Chapter 2
Enzyme Production

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2.1 Enzyme Sources

Enzymes are the catalysts responsible for cell metabolism. Cells from different sources have been, are, and will continue to be the main source of enzymes. Enzymes can be produced from any living organism, either by extracting them from their cells or by recovering them from cell exudates.

Plant tissues and animal organs were the most important sources of enzymes at the onset of enzyme biotechnology; in 1960, about 70% of the enzymes were extracted from plant tissues or exudates and animal organs. Twenty years later the situation had reversed and most industrial enzymes were produced from microbial sources (Lambert and Meers 1983). Nowadays, enzymes from plants and animals, mostly proteases, are still in the market and some of them are of commercial relevance. Catalase (EC 1.11.1.6) from liver (Yildiz et al. 2004), lipase (EC 3.1.1.3), chymotrypsin (EC 3.4.21.1), and trypsin (EC 3.4.21.4) from pancreas (Underkofler et al. 1958) and rennin from calf abomasus are the most relevant animal enzymes, widely used in the food and leather industries (Oberg et al. 1992; Kosikowski and Mistry 1997). Animal derived enzymes represent about 10% of the total enzyme market. Plant derived enzymes, such as papain and bromelain, are still industrially relevant (Balandrin et al. 1985; Tucker and Woods 1995; Uhlig 1998) and they roughly represent 5% of total market. Papain (EC 3.4.22.2), a cysteine protease obtained from the latex of papaya (*Carica papaya; Carica candamarcensis*), is the most prominent plant enzyme marketed today. It is widely used in meat tenderization (Ashie et al. 2002), beer clarification (Monsan et al. 1978), yeast extract production, stain removal (Gebreselassie et al. 2002), and, in highly purified form, in several cosmetic and medical applications (Craig 1975; Pendzhiev 2002). Bromelain, a complex of cysteine proteases extracted from pineapple stems (Rowan et al. 1990) is another relevant plant protease with different applications, mainly in

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A. Illanes (ed.), *Enzyme Biocatalysis*.

the medical area (Taussig and Batkin 1988; Gregory and Kelly 1996) as a wound-healing, anti-inflammatory, digestive-aid and appetite suppressant agent. Plant and animal proteases have been usual components of digestive-aids (Bilton 1984); recently a crude protease extract from kiwifruit has had a considerable market success as a digestive-aid (see http://www.vitalfoods.co.nz/ourproducts/zylax/). Proteases from animals and plants have also been evaluated in the synthesis of peptides in non-conventional media (Riechmann and Kasche 1985; Adlercreutz et al. 1990; Adlercreutz and Clapes 1991; Clapes et al. 1995; Hansler et al. 1995). Some animal enzymes, like urokinase and tissue plasminogen activator are being produced from in-vitro cultivation of established cell lines (Mizrahi 1986; Avgerinos et al. 1990; Zang et al. 1995; Altamirano et al. 2006). Some plant enzymes, like invertase (Pressi et al. 2003), neutral protease (Cimino et al. 2006) and acid phosphatase (Su and Arias 2003) are produced by in-vitro plant cell cultivation. Only high value enzymes for sophisticated applications are potential candidates for in-vitro production with animal and plant cell lines, because of the complexities and high costs of production at large scale (Chu and Robinson 2001; Hood 2002). Glycoenzymes, not easily produced as recombinant proteins in microbial hosts (i.e. glutamine synthetase), are good candidates for in-vitro production with established animal cell lines (Barnes et al. 2000).

Since the early 1960s, microbial enzymes have gradually and progressively replaced those from other sources (Lambert and Meers 1983). In a conservative estimate, they might now represent almost 90% of the total market. This is so because microorganisms are excellent cell systems for enzyme production: they are metabolically vigorous, they are quite versatile and easy to propagate on a large scale by submerged or solid-state fermentation, they are simple to manipulate both environmentally and genetically, their nutritional requirements are simple and their supply is not conditioned by seasonal fluctuations (Blanch and Clark 1997). These attributes have profound technological implications, making the production of microbial enzymes more reliable, simpler and cheaper, independent from side-products and produced irrespectively of the season with reliable supply of raw materials of constant composition, usually free of any harmful substance. Most enzymes are produced by mesophilic organisms; however, the search for enzymes well suited to perform under extreme conditions has prompted an active research on enzymes from extremophiles under the hypothesis that organisms that can thrive in extreme environments have an enzyme machinery adapted to perform under such conditions; enzymes that are stable at high temperatures and those active at low temperatures are now being screened and tested as potential industrial enzymes (Niehaus et al. 1999; Gerday et al. 2000; Vieille and Zeikus 2001; de Miguel Bouzas et al. 2006). Even though such extremophiles are difficult to grow under laboratory conditions, their genes can be cloned into suitable mesophilic hosts as illustrated by the cloning of thermophilic enzyme genes from archaea and bacteria (Bertoldo and Antranikian 2002), and psychrophilic enzyme genes into mesophilic hosts (Feller et al. 1998). In fact, the advances in molecular genetics and genetic engineering in the last decades have made possible to clone and express virtually any gene into a suitable microbial host, so that now enzymes from other microorganisms and also from higher

organisms can be produced in convenient microbial hosts like bacteria, yeasts and fungi. This fact has contributed significantly to increase the number of enzymes that can be produced by microbial fermentation and also to increase the productivity of the fermentation and the quality of the enzyme product. In 1994 it was estimated that about 50% of the industrial enzymes (on a mass basis) were produced from genetically engineered organisms (Hodgson 1994). This proportion must have increased significantly in the last decade because of the advances in recombinant DNA technology and protein engineering and also because of the increasing production of specialty enzymes for the pharmaceutical and fine-chemicals industries (McCoy 2001; Rasor and Voss 2001; Schmid et al. 2001; Thomas et al. 2002).

The evolution of sources for enzyme production has been fast during the last decades. An example that illustrates this evolution is the case of chymosin (EC 3.4.23.4). Chymosin is a very specific aspartic acid protease (the active enzyme is formed from its zymogen, prochymosin, by proteolytic cleavage of the N-terminal 42-residue propeptide) that hydolyzes the Phe_{105}–Met_{106} bond of κ-casein, and, to a lower extent, some other four peptide bonds (Foltmann 1981; Kim et al. 2004); it is the hydrolysis of the 105–106 peptide bond that triggers the clotting of casein in the presence of calcium ions to yield the curd (Budtz 1994). No other enzyme is so specific for milk clotting and chymosin is therefore the best choice for cheese making, because of high yields of curding and proper flavor development in aged cheeses (Duxbury 1989). The traditional source of chymosin is calf rennet, an extract from the inner mucosa of the fourth stomach (abomasus) of suckling calves obtained as a by-product of veal production. Crude rennet contains three chymosin variants: A, B, and C, as well as pepsin which produce a less specific action (Kim et al. 2004). Shortage of rennet as source of chymosin for cheese making became critical (Green 1977) and a continuous biotechnological development has occurred during the last decades to substitute rennet as its source, which is illustrative of the dynamics of knowledge in the field of enzyme production. A first approach to solve the crisis was the partial substitution of chymosin for pepsin; therefore, rennets from varying chymosin: pepsin ratios began to be used despite the sacrifice in quality of the cheeses so produced (Emmons and Binns 1990). In the early 1960s, chymosin activity in microbial sources was being actively pursued (Neelakantan et al. 1999); most successful were the proteases obtained from *Mucor miehei* (Bailey and Siika-aho 1988) and *Mucor pusillus* (Hiramatsu et al. 1989). The so-called microbial rennets were introduced in the market in the 1960s and still represent a significant share of the chymosin market (Duxbury 1989). However, they are not as acceptable as calf chymosin because curd yields are lower and not so pleasant flavor is developed upon maturation. An additional drawback is that microbial rennets are more thermostable and its inactivation at high temperature impairs the texture of the product so that the enzyme requires being chemically modified (van den Berg et al. 1987). With the advent of genetic engineering, the logical option was to express the chymosin gene into a suitable microbial host (Pitcher 1986). Much information accumulated since then on the expression and production of chymosin in microbial hosts (Johnson and Lucey 2006). Initially (pro)chymosin was expressed in *Escherichia coli*; however the enzyme was produced in the form of insoluble inclusion bodies that required

post-transcriptional processing and a complex process of denaturation and renaturation (Emtage et al. 1983; Marston et al. 1984). Better hosts for chymosin expression were then eukaryotic microorganisms, namely fungi and yeasts. Filamentous fungi, such as *Trichoderma* (Harkki et al. 1989) and *Neurospora* (Stuart et al. 1997) have been reported as hosts, but *Aspergilli* have been claimed as the best hosts for chymosin production, since the enzyme can be excreted in higher amounts in active form by using the secretory control regions of some extracellular enzyme coupled to pro-chymosin cDNA (Cullen et al. 1987; Lamsa and Bloebaum 1990; Ward et al. 1990; Tsuchiya et al. 1993). The enzyme from *Aspergillus oryzae* has been in the market for over a decade (Dunn-Coleman et al. 1991). Chymosin has also been successfully synthesized in yeast hosts. Originally, *Saccharomyces cerevisiae* was the organism of choice and the calf chymosin cDNA gene was cloned and expressed, prochymosin being synthesized at a level of 5% of the total yeast cell protein (Dobson et al. 1983). However, the enzyme was poorly excreted in an active form, so that *Kluyveromyces lactis*, a yeast capable of synthesizing and excreting fully active prochymosin, was soon considered as the best option (van den Berg et al. 1990; Morris and Anderson 1991) because of its impressive secretory capacity, its excellent fermentation characteristics at large scale, its food grade status and the availability of both episomal and integrative expression vectors (Swinkels et al. 1993; Bonekamp and Oosterom 1994). The recombinant chymosin from *K. lactis* has been in the market for more than 20 years now (Tramper 1994; van Dijck 1999) with great commercial success and it is probably the most widely used enzyme in cheese-making. Protein engineering has also been applied to improve chymosin performance by conferring the enzyme an increased specificity and better pH profile (Mantafounis and Pitts 1990).

The above example illustrates the evolution of the field of enzyme production. Chymosin is just one case; others exist that are also quite significant, like the production of alkaline proteases for detergents which has made extensive use of the tools of genetic and protein engineering to tailor-make proteases specifically designed to act efficiently under the harsh conditions of laundering (Estell et al. 1985; Aehle et al. 1993; Bryan 2000; Maurer 2004). The rational modification of enzyme structure and function by modern techniques, like directed evolution and site-directed mutagenesis, and the high throughput screening methods for finding novel or improved activities in nature's diversity will certainly impact enzyme biotechnology in the forthcoming years (Ogawa and Shimizu 1999; Panke and Wubbolts 2002).

