentration of solids in the original suspension, will increase during filtration. However, in practice the above expression does not take account of the more complex problems that involve blockage or blinding of the filter medium, thereby increasing R_m. The compressibility of biological solids, especially fungal cell debris and protein precipitates, is a major problem and there is often the temptation to assist filtration by increasing ΔP. This, however, leads rapidly to filter cake structure collapse to a more compressed form, thereby resulting in a fall-off in throughput. Because batch filtration of biological solids suffers from phenomena such as filter-cloth blinding, bed compression and variable pore structure, the success of the operation relies to a considerable extent on the skill of the operator. Filtration aids serve to bridge the relatively large pores of the filter medium for the retention of the fine particles and act as surfaces for solids deposition. However, although invaluable for enzyme isolation they have the disadvantage

of rendering microbial cells unfit for use as animal feed supplements, occlude enzyme-containing liquor, create an additional waste disposal problem, add to the cost of the recovery process and may be conveyed to subsequent isolation steps, causing abrasive damage to process equipment.

The industrial equivalent of the laboratory-scale Büchner funnel is the filter press, which operates under positive pressure and is available from manufacturers such as Fletcher Filtration Ltd, Mirfield, West Yorkshire, and Edwards & Jones Ltd, Stoke-on-Trent, Staffordshire. The filter press consists essentially of a series of filtration chambers, made up in the form of the traditional plate-and-frame arrangement or from recessed-chamber type plates, into which the slurry is fed. The filtration medium covers the surface of each chamber, enabling solid particles to remain within the filter press and filtrate to drain through the filter cloth onto a corrugated filter plate surface and via drainage ports to a filtrate collection header pipe. The pumping pressure is gradually increased as solids build up to compensate for loss of filtration rate. Removal of solids requires parting of the plates, after which the cloths must be cleaned thorougly. Fully mechanized presses are available, providing filtration areas of up to 1000 m^2, featuring electrically operated opening and closing gear, *in situ* filter cake washing and 'squeezing' (dewatering), and automatic cake discharge and cloth washing facilities. Steel reinforced rubber-moulded or polypropylene filter plates are now available, providing corrison and maintenance-free operation.

The use of filter presses with the aid of flocculents for the recovery of bacterial enzymes such as alkaline proteases for use in the detergent industry or for amylase production is now commonplace. Filter presses are eminently suitable for removing fungal cell or cell fractions from enzyme broths. The isolation of an extracellular pectinase complex from a 10 m^3 culture of *Aspergillus ochraceus*, for example, has been described by Nyiri (1969).

The rotary vacuum filter (from, for example, Stockdale Engineering Ltd, Macclesfield, Cheshire) consists essentially of a cyclindrical drum, the lower portion of which rotates through a trough filled with the biological suspension to be filtered. The cylindrical shell of the drum comprises a number of shallow compartments covered with the filter medium. As the drum passes through the trough, vacuum is applied to the lower compartments, into which filtrate passes to be discharged subsequently by a pump. The upper segments of the drum can be devoted to washing and dewatering of the filter cake under vacuum and also to applying air pressure, i.e. 'blow-back', as an adjunct to cake removal. String and knife-cake discharge rotary vacuum filters are employed in fermentation and recovery process steps, although other discharge methods are common in the chemical industry. String discharge filters give continuous and steady cake removal, the endless strings, spaced at 1–2 cm intervals, passing around the drum and an external roller, acting as open conveyers through which filtration takes place and on which filter cake is built up. No blow-back is needed to assist cake removal, and therefore no wire winding is required to secure the filter cloth. Mechanical wear is minimal; a wide choice of cloth is therefore available. This method of harvesting is used widely and successfully for fibrous materials such as mycelial suspensions. In knife discharge, filter cake is dislodged by its own weight or with the assistance of compressed air. Although particularly suitable for friable filter cakes this method is more usually used in conjunction with a precoating of filtration aid, especially for the recovery of yeast (Gray *et al.* 1973) and fungal cell debris. The method relies upon the knife edge being advanced automatically at less than 20 $\mu m\ min^{-1}$ to cut away the cell matter and a very thin layer of precoating thereby presenting a clean surface for further

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filtration. Okabe & Aiba (1974) have constructed algorithms to simulate and optimize rotary precoat filtration, using actinomycete broth to assess the efficiency of their model.