2.2 Production of Enzymes

Enzymes are now produced for a variety of applications, going from bulk high tonnage processes in which the enzymes are considered as commodities (Hodgson 1994) to small-scale applications for refined uses and research where enzymes are considered specialties (Thomas et al. 2002). Level of production and type of application define the kind of process for its production. Specialty enzymes to be used in medicine and health-care products are usually required in high levels of purity

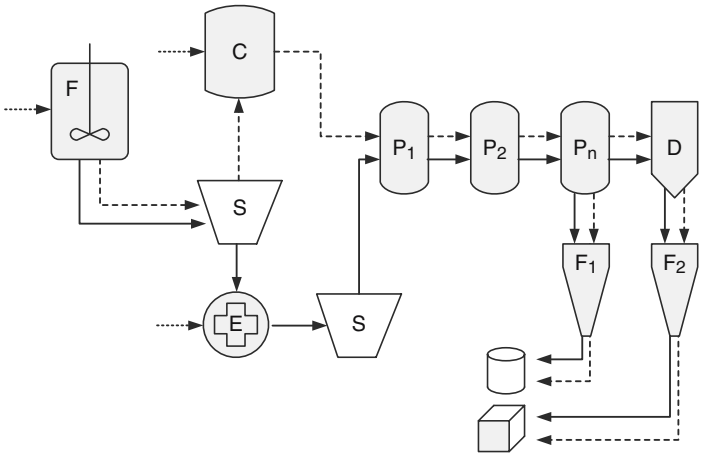

Fig. 2.1 Scheme for the production of enzymes. F: fermentation; S: solid–liquid separation; E: cell extraction; C: concentration; P_i: operations of purification; D: drying; F_i: formulation; - - - - ▶: extracellular enzyme; ——▶: intracellular enzyme; cell tissue or fluid - - - - ▶

and in rather small quantities, while enzymes used in the bulk production of food, feed, fabrics and fuel are usually produced as rather crude preparations in high tonnage (Headon and Walsh 1994). The increasing use of immobilized enzymes for large-scale processes has increased the demand for purer enzyme preparations (Cao 2005). The production process will depend on the source and localization of the enzyme. Enzymes from plant and animal origin will simply be extracted from the corresponding tissues or recovered from the corresponding fluids; microbial enzymes will be produced by fermentation and recovered either from the spent fermentation medium (extracellular enzymes) or from the cell paste after extraction by cell rupture or permeabilization (Aehle 2003). A generalized scheme for enzyme production is presented in Fig. 2.1.

The production process can be divided into four stages:

- Enzyme synthesis: it represents the propagation stage of the producing cells.
- Enzyme recovery: it represents the extraction of the enzyme from the producing cell system and involves solid–liquid separations, cell extraction and/or concentration.
- Enzyme purification: it represents a series of operations after enzyme recovery aiming to remove unwanted contaminants (mainly accompanying proteins).
- Enzyme product formulation: it consists in different operations aimed to give the enzyme product its final presentation; it includes final polishing operations, stabilization and standardization.

2.2.1 Enzyme Synthesis

Plant enzymes from tissues or exudates and enzymes from animal organs or fluids are synthesized as a part of the agricultural processes of plant growth and animal

breeding, so that the subject is outside of the scope of this book. These enzymes are usually byproducts of a main product to whose market is the enzyme production tightly bound.

Microbial enzymes produced by fermentation under controlled conditions constitute now the most relevant option for enzyme synthesis. Microbial strains can produce not only the enzyme proteins coded by their own genetic information, but also those produced by the expression of foreign genes as recombinant proteins. As analyzed before, microorganisms are ideal hosts for enzyme synthesis and only those glycoenzymes which cannot be properly produced in microbial hosts are to be produced in plant (Ma et al. 2003) or animal cell culture (Altamirano et al. 2004; Wurm 2004).

Microbial enzymes are produced mainly by submerged fermentation under tightly controlled environmental conditions (Rose 1980). However, solid-state fermentation (SSF) has also a good potential for the production of enzymes (Raimbault 1998; Pandey et al. 1999), especially those from filamentous organisms that are particularly suited for surface growth. Some enzymes, particularly those related to lignocellulose degradation, are currently being produced by SSF (Dueñas et al. 1995; Pandey et al. 2000; Kanga et al. 2004), but other hydrolases, namely amylases (Bogar et al. 2002) proteases (George et al. 1997) and phytase (Bogar et al. 2003; Vohra and Satyanarayana 2003; Roopesh et al. 2006) are being produced by SSF as well. SSF compares favorably with submerged fermentation in terms of energy requirements, volumetric productivity and product recovery; it represents a good option when production costs should be reduced as is the case of the microbial enrichment of agricultural residues or the production of bulk inexpensive enzymes (Illanes et al. 1992; Pandey et al. 2000). A complete treatment of the subject of solid-state fermentation has been recently published (Mitchell et al. 2006). In most cases, submerged fermentation is the technology of choice for microbial enzyme production (Lambert and Meers 1983; Barredo 2005). Submerged fermentation was vigorously developed after World War II for the industrial production of antibiotics (Mateles 1998) and since then it has represented the most relevant area of bioprocess engineering. Comprehensive reviews on the subject can be found in several textbooks (Aiba et al. 1973; Bailey and Ollis 1977; Wang et al. 1979; Quintero-Ramírez 1981; Blanch and Clark 1997; Nielsen et al. 2003; Acevedo et al. 2004; Bommarius and Riebel 2004). The technology is highly developed and automated and nowadays utilized for the production of most industrial enzymes (El-Mansi et al. 2007).

Submerged fermentation can be conducted in different modes of operation. The most traditional is batch fermentation, in which the bioreactor is filled with medium, inoculated and incubated under controlled conditions to the point in which the product (enzyme) has been synthesized to (or nearly to) its maximum level; then the cells are harvested for enzyme recovery, if intracellular, or else discarded to recover the medium containing the enzyme, if extracellular. Fed-batch fermentation is a variant of the former in which, after certain time of batch cultivation, the bioreactor is fed with nutrients according to a controlled rate profile and up to a final volume and the product is then recovered as above. This mode of cultivation is particularly appealing for the production of enzymes because it allows the control of the metabolic

responses of the producing cells and operation is rather simple (Moon and Parulekar 1991; Acevedo and Gentina 1996; Bojorge et al. 1999; Cereghino et al. 2002). The third mode is continuous culture, in which the medium is fed continuously to the bioreactor and the fermented broth continuously removed at the same rate. Steady state will eventually be obtained, so that the theory of the chemostat is applicable to describe this operation (Smith and Waltman 1995). Continuous culture has been extensively used as a tool to study enzyme regulation (Wiersma and Harder 1978; Egli et al. 1980); however, despite its obvious advantages of higher productivity and better control of operating conditions, the industry has been reluctant to adopt it, mainly because of the hazards of contamination and mutation that can washout the producing strain (Wang et al. 1979; Acevedo et al. 2004).

Some relevant aspects to be considered for developing a fermentation process for enzyme production are now analyzed.

Enzyme localization with respect to the producing microorganism is a key aspect in enzyme production. The enzyme can be properly intracellular, periplasmic or excreted into the medium during fermentation and this will define the downstream operations for its production. In principle, enzyme excretion is an asset as will be analyzed in the forthcoming sections. Most enzymes are intracellular but among extracellular enzymes there are many of technological significance; actually, a significant part of the commodity enzymes are extracellular. There are enzymes that are intracellular in one organism and extracellular in another; for instance, invertase is mainly intracellular in *Saccahromyces* (Illanes and Gorgollón 1986), while a significant portion of it is excreted in *Candida* (Dworschack and Wickerham 1961) and *Streptomyces* (Kaur and Sharma 2005); β-galactosidase is extracellular in molds (Park et al. 1979) while intracellular in yeasts (Mahoney et al. 1974). Intracellular enzymes can be made extracellular by genetic engineering and protein engineering techniques (Becerra et al. 1997).

Specific activity (units of enzyme activity per unit mass of microorganism) is a very relevant parameter for enzyme production by fermentation and much effort has been devoted to increase it by both genetic and environmental manipulations (Parekh et al. 2000). Conventional mutation and selection, genetic engineering, site-directed mutagenesis and directed evolution are powerful genetic tools to obtain high producing microbial strains (White et al. 1984; Arbige and Pitcher 1989; Verdoes et al. 1995; Reetz and Jaeger 1999; Chen 2001; Morley and Kazlauskas 2005; Kaur and Sharma 2006); in some cases, a substantial portion of the total protein synthesized by the organism corresponds to the enzyme. High specific activity not only reduces the cost of fermentation but also the cost of downstream operations. Significant increase in enzyme specific activity can be obtained by adequate environmental manipulations, mainly through medium design and optimization of relevant operation parameters like temperature, pH, agitation and aeration rates (Acevedo and Cooney 1973; Illanes et al. 1994; Barberis and Gentina 1998). Enzyme synthesis is subjected to different types of control by the producing strain (see ahead), so by proper medium design the biological signals that trigger such mechanisms can be put under our control. Most of the present applications of enzymes relate to hydrolases, which are enzymes mainly associated to cell catabolism;

therefore, their synthesis is controlled by induction (Jacob and Monod 1961; Clarke and Brammar 1964) and catabolite repression (Moses and Prevost 1966; Epstein et al. 1975; Shinmyo et al. 1978). Both controls are exerted at the level of transcription; the former requires the presence of the inducer to block the repressor protein and allow the transcription of the structural gene coding the enzyme; the later allows the cell to establish a hierarchy of substrate utilization by repressing the expression of the genes coding the catabolic enzymes of one substrate while the other (supposedly the better) is being consumed. Catabolite repression is related to the substrate consumption rate and not to the substrate structure. In Gram-negative bacteria, control is associated to the level of cyclic AMP that acts as a positive modulator by preventing the blockage by a repressor protein of the structural genes coding the enzymes (Demain 1968; Pastan and Adya 1976; Nandakumar et al. 1999). This is not a universal mechanism: in some enterobacteria cyclic AMP has proven to have no significant effect (Wanner et al. 1978) and in Gram-positive bacteria, yeasts and moulds other signal molecules, like cyclic GMP and polyphosphorylated nucleotides, are involved (Karaolis et al. 2005; Traxler et al. 2006). These control mechanisms have profound influence on enzyme synthesis. Culture medium should have adequate levels of inducer, which can be the substrate, a substrate analogue or the product of the enzyme reaction (Rosenfeld and Feigelson 1969; Kurasawa et al. 1992). Substrate analogues are more potent inducers than the substrate itself because they are not acted upon by the enzymes they induce; in the case of depolymerases, inducers are usually intermediate or end products of hydrolysis, since the substrate as such cannot enter the cell to trigger the mechanism (Illanes and Rossi 1981; Illanes et al. 1988a). Level and time of addition of the inducer affects the level of enzyme synthesized and are operation parameters that should be optimized (Illanes et al. 1994).