Cell disruption

Cell disruption refers to the liberation of enzymes from cells or cellular components by mechanical or non-mechanical breakage of the cell wall or membrane. Many techniques exist for cell disruption (Wimpenny 1967), especially for laboratory-scale work, although not all are

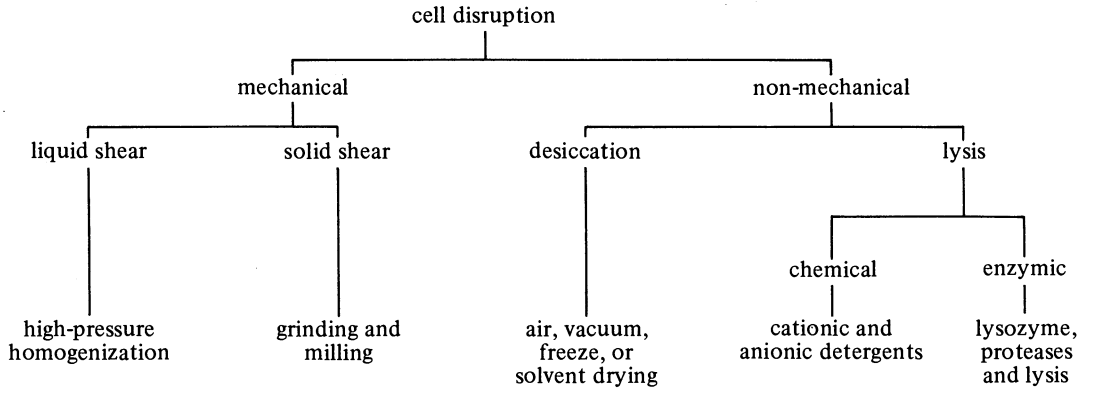

Figure 2. Industrial methods of cell disruption.

suitable or applicable to large-scale processing. This may be due in part to some restriction in available technology or because of the protracted time required for a given method. A classification of methods used on an industrial scale, i.e. processes involving at least kilogram quantities of cell paste, is given in figure 2. The criteria often applied in deciding which method is preferred are hard to generalize, but may be summarized as follows:

(i) susceptibility of the cell to the given method;
(ii) stability of the enzyme during cell disruption;
(iii) ease of enzyme recovery from cell debris;
(iv) rate of enzyme release;
(v) simplicity and cost of technique.

Disruption by mechanical methods

Two principal mechanical methods appear to be employed on an industrial scale for all disruption: high-pressure homogenization, and grinding or milling with abrasives.

Liquid shear disruption with high-pressure homogenization

The APV–Gaulin homogenizer has been used by Hetherington *et al.* (1971) to investigate disruption of *Saccharomyces cerevisiae* cells through a restricted orifice at pressures up to 55 MPa. During disruption three different mechanisms appear to be in action. Intact cells are forced into a homogenizing valve, whereupon they collide with the face of the valve, are deflected at right angles through a small annular gap and undergo high velocity shear. After leaving the annulus the fragmented particles are subjected to cavitational stresses under sudden decompression and accelerate before striking the surface of an impact-ring. To obtain high enzyme

recovery yields it is usually necessary to operate a multipass batch or continuous recycling system, as described by Charm & Matteo (1971); however, the exact method used is influenced strongly by the culture in question. The use of high-pressure homogenizers has been reported for disruption of *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger* (Wang *et al.* 1979). In our experience, especially with filamentous fungi, problems may be encountered due to blockage of the homogenizing valve on the feed side, thereby restricting the effective concentration that can be handled. In addition, insufficiently high operating pressures were found to be available for single-pass disruption of certain *Aspergillus* species, although multiple passes at lower pressures gave generally satisfactory results. The mechanism causing cell disruption in APV-Gaulin type homogenizers is said to be either the size of the pressure drop and rate with which it occurs (Brookman 1974) or oscillation of the cell liquid resulting from turbulent eddies (Doulah *et al.* 1975). However, according to Engler & Robinson (1981), the predominant mechanism causing cell disintegration in high-pressure flow devices appeared to be high-velocity impingement of the suspended cells against stationary surfaces.

Solid shear disruption with grinding and milling techniques

Another method of enzyme release used in an industrial environment involves mechanical disintegration, where the cell suspension is mixed with glass or ceramic beads and stirred intensely. Current grinding and dispersion technology was developed from the pigment and dyestuff industry. Horizontal rotating drums, loaded with material to be ground and the ball charge, led to the introduction of vertical agitator ball mills, which were further developed to include continuous recycling of ground material through the mill. Subsequent improvements include the vertical annular chamber mill, where the grinding charge is agitated by a series of pins, located on a cooled hollow agitator shaft, and positioned so as to rotate between fixed counter pins on the chamber wall.

Protein release from *Saccharomyces cerevisiae* has been examined in detail by Currie *et al.* (1972) with a Netzsch–Molinex agitator mill. The variable-speed agitator comprised sets of circular discs mounted eccentrically on a shaft in helical array. Increased disruption efficiency was obtained at higher speeds, greater loading of grinding elements, and lower rates of cell recycling through the mill. Disintegration of *S. cerevisiae* cells has also been examined more recently by Rehacek & Schaefer (1977). They employed a 20 l continuous high-speed horizontal bead mill of more novel design fitted with disc agitators, alternating radially and obliquely to the shaft, and achieved a 90% cell disruption for a residence time of $2\frac{1}{2}$ min. Temperature control of the vessel was effected by circulating coolant through the agitator shaft and vessel casing. The maximum flow rate through the machine was 0.4 $m^3 h^{-1}$. Netzsch horizontal mills are available with throughputs of up to 2 $m^3 h^{-1}$ by using the agitator system described above. Alternatively, low-speed cooled-pin type or uncoiled eccentric-ring agitators may be used. Another type of continuous-flow disruptor, the Dyno-Mill, has been examined by various workers (Marffy & Kula 1974; Mogren *et al.* 1974; Deters *et al.* 1976; Limon-Lason *et al.* 1979). According to Limon-Lason *et al.* (1979), disruption kinetics for *S. cerevisiae* followed a first-order rate expression, being a function of impeller tip speed and yeast concentration. The high agitator speeds (typically 1500–2250 rev. min^{-1}) gave high disruption rates, although concomitant heat production rates required very effective temperature control.

In an ideal situation the most efficient mechanical cell disruption method would be one that

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liberated the enzyme by puncturing the cell walls, leaving relatively intact 'ghost' cells that could be recovered with a reasonable degree of ease. In this connection the fine particle size produced during grinding often renders ground cell suspensions far more difficult to separate than homogenized-type material. High pressure homogenization therefore tends to find wide application, helped further by the availability of large capacity machines enabling continuous processing of up to 53 $m^3 h^{-1}$.

Disruption by non-mechanical methods

Microorganisms may be disintegrated efficiently to release cellular constituents on a commercial scale by a wide range of mechanical methods. However, these often incur high capital expenditure and operating costs. Several non-mechanical cell disruption methods, developed in the laboratory, have become the basis for patented procedures of large-scale potential, often sharing the common feature that they may be applied to the extraction of a particular enzyme from a given organism.

Physical lysis

According to Cedar & Schwartz (1967) a sudden increase in cell osmotic pressure after a rapid change in salt concentration in the surrounding medium constitutes osmotic shock. This method is effective for animal cell disruption, but most microorgnisms, with the exception of Gram-negative bacteria, are affected much less. On an industrial scale the disadvantages tend to be the large amounts of cold water (typically 4 °C) required, a reduction in impact of the effect brough about by occluded cell-bound salts and the time taken for cell resuspension. Freezing and thawing, a slow method of cell disruption, produces effects on the cell similar to those observed during osmotic shock and may result in loss of enzyme activity (Cowman & Speck 1969).