Specific growth rate of the producing strain is also a relevant parameter for enzyme production by fermentation. Many enzymes are synthesized as growth-associated metabolites so that cell specific growth rate has a direct impact on enzyme specific rate of synthesis as shown by the non-structured model of Luedeking and Piret (1959):

$$\frac{dp}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X \tag{2.1}$$

$$q_P = \frac{1}{X}\frac{dp}{dt} = \alpha \cdot \mu + \beta \tag{2.2}$$

where α prevails over β for growth associated metabolites. Conditions that maximize the specific cell growth rate are often in compromise with those that maximize the specific rate of enzyme production (Gordillo et al. 1998). In fact, it is usual that pH (Illanes et al. 1988a; McDermid et al. 1988), temperature (Akinrefon 1969; Feller et al. 1994) and the level of dissolved oxygen (García-Garibay et al. 1987; Barberis and Gentina 1998) optimal for growth differ from the corresponding optima for enzyme production. Compromise values are often used, but impressive increases in enzyme productivity have been reported by profiling these variables during cell culture (Mukhopadhyay and Malik 1980; Mukhopadhyay 1981;

Ioniţă et al. 2001). In the case of enzymes whose synthesis is non-growth associated, a two stage culture can be envisaged and the variables optimized for each stage.

Genetic stability and safety of the producing microbial strain are also relevant aspects to be considered in enzyme production. This is particularly so in the case of recombinant enzyme proteins because of structural and segregational instability of the cloning vector (Vehmaanperä and Korhola 1986; Impoolsup et al. 1989; Murooka and Imanaka 1994; Ansorge and Kula 2000). Depending on the use of the enzyme, the producing strain must be considered safe for its application. For instance, enzymes used in the food industry in USA should have the GRAS (generally recognized as safe) status conferred by the Food and Drug Administration (FDA). A list of GRAS enzymes and the corresponding producing strains can be found in http://www.cfsan.fda.gov/∼dms/opa-enzy.html. To obtain a GRAS status for an organism is costly and time-consuming so that sometimes it is a better option to clone the enzyme structural gene into a GRAS host (White et al. 1984; Domínguez et al. 1998).

Morphological and rheological properties of the producing strain are also relevant for enzyme production, especially for the case of mycelial microorganisms (Jayanta et al. 2001; Kim et al. 2003). Viscosity increase and non-Newtonian rheology may reduce oxygen transfer rates and enzyme synthesis is usually related to one particular growth morphology (Olsvik et al. 1993; Bhargava et al. 2005).

2.2.2 Enzyme Recovery

Once the enzyme has been synthesized by the producing organism, it must be recovered, which implies its separation from the cell system (see Fig. 2.1).

2.2.2.1 Solid–Liquid Separation

First downstream operation is the solid–liquid separation of the cells from the spent fermentation medium. Separation can be done conventionally by centrifugation or dead-end filtration. Filtration is more adequate for multicellular organisms of filamentous morphology, like molds and actynomycetes, but filter-aid should be used because of the compressible nature of the cell cake formed. Plate and frame and rotary filters are frequently used. Centrifugation is more adequate for unicellular organisms, like bacteria and yeasts, but usually prior flocculation is required because of the small size of the individual cells. Tubular or disk-type centrifuges are the most used. These operations, though widely employed in bioprocesses, suffer from some drawbacks because microbial cells are small, compressible, and their density is similar to that of the spent medium. Therefore, alternatives to these operations have been developed. Among them, cross-flow microfiltration is outstanding: flow is tangential to the membrane which severely reduces cake formation and membrane clogging, operating costs are lower and the operation is

modular and scalable (Quirk and Woodrow 1983; Le and Atkinson 1985; Nagata et al. 1989). Variables in cross-flow microfiltration for enzyme recovery have been thoroughly analyzed (Kroner et al. 1984). Improvements of the original design of microfilters have been developed and proven useful for enzyme recovery (Lee et al. 1995; Frenander and Jönsson 1996). A comprehensive review on conventional solid–liquid separation operations for biological products has been published by Medronho (2003).

If the enzyme is excreted during cell growth, recovery will proceed from the liquid phase; if the enzymes remains associated or within the cell, recovery will proceed from the solid phase (see Fig. 2.1). Excretion is desirable from a process perspective, so efforts have been made for genetically engineered cells to export otherwise intracellular enzymes (Hatti-Kaul and Mattiasson 2003a).

2.2.2.2 Concentration of Extracellular Enzyme Crude Broths

Excretion of the enzyme is favorable because of cell membrane selectivity that acts as a powerful purification step, since only few proteins are excreted. Therefore, the clarified fermentation broth has a rather high degree of purity that in many cases suffices the requirements for a particular enzyme use so that further purification steps are not required. The main concern here is the low protein concentration. This represents the main drawback of extracellular enzymes, since concentration of the starting material has a profound impact on final production cost (Knight 1989). Even under dense culture conditions, protein concentration rarely exceeds a few grams per liter (Liu et al. 2000) so that the enzyme broth has to be concentrated at least by one order of magnitude prior to further purification steps or formulation.

Many of the operations suitable for enzyme concentration have some potential for protein fractionation (purification). Only those mainly devoted to concentration will be analyzed here; the others will be considered in the section 2.2.3.

The most obvious operation for carrying this out is vacuum evaporation: technology is conventional and has been traditionally applied for the concentration of foodstuffs (Conrad et al. 1993; Singh and Heldman 2001) and pharmaceuticals (Manzatu et al. 1999; Shire et al. 2004). Enzymes are labile molecules so that evaporation must be conducted under rather high vacuum to prevent denaturation (Lambert and Meers 1983; Schaffeld et al. 1988). Other gentler methods of concentration are becoming more relevant. Concentration by water freezing has also been considered (Darbyshire 1981; Whitaker 1994) and high throughput crystallyzers have been designed (Janson et al. 1974). The method is not competitive with evaporation in economic terms and protein occlusion within the ice-water crystals reduces yield so that it is not a viable option except for the case of very labile enzymes. Water removal by freeze drying is acceptable as a final polishing step for highly purified enzymes, but it is not economically viable at that step (van den Tweel et al. 1994); besides, the presence of dissolved salts produce eutectic mixtures and foaming is a problem because of enzyme denaturation. Concentration by foaming has also been performed taking advantage of the tensoactive properties of proteins (Lalchev and

Exerowa 1981; Ekici et al. 2005). Foaming can be induced by air or inert gas bubbling through the enzyme solution and proteins will accumulate at the gas–liquid interface as predicted by Gibbs law (Thomas and Winkler 1977):

$$-\frac{d\sigma}{dc} = \frac{R \cdot T}{c} \cdot \Gamma \tag{2.3}$$

This technique has also the potential for protein fractionation (Sarkar et al. 1987; Miranda and Berglund 1993; Varley et al. 1996; Brown et al. 1999a,b), but this has not been fully appreciated, maybe because yields of recovery are low due to enzyme inactivation at the gas–liquid interface.

The most relevant operation for enzyme concentration is ultrafiltration. Ultrafiltration is a membrane filtration operation, where pressure difference across the membrane is the driving force. Molecules are separated on the basis of their size in the range from 1,000 to 100,000 Da, so covering most of the enzymes. Despite its potential of fractionation, ultrafiltration has been mainly used to concentrate enzymes by removing solvent (water) and smaller size solutes (Ehsani et al. 1996; Euzenat et al. 1998). Ultrafiltration membranes are made of different materials, among which polyacrilonitrile, polisulphone, polyamide and polyvinylidene fluoride are outstanding in terms of stability, mechanical strength and chemical compatibility. Ultrafiltration membranes are characterized in terms of its molecular weight cutoff and can be isotropic or anisotropic, the latter being best in terms of flowrate and control of operation (van den Tweel et al. 1994). Rejection coefficient, this is, the fraction of the solute (protein) concentration that is retained by the membrane is also used to characterize it. Membranes are composed by a selective skin of about 0.2 μm width supported by a sponge-like structure of about 50 to 150 μm width that confers mechanical strength. Ultrafiltration equipments come in different formats and sizes. Large size units are usually composed by stacked flat sheets or hollow fiber bundles of about 1 mm in diameter. Units are quite compact, exhibiting large area to volume ratios, and are modular so that they can flexibly adapt to process requirements. Operating pressures range from 100 to 500 KPa and capacities range from 10 to $200\,L/m^2 \cdot h$ (Aroca and Zúñiga 2004). Ultrafiltration is a mild operation; therefore, enzyme inactivation is kept to a minimum. Its main problem is the phenomenon of concentration polarization (Sablani et al. 2001), which refers to the buildup of protein near the membrane as a consequence of the flow through it. Increase in protein concentration near the membrane establishes a gradient that promotes back diffusion of the protein away from the membrane surface. At high protein concentration or flux, the membrane can be saturated and transmembrane flow determined by the protein back diffusion rate, which is slow enough to severely affect the operation. A secondary membrane composed by retained solutes (proteins) builds up increasing the hydraulic resistance and reducing membrane selectivity (Goosen et al. 2005). This latter aspect is of major concern when using ultrafiltration for protein fractionation (Ghosh and Cui 1998). To minimize concentration polarization, the judicious selection of the membrane and the flow regime near the membrane are important to reduce the film thickness and to scour deposits; low flux and low protein concentrations are advisable if practical. The effect of

concentration polarization can be managed by offering a large area for filtration as it occurs in the very compact modules now available. Selective removal or replacement of low molecular weight solutes in the enzyme preparation can be done by diafiltration in which ultrafiltration proceeds against a solution of defined composition (Pacheco-Oliver et al. 1990). Ultrafiltration can be considered now as the best option for enzyme concentration in most cases: it is a mild operation that causes no significant losses and the advances in the field of material science and process engineering provide now very flexible options to select the most adequate membrane and equipment for each particular case. A comprehensive review on protein isolation by membrane technology has been reported by Ulber et al. (2003).

2.2.2.3 Extraction of Intracellular Enzymes

Enzyme extraction will be determined to a great extent by the type of container cells and the location of the enzyme within the cell structure (Balasundaram and Pandit 2001). Enzymes from animal cells or tissues are easy to extract since such cells are devoid of cell wall; therefore, osmotic shock and other rather mild extraction methods are quite effective (Shin et al. 1994). Enzymes from plant cells or tissues require more rigorous conditions because they are endowed with a thick cellulose wall; however they have a rigid structure that can be efficiently ruptured by applying shear forces (Pierpoint 2004). Microbial cells, especially bacteria and yeast, are particularly difficult to disrupt because of the resilient nature of their cell envelopes. However, some are periplasmic and can be extracted rather easily by gentle procedures like osmotic shock; actually, a differential release can be obtained in such cases aiding substantially to enzyme purification (Fonseca and Cabral 2002). On the other extreme, some enzymes are bound or contained within membranes or other internal cell structures; in those cases, extraction not only requires cell disruption but also special extractive procedures involving detergents or solvents that can certainly be harmful for the enzyme (van den Tweel et al. 1994; Whitaker 1994). Notwithstanding, most intracellular enzymes are cytoplasmic and their recovery implies cell disruption or permeabilization, which is not an easy task. Methods for intracellular enzyme recovery can be divided into those that produce cell rupture by mechanical forces (Chisti and Moo-Young 1986; Kleinig and Middelberg 1998) and those that produce cell permeabilization by membrane damage (Bansal-Mutalik and Gaikar 2003; Cheng et al. 2006). Some new methodologies have arisen, like the extraction of thermostable enzymes by thermolysis (Ren et al. 2007). The most relevant methods for intracellular enzyme extraction are listed in Table 2.1. Not all of them are amenable for large-scale operation, whether for economical or technological reasons. Only those with potential application for the large-scale recovery of enzymes will be shortly reviewed here. Comprehensive reviews on the subject can be found elsewhere (Asenjo 1990; Doelle 1994; Cumming and Iceton 2001).

Mechanical disruption methods are the most studied and frequently represent the best option in terms of process economics. They are well endowed for large-scale operation, validation is rather simple and conditions of operation can be optimized

Table 2.1 Methods of Extraction of Intracellular Enzymes

Method	Principle	Application at Large Scale
Cell rupture		
Pressure	Compression, shear stress	Moderate
Homogenization	Shear stress, cavitation	Highly feasible
Milling	Compression, shear stress	Highly feasible
Sonication	Cavitation	Moderate
Decompression	Decompressive explosion	Moderate
Freezing–thawing	Shear stress	Unlikely
Dispersion in water	Osmotic shock	Unlikely
Thermolysis	Cell wall rupture	Moderate
Cell permeabilization		
Alkali treatment	Cell wall digestion	Unlikely
Solvent treatment	Membrane digestion	Moderate
Enzymatic lysis	Cell wall digestion and osmotic rupture	Feasible
Autolysis	Cell wall digestion and osmotic rupture	Feasible

(see ahead). Their main drawback is that drastic conditions are required for efficient cell breakage so that enzyme inactivation during operation is a major concern. Depending on the method, temperature may rise significantly and very efficient heat transfer is required, which is not easy to attain especially at large scale of operation (Foster 1995). Among the many options available, the use of homogenizers and bead mills outstand. High-pressure homogenizers are intensively used in the food industry. Actually, equipments similar to those used in milk homogenization can be used for cell disruption and are available in all kind of sizes and design (see www.directindustry.com). They have been extensively used for enzyme and recombinant protein extraction (Chi et al. 1994; Leser and Asenjo 1994; Choi et al. 1997; Fonseca and Cabral 2002) and perform well especially with bacterial cells being also efficient with yeast cells, but not applicable with highly filamentous organisms. Homogenizers work by forcing the cell suspensions under high pressure through a very narrow passage in the valve, which then impinges on a hard-impact ring. Disruption of the cell wall occurs by a combination of compression, highly turbulent eddies and strong shear forces (Kleinig and Middelberg 1998; Middelberg 2000). Most of the studies on mathematical modelling of cell disruption (see ahead) have been performed in homogenizers, presumably because the number of relevant operational variables is small: pressure is the key variable and also temperature and the number of passages through the homogenization valve are relevant. Bead milling is another important method for cell disruption which is considered one of the most efficient techniques for cell disruption (Darbyshire 1981). It works very well with yeast (van Gaver and Huyghebaert 1991; Zúñiga et al. 1992; Garrido et al. 1994; Illanes et al. 1995; Illanes et al. 1996) and is also effective with bacteria (Veide et al. 1983; Santos et al. 1996; Bierau et al. 2001; Singh et al. 2005) and filamentous organisms (Baldwin and Moo-Young 1991a,b). Cells in the form of a paste or slurry are mixed with small size beads (made of glass, ceramic or metal) that act as abrasives, and subjected to intense stirring, rupture being produced by a combination of compression and shear forces. Process can be continuous or discontinuous and temperature

Table 2.2 Components of Microbial Cell Envelopes

Microorganism	Cell Structure	Components
Gram-negative bacteria	Cell wall	Peptidoglycans
	External membrane	Lipopolysaccharides, proteins
	Cytoplasmic membrane	Phospholipids, proteins
Gram-positive bacteria	Cell wall	Peptidoglycans
	Cytoplasmic membrane	Phospholipids, proteins
Yeasts	Cell wall	Mannanoproteins, β-glucans
	Cytoplasmic membrane	Phospholipids, proteins
Molds	Cell wall	Cellulose, chitin, β-glucans, proteins
	Cytoplasmic membrane	Phospholipids, proteins

control can be exerted by an outer jacket or by recycling through a heat exchanger. There are a number of equipments that have been specifically deigned for cell disintegration and are available in different sizes and designs (see: www.glenmills.com). Mathematical modelling of this type of operation has been hampered by the large number of variables involved (see ahead).

Digestion of cell envelopes is a more selective technique for the release of intracellular compounds. Contrary to mechanical methods, cell rupture is not required and in most cases cells are merely pemeabilized. The system of choice should be dictated by the chemical composition of the cell envelopes. Among cell permeabilization methods, those involving alkali (Wiseman 1995) or organic solvent treatment (Decleire et al. 1987; Bachhawat et al. 1996; Panesar et al. 2007) are usually too harmful, costly and non-specific, so that their use is limited.

Cell permeabilization by selective enzyme digestion is a more promising technology that can be of interest for selectively recovering labile and expensive intracellular enzymes. Lysozyme has been routinely used for disrupting bacterial cell wall peptidoglycans (Chen and Chen 1996), though it has been postulated that can also act as a an activator of pre-existing autolytic wall enzymes (Wecke et al. 1982); it has also been used in combination with β-glucanase for the degradation of yeast cell wall (Knorr et al. 1979). Helicase from *Helix pomatia* has been routinely used for cell wall degradation of yeasts (Koch and Rademacher 1980) and molds (Anné et al. 1974) The chemical composition of the cell envelopes, shown in Table 2.2 (Aroca and Zúñiga 2004), can be used to judiciously select the appropriate enzyme or enzyme cocktail.

Former lytic enzyme preparations were expensive, non-specific and not readily available in large quantities (Kula and Schütte 1987), but more specific microbial lytic enzymes have been developed that can be produced more economically on a large scale (Asenjo and Andrews 1990). β-Glucanases from *Cytophaga* and *Oerskovia* have proven to be quite effective in yeast cell wall degradation (Hunter and Asenjo 1987a). Selective extraction of recombinant proteins and enzymes have been performed with such enzymes with considerable success; in many cases, no more than 20% of the intracellular protein has been released which is a major saving in terms of further purification requirements (Huang et al. 1991; Asenjo et al. 1993). Actually, the potential of this method relies very much on that selectivity,

but enzyme cost is still a major restriction that can be overcome by screening better lytic enzymes by directed evolution (García et al. 1988; Salazar et al. 2006) and protein engineering (Salazar et al. 1999). Kinetics of extraction with lytic enzymes has been modelled and the process optimized (Hunter and Asenjo 1987b; Liu et al. 1988). The subject of enzymatic lysis of microbial cells has been recently reviewed (Salazar and Asenjo 2007).

Autolysis is a very appealing method for enzyme extraction in those organisms prone to autolysis, like yeasts (Knorr et al. 1979; Kollar et al. 1991) and bacilli (Svarachorn et al. 1991). Under certain stress conditions (i.e. drying, high organic solvent or electrolyte concentration) lytic enzymes (proteases, nucleases and glucanases) are induced that partially digest its own cell wall being then the intracellular content easily extracted by osmotic shock (Reed and Peppler 1973; Breddam and Beenfeldt 1991; Kollar et al. 1993). Autolysis has been used for long in the extraction of cellular proteins; it is the basis for commercial production of some types of yeast extracts (Nagodawithana 1992; de Palma Revillion et al. 2003) and it has been used efficiently to extract yeast enzymes (Amrane and Prigent 1998). Invertase, both periplasmic and cytoplasmic, was efficiently extracted from bakers' yeast cells subjected to autolysis by drying. At a critical moisture content autolysis was triggered, the efficiency of protein extraction and enzyme recovery depending on the time that the cells remained below such critical moisture (Illanes and Gorgollón 1986). The process was scaled up to pilot level and the crude extract was used as raw material for the production of a biocatalyst employed in the continuous inversion of sucrose syrup (Illanes et al. 1988b). The process of autolysis has been optimized, the addition of exogenous papain notably increasing yield and productivity by reinforcing the autolytic effect (Gutiérrez 1993). It is an interesting alternative for extraction of yeast enzymes: it is cheap, simple and readily scalable, the main drawback being its low productivity as a consequence of the prolonged time required for autolysis.