Chemical and enzymic lysis

Chemical lysis-promoting agents have been used to extract enzymes from fungal cells. Alkali treatment provides a cheap method of cell lysis that is readily adaptable for use on the large scale, although the success of the treatment depends on the stability of the particular enzyme to high pH. Enzymes may be extracted from microorganisms by the use of ionic detergents, e.g. sodium dodecyl sulphate, or non-ionic detergents, e.g. Triton X-100. The former tend to be more reactive than the latter, giving rise to possible protein denaturation. In addition, subsequent salt precipitation procedures are made difficult by the presence of detergents, necessitating their removal by ion-exchange chromatography (Tzagoloff & Penefski 1971), which introduces a further processing step.

A wide range of enzymes capable of promoting cell lysis are known that are of potential commercial interest due to their gentle and selective nature. However, their current cost and low possibility of reuse may prohibit their application on the industrial scale. A number of reports have shown that various microorganisms accumulate enzymes exhibiting lytic activities against yeast cell walls (Knorr *et al.* 1979; Asengo *et al.* 1981; Asenjo & Dunnill 1981). In this connection, Hartmeier (1977) has given details of a patented process for releasing invertase from spray-dried baker's yeast, with the use of a mixture of cellulase, papain and mould lipase. The use of relatively inexpensive, lysozyme-rich, dry egg-white is effective only for a narrow range of microorganisms (Wang *et al.* 1979).

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Concentration

A variety of concentration procedures have been devised to attain the primary objective of water removal. Some of the methods also allow simultaneous partial purification of the enzyme in question.

Ultrafiltration and reverse osmosis

Membrane separation is a developing technology in the enzyme production industry, enabling enzyme concentration to occur at more ambient temperatures and thus avoiding protein degradation, which often accompanies the phase change involved in more traditional methods such as evaporation or freeze drying.

Reverse osmosis refers to the flow of permeate, often pure water, through a semi-permeable membrane by the application of hydraulic pressures, in excess of the natural osmotic pressure of the solution, at up to 8 MPa. Although used extensively in the dairy industry, it is seldom used in enzyme production. An extension of reverse osmosis is ultrafiltration. This uses lower pressures, up to 1 MPa, and more open membranes that allow the passage of some dissolved salts and small molecules with the permeate. The potential applications of ultrafiltration in enzyme purification are concentration, removal of salts and low molecular mass species, and enzyme fractionation on the basis of molecular mass. However, the practical use of ultrafiltration on an industrial scale centres on enzyme concentration with concomitant desalting. Enzyme fractionation is rarely attempted on an industrial scale owing to problems encountered with concentration polarization. This refers to an increase in the concentration of solute adjacent to the surface of the membrane, the effects of which become more pronounced as retained solute concentration increases, as hydraulic driving force is raised, and as the temperature is reduced. The phenomenon may be controlled by increasing the transport of solute back into the bulk of the solution by inducing turbulent flow in chambers of large cross section or by using high-velocity laminar flow in thin-channel systems (Van Altena 1973; Melling 1974). Butterworth & Wang (1972) found that separation of solutes is not necessarily ensured if the porosity of the membrane is chosen on the basis of differences in molecular mass alone: the efficiency of ultrafiltration is, they concluded, also affected by the presence of finely divided particles, solute concentration, pH and ionic strength.

Ultrafiltration membranes comprise very thin membranes, with defined porosity, bonded to coarser materials, 125–250 μm thick, which impart mechanical strength to resist the hydraulic forces. The thin active membrane should ideally permit high permeate flux rates with a sharply defined molecular mass cut-off. Commercial units are available in modular form, typically skid-mounted, which offer ultrafiltration areas of between 27 and 62 m^2, depending on the manufacturer. Various replaceable-type membrane cartridge configurations are used, such as the fibre system (Romicon Inc., Massachusetts, U.S.A.), the parallel-plate system (Dorr-Oliver Co. Ltd, Croydon, Surrey), the tubular system (Paterson Candy International, Whitchurch, Hampshire) and the plate-and-frame system (De Danske Sukkerfabrikker, Nakskov, Denmark).