There are many efficient methods for disrupting cells for the release of its intracellular content. The problem with enzymes is that the method must be sufficiently rough to disrupt or distort the cell envelopes, but gentle enough to preserve activity. This poses a compromise so that the process can be optimized. A suitable objective function for optimization is the amount of active enzyme recovered:

$$E = p_R \cdot a \tag{2.4}$$

Protein extraction and enzyme inactivation follow their own kinetics so that:

$$p_R = f(t) \tag{2.5}$$

$$a = \varphi(t) \tag{2.6}$$

$$E = f(t) \cdot \varphi(t) \tag{2.7}$$

and the optimum condition will be given by:

$$\frac{dE}{dt} = f(t) \cdot \frac{d[\varphi(t)]}{dt} + \varphi(t) \cdot \frac{d[f(t)]}{dt} = 0 \tag{2.8}$$

In principle, any extraction method can be optimized accordingly as long as suitable and validated expressions for protein release and enzyme inactivation rates are available. In practice Eqs. 2.5 and 2.6 can be complex and depend on many operational variables (Currie et al. 1972). If both protein release and enzyme inactivation are assumed to proceed according to first order kinetics:

$$\frac{dp_R}{dt} = k_R \cdot (p_T - p_R) \tag{2.9}$$

$$-\frac{da}{dt} = k_D \cdot a \tag{2.10}$$

From Eqs. 2.8 to 2.10, the optimum $(^*)$ conditions for extraction are obtained (Illanes 1994):

$$p_R^* = \frac{k_R}{k_R + k_D} \cdot p_T \tag{2.11}$$

$$a^* = a_0 \cdot \left(\frac{k_R + k_D}{k_D}\right)^{-\frac{k_D}{k_R}} \tag{2.12}$$

$$t^* = \frac{\ln \frac{k_R + k_D}{k_D}}{k_R} \tag{2.13}$$

$$E^* = a_0 \cdot p_T \cdot \frac{k_R}{k_R + k_D} \left(\frac{k_R + k_D}{k_D}\right)^{-\frac{k_D}{k_R}} \tag{2.14}$$

Protein release by bakers' yeast and *Bacillus brevis* cell disruption in an industrial homogenizer has been carried out and modelled. The most relevant variable was pressure drop across the homogenizer valve; k_R depended on the pressure at exponents of 2.9 and 1.8 for the yeast and the bacteria respectively (Follows et al. 1971; Augenstein et al. 1974). In the latter case, the recovery of a labile intracellular enzyme was optimized by combining protein release and enzyme inactivation kinetics. Protein release was also modelled in an industrial bead mill, but in this case k_R depended on too many variables: bead size, cell concentration, beads to cell paste volumetric ratio, temperature, agitation speed, agitator design, and recycling ratio (Schütte et al. 1983).

2.2.2.4 Removal of Cell Debris

After extraction, the enzyme preparation is contaminated with undisrupted cells and cell debris that have to be removed before purification. Common operations of solid–liquid separation, like centrifugation and dead-end filtration, can be used but the drawbacks already mentioned with respect to cell separation are augmented by the very small size of the cell fragments. Microfiltration is a better option that has been used successfully (Vaks et al. 1984; Chan et al. 1991).

Aqueous two-phase extraction is the most promising operation for cell debris removal that, despite its potential for protein fractionation (Marcos et al. 1999;

Mohamadi and Omidinia 2007), will be revised in this section. It is basically a liquid–liquid extraction system that considers two aqueous phases comprising either two polymers or a polymer and a salt (Kroner and Kula 1978; Kula 1979; Hustedt et al. 1985; Abbott et al. 1990). Phase separation is produced due to the phenomenon of polymer incompatibility (Albertsson et al. 1990) that produces two-phases by mutual exclusion. However, each phase is aqueous in nature, so in principle not detrimental for enzyme activity as in conventional water-solvent two-phase systems (Andersson and Hahn-Hagerdal 1990). Other advantages are the variety of polymers that can be used, its environmental benignity and the availability and suitability of equipment for liquid–liquid extraction, which is a conventional operation in the chemical and pharmaceutical industry (Podbielniak et al. 1970; Raghavarao et al. 2003). The most common systems are polyethyleneglycol-dextran and polyethyleneglycol-salt. The former is expensive, the medium is highly viscous and the process is hard to validate when crude dextran fractions are used so the latter is preferred especially for large-scale operation, as long as the enzyme withstands the high ionic strength required in the salt phase (Gupta et al. 1999; Banik et al. 2003). Several other biphasic systems have been tried with the purpose of using less expensive an more environmentally benign polymers (Tjerneld et al. 1986; Andersson and Hahn-Hagerdal 1990; Miranda and Berglund 1990; Kepka et al. 2003; Raghavarao et al. 2003; Sarubbo et al. 2004; Rosso et al. 2005; Bezerra et al. 2006), but the polyethyleneglycol-salt system is still the most used (Sarmento et al. 1997; Iwamoto and Shiraiwa 2005; Dolia and Gaikar 2006; Jaw et al. 2007). Two-phase partitioning can be smoothly integrated to the extraction step (Zhou et al. 1997; Su and Feng 1999; Chang and Su 2005) and can also be improved by combining with affinity ligation (affinity partition) (Xu et al. 2003; Lam et al. 2004; Teotia and Gupta 2004; Castell et al. 2006).

The two-phase system can be represented by a phase diagram, as the one shown in Fig. 2.2 for the polyethylene glycol (MW 4000)-dextran (T500) system where the equilibrium is represented by the curved line.

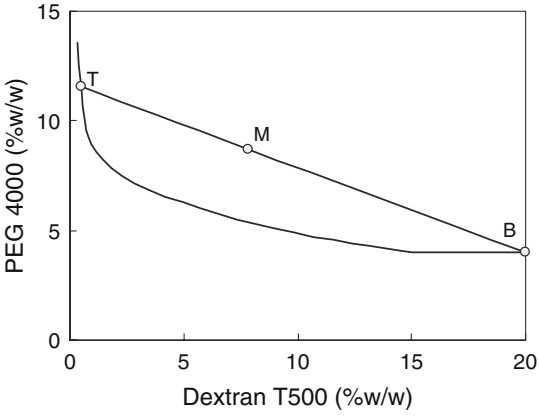

Fig. 2.2 Phase diagram of the polyethylene glycol 4000-dextran T500 system

All mixtures corresponding to points over the equilibrium curve (M) separate into two phases whose composition is defined by points T and B, corresponding to the top and bottom phases respectively. The volume ratio of top phase to bottom phase will be given by the relative values of the traces BM and MT over the corresponding tie-line:

$$\frac{V_T}{V_F} = \frac{\overline{BM}}{\overline{MT}} \tag{2.15}$$

Proteins and cell debris are distributed between the two phases according to their partition coefficient. For a specific protein, its partition coefficient is:

$$K_P = \frac{c_T}{c_B} \tag{2.16}$$

When used for cell debris removal, proteins invariably concentrate in the top phase (PEG in the case of PEG/dextran and PEG/salt), while fragments do it in the bottom phase. Therefore yield of protein recovery is:

$$Y_T = \frac{V_T \cdot c_T}{V_T \cdot c_T + V_B \cdot c_B} = \frac{1}{1 + \frac{V_B}{V_T} \cdot \frac{1}{K_p}} \tag{2.17}$$

Yield will obviously increase with K_P, but also with the top to bottom volume ratio. K_P depends on many factors related both to the biphasic system (i.e. molecular weight of polymers, concentration of polymers and salts, pH, temperature, concentration of cell fragments), and to the target protein (i.e. hydrophobic amino acid residues, number of carboxyl and amino side chains). General rules on how to increase or decrease K_P have been set up by Huddleston et al. (1991).

Most large-scale equipment for liquid–liquid extraction is available for two-phase extraction and usually multistage systems have to be used for obtaining high yields. Kühni type columns, Podbielniak centrifugal extractors and disk and bowl centrifuges have been used (Kula et al. 1981; Veide et al. 1983; Cunha and Aires-Barros 2002).

2.2.3 Enzyme Purification

The crude extract or clarified broth containing the enzyme is then subjected to purification, conceived as a sequence of operations (see Fig. 2.1) aimed to remove all contaminants that can interfere with its intended use. Purification can also serve to the purpose of increasing the specific activity of the biocatalyst in the case of enzyme immobilization. The situation will be radically different for an intracellular than for an extracellular enzyme. In the first case, the enzyme extract is a complex mixture of proteins, nucleic acids and other cell constituents, while in the latter the enzyme concentrate contains only some extracellular proteins and small molecular weight solutes, since the cell membrane acts as a powerful barrier to retain most of the cell constituents aiding powerfully to purification. Many extracellular hydrolases

sold as commodities are in fact rather crude preparations, hardly subjected to any purification step. In the case of intracellular enzymes, the extract is heavily contaminated not only with other proteins but also with nucleic acids, so that a previous step for nucleic acid removal is customary. Several options exist, including nuclease treatment and precipitation with different agents (Harve and Bajpai 2000). Ammonium sulfate is effective in removing nucleic acids but precipitates protein as well, so more specific precipitants are required. They are usually positively-charged materials which form complexes with the negatively-charged phosphate residues of the nucleic acids. These include polyethyleneimine, the cationic detergent cetyltrimethyl ammonium bromide, streptomycin sulfate and protamine sulfate (Burgess 1969; Yang et al. 1987; Cordes et al. 1990). All these procedures are expensive and may have some detrimental effect upon enzymes so they are used at a large scale only when contamination of the enzyme product is unacceptable. Treatment with bovine pancreatic nucleases has been considered as the most cost-effective method of nucleic acid removal (http://www.lsbu.ac.uk/biology/enztech/nucleicic.html).

Small molecular weight solutes, particularly ions, might interfere with the initial purification steps and in such case they should be previously removed either by diafiltration or size exclusion chromatography. This is not usual, so that removal of ions (desalination) is rather used as a final polishing step.

Enzyme purification is aimed mainly to the removal of contaminant proteins; therefore, enzyme purification is in essence a series of operations of protein fractionation. A compromise exists between purification and yield of recovery. Each operation intended for purification produces an increase in purity (conveniently expressed in terms of specific activity: units of activity per unit mass of protein) but inevitably some enzyme activity is lost so that yield of recovery is lower than 100%. Purification factors and yields for a given operation (i) and global values for N consecutive operations are then:

$$(\mathrm{PF})_i = \frac{a_i}{a_{i-1}} \tag{2.18}$$

$$\mathrm{PF} = \frac{a_N}{a_0} = \prod_{i=1}^{N} (\mathrm{PF})_i \tag{2.19}$$

$$Y_i = \frac{E_i}{E_{i-1}} \tag{2.20}$$

$$Y = \frac{E_N}{E_0} = \prod_{i=1}^{N} Y_i \tag{2.21}$$

There is clearly a compromise between purification and yield of recovery, since increasing levels of purity require a higher number of operations, each of them having its own yield of recovery. Even if the yield of recovery per operation is high (i.e. 80%), if purification requires several (i.e. five) consecutive operations, global yield of recovery will be low (i.e. only 33%). If average purification factor is 2.5 per operation, after five operations it will be 98. This can be appreciated in Fig. 2.3.

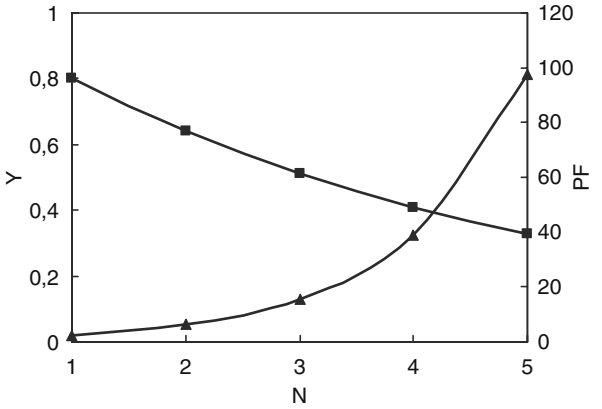

Fig. 2.3 Purification factor (PF) and yield of recovery (Y) as a function of the number of purification steps (N)

For the case of industrial enzymes, which are produced at a large scale as commodities, the criterion of purification is the minimum compatible with its intended use. This is so, because purification at large scale is cumbersome and expensive and usually not compensated for the benefit of producing a purer protein. In such cases, yield of enzyme recovery rather than enzyme purity is the objective function. The situation is different for the case of specialty enzymes, where purity cannot be sacrificed for yield considerations. However, there are increasing incentives for purification even for bulk enzymes, especially in the case of immobilized enzymes where purity might have a significant impact in process economics and also because of recent advances in the field of protein purification on a large scale (Ladisch et al. 1998). Besides yield of recovery and resolution, capacity and speed are relevant characteristics that can be considered when selecting a given operation of purification.

In principle, any method intended for protein fractionation can be used for enzyme purification. However, the methods applicable for production purposes are restricted to those amenable for scale-up at a reasonable cost. Only those will be reviewed here. Comprehensive reviews on protein purification can be found elsewhere (Asenjo 1990; Janson and Rydén 1998; Roe 2001; Hatti-Kaul and Mattiasson 2003b; Rosenberg 2004) and handbooks on the subject are also available (http://www.biochem.uiowa.edu/donelson/Database%20items/protein_purification_handbook.pdf).

Some general guidelines can be helpful when designing a purification system (Wheelwright 1987). Operations based on physical properties (i.e. molecular size, solubility, surface charge distribution) or biological properties (ligand specificity) should be selected to fully exploit those in which marked differences exist between the target enzyme and the rest of the contaminant proteins (Watanabe et al. 1994). Each operation should be based on a different property. More bulky operations should be conducted first to early reduce the processing volume; more expensive operations should be performed last to act on a reduced volume of product stream.

Damaging contaminants, like proteases, should be early removed. All operations developed at laboratory scale should be judiciously analyzed for its scalability. Number of operations should be as few as possible. Use of additives should be minimized to avoid further purification steps. The famous KISS rule (keep it simple, stupid – recalling Nobel laureate William Shockley) certainly applies.

Rational design and optimization of protein purification processes have been developed and optimal operation sequences determined by using expert system (Asenjo et al. 1991; Leser and Asenjo 1992; Mao and Hearn 1996; Lienqueo et al. 1999; Vásquez-Alvarez et al. 2001; Simeonidis et al. 2005).

Most reported protocols for protein purification at laboratory scale are hardly applicable for production purpose since a large number or complex operations are considered and very low yields of recovery (usually below 10%) and productivities are obtained at that scale (Saha 2004; Hanson et al. 2005; Sian et al. 2005; Patel et al. 2006). Only highly priced recombinant proteins for therapeutic use that are produced on a small scale can be obtained with such low yields since in such cases very high purification factors are required that demand many and complex operations (Schaap and Parker 1990; Hua 1997; Bond et al. 1998).

Some of the most relevant methods of enzyme purification by protein fractionation applicable at large scale will be reviewed. Such methods can be roughly divided into those based on fractional precipitation and those based on differential retention in a solid matrix.

The formers are based on the reduction in solubility as a consequence of the addition of salts (*salting-out*), organic solvents or polymers. These methods have been traditionally used as initial purification steps because they are simple and readily scalable. However, because of its low selectivity, purification factors are modest so they are used mostly as an initial concentration step that will facilitate subsequent more selective operations. The solubility of proteins varies with the ionic strength and hence with the salt concentration in the solution. At low concentrations of salt, the solubility of the protein increases with salt concentration (*salting-in*), but as the salt concentration increases further, the solubility of the protein begins to decrease and protein precipitates from solution (*salting-out*). Salting-out is a traditional operation for protein fractionation, usually performed first (Coulon et al. 2004), which is based on the decrease in protein solubility produced at high concentrations of dissolved salts. The ability of a salt as precipitating agent is well correlated to the Hoffmeister series (Hoffmeister 1887). In general, the more effective are the salts of univalent cations and multivalent anions, which are more antichaotropic and increase hydrophobicity promoting protein aggregation (Erson et al. 1998). Protein precipitation at high salt concentration has been explained by ion solvation that reduces the availability of water for protein dissolution (Dixon and Webb 1961). Salting-out has been described by Cohn's equation:

$$\log S = \beta_S - K_S \cdot \mu' \tag{2.22}$$

where:

$$\mu' = \frac{1}{2} \sum m_i \cdot Z_i^2 \tag{2.23}$$

β_S represents the logarithm of the solubility of the protein at zero ionic strength and is a strong function of temperature and pH. K_S is the slope of the solubility curve and depends on the nature of the protein and the salt, but not on pH or temperature. The magnitude of K_S defines the range of ionic strength at which the protein precipitates; then, a high K_S value is convenient in terms of purification because the protein will precipitate in a narrow range of ionic strength. β_S defines the magnitude of ionic strength at which protein precipitation starts so that a low value of β_S is convenient because the lower its value the lower the ionic strength at which the protein precipitates. Cohn's equation does not contain any protein concentration dependent term and in fact, the precipitation curve will not change with protein concentration, its value simply determining the ionic strength threshold value for precipitation (Illanes 1974). For any given salt, K_S may vary significantly according to the size of the molecule, being larger for large asymmetric proteins. Ammonium sulfate, even though not being the best salt according to the Hoffmeister series, is usually the choice since it is harmless to the enzyme (it is actually used for enzyme preservation) and quite soluble allowing ionic strengths high enough to precipitate almost any protein. Salting-out precipitation is very fast, equilibrium being reached after a few minutes, but particle size is small (from 0.5 to 5 μm) which hinders recovery since high centrifugal forces are required to obtain a good separation (Foster et al. 1976). As any precipitation method, salting out is not very selective and purification factors below 10 are obtained even under optimized conditions (Illanes 1974). It is however quite useful as an early concentration step since the precipitated protein can be readily dissolved in a small amount of water.

Water-miscible organic solvent precipitation has been also used traditionally in the early steps of enzyme purification (Drapeau et al. 1972; Omar et al. 1987; Iizumi et al. 1990). Some drawbacks of them are that operation must be conducted at low temperatures (near or below 0 °C) because these solvents are protein denaturants at room temperature, and explosion-proof motors should be used. Ethanol and acetone have been the most used solvents; ranges of precipitation are narrower with acetone but it is quite harmful, so very low temperatures have to be used. Despite this, organic solvent precipitation has some advantages over salting-out, since it is more selective and strictly based on the physicochemical properties of the protein (Askonas 1951); additionally, it produces less amorphous precipitates, easier to recover by conventional centrifugation (Illanes 1974). Precipitation is promoted by protein–protein Coulombic interactions at low ionic strength, which are magnified by the decrease in dielectric constants promoted by the water-miscible solvent. An empirical correlation, analogous to the salting-out equation of Cohn, has been found useful for evaluating protein precipitation by water-miscible organic solvents:

$$\log S = \log S_0 - K'_S \cdot \varepsilon^{-2} \tag{2.24}$$

The explanation above has been questioned since when working with ethanol at very low temperatures, reduction of dielectric constant is not significant with respect to water; in that case it has been demonstrated that van der Waals forces of attraction are enough to promote protein aggregation (van Oss 1989). Protein solubility has proven to correlate well with solvent polarity and the Hildebrand solubility

parameter has been used to predict protein precipitation by organic solvents (Hwang et al. 2007). Salt and solvent precipitation have been combined in a three phase partition system where the precipitated enzyme concentrates in the water-solvent interface obtaining high purification factors at acceptable yields of recovery (Sharma et al. 2000; Sharma and Gupta 2002).

Non-ionic soluble polymers have been used also as precipitants in enzyme purification (Gupta et al. 1994; Yu et al. 1994; Gupta et al. 1997). Protein–protein interactions promoted by the polymers have been explained in terms of the volume-exclusion potential of Asakura and Oosawa (Mahadevan and Hall 1990), whereby the depletion of solvent between the protein molecules causes attractive forces that are primarily responsible for protein aggregation and phase separation (Vlachy et al. 1993). As opposed to organic solvents, polymers like polyethylene glycol are harmless to the enzyme structure and can be used at room temperature without any detrimental effect on enzyme structure (Haire et al. 1984; Gupta et al. 1997). Some difficulties associated to polymer precipitation are the increase in viscosity and the complex removal of the polymer after precipitation. Solubility of proteins decays sharply with polymer concentration and it has been modelled according to an exponential decay equation (Atha and Ingham 1981), which is analogous to the Cohn's salting-out equation:

$$\log S = \log S'_0 - K''_S \cdot c_P \tag{2.25}$$

Some other techniques of protein precipitation, like differential inactivation by temperature or pH, have been occasionally used for those cases where abnormal stability allows the enzyme to retain its functional structure while contaminant proteins are precipitated by irreversible unfolding (Prado et al. 1999; Harris 2001).

The low selectivity of most methods of fractionation by precipitation represents its main drawback. The addition of specific ligands to polymers can be a powerful tool for purification (affinity precipitation) and "smart" polymers (i.e. those whose solubility changes dramatically with small changes in the environment) are increasingly being used for protein purification, since it allows to include a highly selective operation at the very beginning of the purification process (Galaev and Mattiasson 2002; Kumar et al. 2003).