A problem encountered frequently when using ultrafiltration is ensuring that proper cleaning and maintenance routines are followed meticulously. Insufficient attention to cleaning may result in poor flux rates, cross-contamination with other enzyme products or the introduction of microbial infection problems. Poor maintenance through faults such as leaking pump seals

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can result in expensive and disastrous loss of enzyme product. In summary, the best results in using this technique follow as a consequence of due consideration to and supervision of every aspect of the entire unit operation.

Evaporation

Various convential rotary, shell-and-tube, plate falling or climbing film evaporators may be used for concentration and final drying of enzyme products. Until the mid-1960s low-temperature vacuum evaporation was essentially the only method in common use for concentrating bulk enzyme solutions. Since then, however, many of these hitherto traditional evaporative methods have been replaced by techniques such as ultrafiltration. Most evaporative methods in current use are operated so as to allow a short contact time of the process liquor with the heating medium, often at temperatures below 30 °C to minimize losses of activity, through heat.

The Alfa-Laval Centri-Therm ultra-short contact time evaporator is an example of progress in evaporation technology for the concentration of extremely heat-sensitive liquids. The heating surface comprises a stack of hollow conical discs rotating on a common spindle. Process liquid enters through a stationary distributor pipe and is sprayed onto the undersides of the rotating cones, where centrifugal force spreads it over the surface in a thin layer less than 0.1 mm thick. The liquid is in contact with the steam-heated surface for less than a second and results in instant boiling; the vapour escapes and rises through the central column into the surrounding shell, from which it is drawn off to a condenser.

Precipitation

Precipitation may be effected by the addition of inorganic salts, organic solvents or high molecular mass polymers. Differential precipitation may be used so that the desired enzyme remains in solution or is collected in the precipitate, thereby removing much contaminating protein.

The use of neutral salts, such as ammonium or sodium sulphate, to 'salt-out' proteins offers one of the oldest and most widely used methods of enzyme recovery. Ammonium sulphate is used commonly because of its cheapness, high solubility, lack of toxicity to most enzymes and stabilizing effect on many enzymes, although it does present a disposal problem, being highly corrosive to metals and concrete. The use of sodium sulphate does not have these disadvantages, but requires temperatures of between 35 and 40 °C to attain adequate solubility. The theory of enzyme concentration by salting-out has been discussed by Dixon & Webb (1961) and Foster *et al.* (1971). Foster *et al.* (1976) have shown that the precipitation phenomenon differs depending on whether a batchwise or a continuous process is employed.

The addition of organic solvents to aqueous solutions of proteins enabling them to react more closely with each other, leading to complexing of the oppositely charged molecules and subsequent precipitation, is thought to be a consequence of a reduction in the dielectric constant of the medium (Green & Hughes 1955). A serious drawback is that enzyme denaturation may occur at temperatures above 4 °C, owing to the protein molecules refolding into an inactive form. Various alcohols, particularly methanol, ethanol and isopropanol, are used on an industrial scale and some precipitation processes use acetone or diethyl ether. Disadvantages, such as maintaining low tempratures (often below 0 °C) to avoid adverse enzyme structural changes and the economic necessity for distillation plant to recover expensive solvents, have

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resulted in the employment of more modern techniques. The capital cost of equipment tends to be high because of the mandatory requirement to protect against potential explosion hazards by providing flameproof areas, motors, switchgear and protective systems. Aerosol containment and sparkproofing of centrifuges are also necessary during subsequent solid–liquid separation processes.

Precipitation may also be realized by the use of high molecular mass polymers such as dextrans or polyethylene glycol.

Drying

When selecting an enzyme drying technique the chosen method should ideally represent the least expensive alternative that allows concentration without an accompanying loss in enzyme yield or stability. The general methodology of industrial-scale drying for biological materials has been reviewed by Blakebrough (1969). Typically, the more robust enzymes may be spray-dried or vacuum-dried, and the more delicate ones are freeze-dried.