Liquid chromatography is a very powerful system of enzyme purification by protein fractionation. It consists in the passage of a mixture of solutes (proteins in this case) dissolved in a mobile phase through a stationary phase (usually a solid matrix) with which the solutes interact at varying strengths so that a differential retardation is produced that allow their separation by a suitable eluent. Chromatography is a very powerful analytical system, but preparative chromatography has been extensively used for laboratory scale enzyme purification. The most common configuration is column chromatography, in which the stationary phase is packed into a column through which the mobile phase containing the protein and later on the eluent are pumped. Scale-up of chromatographic methods for enzyme production is cumbersome since the system must be robust, have a reasonably high throughput and a reasonable cost, which is by no means an easy task. Liquid chromatography for protein fractionation has been extensively treated and only the most relevant options for enzyme purification will be shortly reviewed here with especial consideration to

its application at a productive scale. A comprehensive coverage of the subject can be found in several publications (Janson and Rydén 1998; Kastner 2000; Ahuja 2003; Cutler 2004).

Several types of chromatography are available for protein fractionation, among which the most relevant are, according to the principle of separation involved: size-exclusion, ion exchange, hydrophobic and affinity chromatography.

Size exclusion chromatography (also not so correctly termed gel filtration or gel permeation), separates proteins according to their molecular size. Smaller molecules enter the porous structure of the matrix and are the most retarded, while larger molecules are progressively excluded from the stationary phase and leave the column earlier. Resolution is high (it is used for molecular mass determination) but throughput is low and therefore is commonly used at a later stage of purification or as a final polishing step (Singh et al. 2007). Elution is simple and does not require gradients of any kind. Several types of matrices are available among which cross-linked dextran, polyacrylamide, polymethacrylate, polyvynil alcohol and agarose gels are prominent. Several particle sizes and degrees of cross-linking produce different resolutions in different molecular size ranges, allowing protein fractionation as well as desalting for which size-exclusion chromatography is very effective and widely used. The range of molecular size fractionation is closely related to the rehydration value of the gel which is a very relevant property that should be considered when choosing the appropriate stationary phase. When scaling-up the column, height to diameter ratio is decreased to reduce the pressure drop and hence bed compaction. This usually implies a sacrifice in resolution, which is roughly proportional to the square root of the bed height. Resolution is also decreased if concentrated protein solutions are fed to the column. Small particle size gels produce a higher resolution but also a higher pressure drop. Stacked columns are frequently used on a large scale to avoid that problem. As seen, scaling up to production level may severely reduce resolution (Simpson 1994). Complete information on operating procedures, types of supports and columns for size-exclusion chromatography is available in the format of handbooks (Anonymous 1999; Wu 1999).

Ion-exchange chromatography has been extensively used for the purification of enzymes of commercial significance (Yang et al. 1987; Chauthaiwale and Rao 1994; Falco et al. 2000; Aguilara et al. 2006; Liu and Xi 2006). The amphoteric nature of proteins means that they will be cationic or anionic depending on the pH of the solution and separation is based on the reversible ionic interaction between charged amino acid residues in the protein molecule and a chromatographic support (often an ion exchange resin) of opposite charge. As they pass through the column, proteins bind differentially according to their surface charge distribution at the pH of operation. When above its isoelectric point, proteins exhibit a net negative charge and bind to anion exchangers; when below, they exhibit a net positive charge and bind to cation exchangers. The retained proteins are eluted differentially by titration, decreasing the pH to approach the isoelectric point in the first case, and increasing the pH to approach the isoelectric point in the second case. In both cases differential elution can be performed by increasing ionic strength, which is sometimes preferred for not submitting the enzyme to deleterious pH values (see Fig. 2.4).

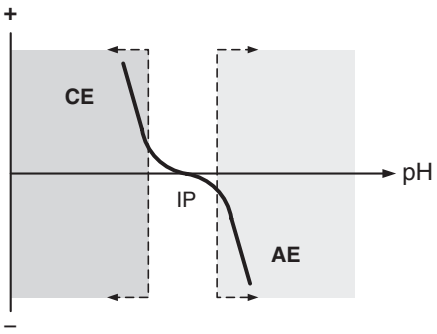

Fig. 2.4 Principle of ion-exchange chromatography. Above the isoelectric point (IP) proteins bind to anion exchangers (AE) and are eluted by a decreasing pH, and below it they bind to cation exchangers and are eluted by a increasing pH. In both cases proteins can be eluted by increasing ionic strength

Protein fractionation is produced in the elution step where a pH or salt gradient is applied; gradient can be performed stepwise or continuously. Ion-exchange chromatography is usually designed to retain the target protein, but it can be designed to retain impurities as well. Most common matrices and ion exchange groups for ionic exchange chromatography are shown in Table 2.3. Exchange capacity of the support is determinant for column performance and can be as high as 5 meq/g (Hostettmann et al. 1998). Ion-exchange chromatography allows higher throughputs than size exclusion chromatography and can be an early operation in the purification process, provided that the ionic strength of the crude enzyme preparation is low or has been reduced by previous desalting. Ion-exchange chromatography is a very powerful operation for enzyme purification and even proteins of similar isoelectric points can be conveniently separated because interaction with the support is determined by the surface charge distribution of the protein rather than by its net charge (Simpson 1994). Scale-up considerations for ion-exchange chromatography are similar to those mentioned for size-exclusion chromatography; however it is a more flexible operation allowing higher throughputs with similar or even better

Table 2.3 Commonly Used Ion Exchange Chromatographic Systems

	Ion exchanger		
Support	Anionic	Cationic	Supplier
Cellulose	DEAE; TEAE; QAE	CM; P	Amersham-Pharmacia; Bio-Rad; Whatman
Dextran	DEAE; TEAE; QAE	CM; P; SP	Amersham-Pharmacia
Agarose	DEAE; PEI	CM	Amersham-Pharmacia; Bio-Rad;
Acrylic copolymers	DEAE	CM; SP	Pall Ind. Biologique Française
Polymer-based Toyopearl and TSK-GEL	DEAE	CM; SP	TosoHaas

DEAE: diethylaminoethyl; TEAE: triethylaminoethyl; QAE: quaternaryaminoethyl; PEI: polyethyleneimine; CM: carboxymethyl; P: phospho; SP: sulfopropyl

resolutions, so that it can be an early or final step of the purification process. Complete information on operating procedures, types of supports and columns for ion exchange chromatography is available in the format of manuals (Westerlund 2004; Wu 1999).

Hydrophobic chromatography has gained increasing importance for the purification of enzymes (Gupta et al. 2002; Aehle 2003; Coulon et al. 2004; Lee et al. 2006). It is based on the interaction of the hydrophobic regions of the protein with a hydrophobic gel matrix; however, the principle underlying protein separation is not clear. Most common matrices are gels coated with hydrophobic aryl or alkyl (frequently butyl, octyl or phenyl) groups. For efficient fractionation ionic strength must be high, since it increases hydrophobic interactions among protein molecules; therefore, this operation is ideal when a previous salting-out operation is considered. Elution is achieved by changing the pH or the ionic strength or by modifying the dielectric constant of the eluent. High throughputs can be obtained but resolution is somewhat lower than in ion-exchange chromatography. The method is, however, quite useful because it is based on a completely different mechanism and can be efficiently combined with other chromatographic operations (Simpson 1994). Detailed information on hydrophobic chromatography is available (Anonymous 1999; Simpson and O'Farrell 2004).

Affinity chromatography is probably the most powerful purification technique in terms of resolution since it is based in the functional rather than the physicochemical properties of the protein (Brocklehurst et al. 2004). Nowadays, this operation is considered in most protocols of recombinant protein purification (Gottstein and Forde 2002; Sahina et al. 2005). Affinity chromatography is especially pertinent for enzymes, that can be purified quite specifically based on their catalytic properties and also, as any other protein, on their immunogenic properties. In many cases a single operation can bring the enzyme to the final desired level of purity (Ito et al. 2004; Mendu et al. 2005; Melissis et al. 2007). This is highly desirable since yield of recovery is expected to be high. However, scale-up to production level is in most cases precluded by its high cost (Robinson et al. 1974; Yang and Tsao 1982), so it becomes relevant mostly for the small-scale production of high value specialty enzymes (Clonis 1987). As materials and operational costs are lowered and more experience is gained in the large-scale operation, affinity chromatography is becoming more relevant for enzyme production. A support for affinity chromatography is composed of a ground matrix which is (usually) linked to a space arm that in turn is linked to the ligand that interacts specifically with the protein. Ground matrix is a polymer that should not interact with the protein to permit a highly specific retardation by the ligand (agarose gel is extensively used). Space arm serves to the purpose of projecting the ligand away from the matrix surface to favor ligand-protein interaction; aliphatic hydrocarbons are usually employed with chain lengths from five to seven carbon atoms, long enough to be effective but not so long to become fragile or favor non-specific interactions (i.e. hydrophobic). Biospecific ligands for enzymes can be any molecule that interacts specifically with the enzyme structure: substrates, coenzymes or cofactors, and the corresponding analogues. The degree of specificity is variable: substrates, inhibitors and their corresponding analogues

are expected to be highly specific for a particular enzyme while coenzymes, cofactors and their corresponding analogues are specific for a group rather than a specific enzyme (i.e. NAD^+ for dehydrogenases; ATP for kinases). Judicious selection of the ligand is crucial for a successful operation. The ligand should bind to the enzyme strongly enough to allow its efficient retardation in the chromatographic column, but not as strongly to preclude elution; values of dissociation constants between 10^{-4} and 10^{-8} M are recommended (Fonseca and Cabral 1996; Wu and Liu 1996). From this viewpoint and also because of economic considerations analogues are often preferred. Substrates are not recommended as ligands, since non-reactive conditions are required to avoid enzyme detachment; some inhibitors can be too weak (high dissociation constant) to effectively retard the enzyme. Cofactor and cofactor analogues, despite their lower selectivity, have been intensively used as ligands for meeting the above criteria (Mosbach et al. 1972); in the latter case, analogue chemicals (i.e. commercial dyes) have the advantage of lower costs (Clonis 2006). Commercial triazine dyes (i.e. Procion red; Cibacron blue) have been used for the purification of several NAD^+ and FAD^+ dependent dehydrogenases (Schneider et al. 1983). In the case of Cibacron blue, ligation is less specific and other classes of enzymes have been purified as well (Subramanian 1984; Koch et al. 1998). Biomimetic analogues of industrial triazine dyes have been successfully used for the purification of the FADH dependent formate dehydrogenase (Labrou et al. 1995). Special affinity chromatography matrices for His-tagged recombinant proteins have been developed and successfully applied for the one-step purification of industrially relevant enzymes (Zheng et al. 2007). Many eukaryotic enzymes are glycoproteins and in this case lectin affinity chromatography has been used where a ligand specific for carbohydrates (i.e. Concavalin A) is used (Mislovičová et al. 1996; Mateescu et al. 2002; Bahar and Tuncel 2004). Immunoaffinity chromatography has also been used for enzyme purification although on a rather small scale, mainly for research and therapeutic enzymes (Ehle and Horn 1990; Thompson et al. 1990). The enzyme retained by its interaction with the ligand is then recovered by elution, which can be specific (gradient of ligand or ligand analogue) or non-specific (gradient of pH or ionic strength). Affinity chromatography is suggested as an intermediate operation within the purification process, but it can be an early (and even the only one) operation (Simpson 1994; Anonymous 1999). A review on practical and fundamental aspects of affinity chromatography can be found elsewhere (Harakas 1994; Hostettmann et al. 1998; Prado et al. 1999). Purification of recombinant proteins for therapeutic use by affinity chromatography has been reviewed by Hage (1999). The same author has recently published a comprehensive review on the subject (Hage 2006).