Freeze-drying is used widely for highly labile enzymes, effecting minimal loss in activity and resulting in open product structures, therefore enabling fast and complete rehydration. The disadvantages of freeze-drying include the high capital and operating costs required. This method has been used by Belyauskaite *et al.* (1980) for recovering extracellular acid proteases from pilot-scale cultures of *Mucor renninus*.

Enzyme feeds for spray-drying often require additives such as inorganic salts as carrier for enzyme stabilization. The method relies on the dispersion of the liquid feed, in the form of a mist of fine droplets, into a stream of hot gas. Moisture is vaporized from the droplets leaving residual particules of dry solid, which are then separated from the gas stream. Fluidized-bed driers tend to be used for final drying at cooler temperatures. Most spray-dried industrial enzymes include detergent proteases (in large quantities), amylases for starch dydrolysis and pectinases for fruit juice clarification. The production of encapsulated enzymes is often required by industrial users to avoid inhalation of enzyme dusts, to produce a free-flowing product and to increase product stability. Encapsulation of wash-active enzymes uses water-soluble waxes with titanium dioxide added to give the final product the same colour as the detergent, and sodium sulphate as stabilizer and diluent to produce the desired activity. An excellent review of spray-drying technology is given by Masters (1979).

As mentioned earlier, vacuum evaporation is often used for concentrating enzyme solutions; in addition it may also be used for final drying operations. A disadvantage of using evaporation, especially in the preparation of liquid formulations of food-grade enzymes containing additives and stabilizers, is the problem sometimes encountered owing to an increase in viscosity, which tends to limit the degree of concentration that may be achieved. The tendency for foaming to occur, causing considerable fouling of equipment, may be reduced by ensuring that enzyme solutions are free from particulate matter and entrained air.

Vacuum drying ovens and fluidized-bed driers such as those manufactured by Calmic Engineering, William Boulton Ltd, Stoke-on-Trent, are used extensively throughout the enzyme industry.

Conclusion

This review has covered the main unit operations generally used in industrial enzyme manufacture. Although not as well publicized as advances in genetic engineering, it is clear that many relevant improvements to production methods are constantly taking place. Equipment

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manufacturers have machines available that are markedly improved compared with those available 10 years ago. The evolutionary changes in production techniques continue and it is hoped that this short paper indicates clearly the current state of the art. It is also hoped that readers are becoming aware of the essential importance of enzyme extraction techniques. This part of enzyme technology is often neglected in favour of fermentation studies, but remains an area where further advances could lead to significant improvements to enzyme production technology.

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Discussion

D. J. Best (*Biotechnology Centre, Cranfield Institute of Technology, U.K.*). I find it rather surprising that in a meeting of this nature no mention has been made of the use of aqueous two-phase partition systems for the bulk production of enzymes. The obvious advantages of such systems are the economics of using cheap, readily available chemicals such as polyethylene glycols, dextrans and inorganic salts, and the minimization of steps involving centrifugation. The purification of formate dehydrogenase by such a procedure (Kroner *et al.*, *J. chem. Technol. Biotechnol.* **32** (1), 130–137 (1982)) demonstrated the drastic reduction in process time required, higher activity yields and a 40-fold reduction in the cost index of the enzyme over standard procedures. Could Dr Lambert give his evaluation of the use of such processes in the large-scale production of commercial grade enzymes?

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P. W. Lambert. Liquid–liquid partition of enzymes in aqueous two-phase systems appears to be a very versatile and efficient method for enzyme recovery. The method may be suited especially to the extraction of intracellular enzymes from liquors containing broken cells and debris, thereby changing the traditional solid–liquid separation process to one involving liquid–liquid separation. However, this requires a two-phase system capable of suspending broken cell components in one phase with partition of the desired enzyme in the opposite phase. Existing chemical engineering skills and commercially available plant may be employed to accomplish partition, which, in addition, can be adapted to continuous processing.

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