The principles of affinity chromatography can be combined with other operations of purification to improve them (Labrou and Clonis 1994). Affinity partition combines the selectivity of affinity ligation with aqueous two-phase extraction (Kamihira et al. 1992; Köhler et al. 1991) and has been successfully employed in enzyme recovery and purification (Johansson and Tjerneld 1989; Schustolla et al. 1992) obtaining impressive increases in the partition coefficient (Eq. 2.16) and therefore in yield of enzyme recovery (Eq. 2.17). Affinity partition has also been combined with membrane separation (affinity ultrafiltration), where a soluble

polymeric biospecific ligand is bound to the enzyme prior to its separation by ultrafiltration (Galaev 1999; Romero and Zydney 2002). Several enzymes of commercial relevance have been purified by affinity ultrafiltration (Male et al. 1990; Filippusson and Sigmundsson 1992). Continuous affinity recycle extraction is based on the combination of affinity chromatography with molecular filtration. The system is simple, scalable and useful as a front or final purification step and has been used for the purification of β-galactosidase (Pungor et al. 1987) and the process modelled and optimized (Gordon et al. 1990; Sun et al. 1995; Dechechi et al. 1997).

Other protein fractionation methods are available for enzyme purification when a high degree of purity is required. These techniques are mostly oriented to the preparation of highly pure protein samples for characterization, and are usually difficult or extremely costly to scale-up, its application being restricted to specialty proteins and peptides for therapeutic use, clinical diagnosis or research. High pressure liquid chromatography (HPLC) is a high resolution system mostly used for analytical purposes; however, it has been used at a preparative scale for the purification of recombinant proteins and enzymes (Smith et al. 1999). Reverse phase HPLC, where proteins are differentially retarded by hydrophobic interaction with the carrier and then eluted by increasing the organic solvent concentration in the mobile phase, is particularly suitable for protein fractionation. However, protein unfolding during HPLC purification is a potential problem to deal with. Preparative HPLC will have increasing importance as equipments suitable for robust large-scale operation are evolved and their costs reduced (Hostettmann et al. 1998). Electrophoresis is another technique mostly used for analytical purposes that has been developed to preparative scale but mostly for characterizing pure enzymes (Fountoulakis and Juranville 2003; Gul-Guven et al. 2007).

2.2.4 Enzyme Formulation

Once the enzyme has been purified to the required level, the preparation must be formulated according to its intended use. Formulation of enzyme preparations is very much like an art and details are kept secret by enzyme producers or revealed to the customer under an agreement of confidentiality. Even though not much attention is given to this production stage in the open literature, formulation is a crucial step in enzyme production especially in the case of industrial enzymes, since it is usually this step of production that confers the producer the competitive edge (Chaplin and Bucke 1990). However, formulation is a main issue not only for bulk commodity enzymes but also for specialty enzymes that must comply with stringent regulations. Regulations will be determined to a great extent by the end use of the enzyme. For instance, enzymes used in detergents, textiles, leather, pulp and paper are regulated as any other chemical used in the manufacturing process; enzymes used in the food and pharmaceutical sectors are regulated by the corresponding agencies (Food and Drug Administration in the case of the USA and the corresponding agencies in other countries). Therefore, regulations vary throughout the world and even between community countries. Even though enzymes are by their very own nature non-toxic and

non-hazardous, they can be allergenic and the producing organisms must be labelled as safe to be approved as producing strains. The risk of enzyme allergy in the detergent industry is well evaluated now since the critical episodes at the end of the 1960s that threatened the detergent industry and wipe off the use of detergent enzymes for several years in the USA (Vanhanen et al. 2000); allergenic problems have also been found for the case of amylases used in bakery (Baur et al. 1994). A subject of major concern now is the production of enzymes from genetically-engineered organisms. There is no consensus on that issue: while some countries prohibit or strictly regulate the production of such enzymes, others require only that production complies with good manufacturing practices (Uhlig 1998; Aehle 2003). Health and safety aspects related to enzyme production and use was revised by Flindt (1978). A comprehensive review on safety regulation for food enzymes have been published recently (Spök 2006). General guidelines for regulatory and safety aspects on enzyme production have been put forward by Chaplin and Bucke (1990).

Enzyme formulation includes final polishing operations, stabilization and standardization (see Fig. 2.1). Final polishing refers to the elimination of contaminants not previously removed. For the case of small-scale production of specialty enzymes for medical applications, polishing includes several key operations for the removal of trace contaminants like pyrogens, endotoxins, nucleic acids and viruses (Anonymous 1999). For the case of bulk industrial enzymes, final polishing usually considers the removal of salts and adjustment of pH if the enzyme is produced in liquid form and drying if produced as a solid preparation. The choice between solid or liquid is by no means trivial. Solid preparations have the benefit of easy handling and transportation and a comparatively higher shelf-life; however, the problem of dust formation in the production facilities is a serious problem so that containment is necessary to reduce risks of allergy among workers. Liquid preparations have also some benefits: containment is simpler, final drying is avoided and dosage can be easier. Each producing company has its own philosophy in this respect, but in general it is the end-use of the enzyme and the customer needs that prevail. Enzymes that will be a part of a solid product (i.e. detergent enzymes) are usually produced as solids, while for application to liquid products (i.e. enzyme saccharification and isomerization for high-fructose syrup production) a concentrated liquid enzyme preparation is more adequate. Vacuum drying and spray drying are the most used operation for producing solid preparations of robust enzymes (Werner et al. 1993; Fickers et al. 2006) while freeze-drying, a more expensive operation, is used for more labile specialty enzymes (Lambert and Meers 1983). With the increasing use of enzymes in non-aqueous media, the requirement of lyophylized enzymes that will act as insoluble catalysts in such media is increasing (Clark 2004; Gupta and Roy 2004). In some cases, special operations are required, as in the case of detergent proteases where encapsulation of the granulated enzyme powder is required to avoid dust formation during production and prevent direct contact of the enzyme with the end user (Maurer 2004). Liquid enzyme preparations are quite common and enzyme concentrates may require some polishing operations like desalting or final concentration. Desalting can be performed by diafiltration (Lambert and Meers 1983; Charcosset 2006) or size exclusion chromatography (Mischitz et al. 1995; Sajedi et al. 2005).

Enzymes are sold in terms of its specific activity so that the producer must ensure certain minimal value to its customer; it is quite common that the labelled specific activity is lower than the actual value obtained at the point of use because of the margin of security with which the enzyme producers work. Stabilization is then a major concern when producing enzymes, since the enzyme product must withstand storage and transportation conditions without significant activity losses. Liquid enzyme products may lose 10–20% of its activity over a period of 4–6 months at room temperature; therefore, refrigerated storage is recommended to increase its shelf-life for a period of a year or longer. Several additives and strategies are used to improve enzyme storage stability. They are intended for preventing microbial contamination and/or preserving enzyme structure. Microbial contamination is a problem in liquid enzyme products since wild proteases can degrade the enzyme to a considerable extent. Absolute filtration and the addition of accepted preservatives (microbicides or microbistats) are the alternatives to ensure the microbiological quality of the enzyme preparation and to prevent spoilage. However, the key issue for enzyme storage stabilization is the preservation of enzyme conformation to prevent aggregation, unfolding or any deleterious change in its native three-dimensional structure. Several strategies are intended for such goal. Proteins are more stable in concentrated solutions and at high ionic strength. Concentration is then not only a way of removing inert material but also an aid in preservation. Some neutral salts can act as stabilizers by promoting hydrophobic interactions in the enzyme molecule, while others can promote chaotropic effects that destabilize the protein structure so that a judicious choice of the salt is required (Lecker and Khan 1998). Hoffmeister series is a good guide to select salt preservants: multivalent anions (like citrate or phosphate) and monovalent cations (like ammonium or alkaline metal ions) are adequate (Chaplin and Bucke 1990). Some cations that are part of the active site can act as stabilizers; for instance: Mg^{++} or Co^{++} in the case of glucose isomerase (Tashpulatova and Davranova 1992), and Ca^{++} in the case of α-amylase (Yutaki et al. 1969; Ogasahara et al. 1970). Low molecular weight polyols, like glycerol and sorbitol (Larreta-Garde et al. 1988; Breccia et al. 1998), and sugars (Chaniotakis 2004) can also act as stabilizers by reducing water activity and preventing protein unfolding (Gianfreda and Scarfi 1991; Joo et al. 2005). Some hydrophilic polymers like polivinyl alcohol, polyvinylpyrrolidone and hydroxypropylcelluloses can stabilize enzymes by substituting the enzyme–enzyme and enzyme–water interactions by less potentially denaturing enzyme-polymer interactions (Chaplin and Bucke 1990). Synthetic polymers are good enzyme stabilizers (Alfani et al. 1984; Bryjak 1995) and are common in enzyme formulation. They may also act by stabilizing the hydrophobic effects within the enzyme molecules. Viscous glass-forming compounds, like trehalose or glycerol, can also exert protection by enveloping the protein structure (Soles et al. 2006). Glycerol may also be used to protect enzymes against denaturation due to ice-crystal formation at sub-zero temperatures. Substrates and inhibitors are used as specific enzyme preservatives by conferring protection to the active site; actually this is a common practice that requires the enzyme preparation to be dialyzed prior to use. Enzymes that have oxidizable amino acid residues in the active site require special protection to avoid inactivation. This is the case of cysteine proteases like

papain (Sanner and Pihl 1963; Klein and Kirsch 1969) and other industrially relevant enzymes like yeast β-galactosidase (Mahoney and Whitaker 1978; Mahoney 1980; Illanes et al. 1998). Thiol compounds, if allowed, may be used as protective agents or else special precautions should be taken to reduce the level of dissolved oxygen in the enzyme product. Some of the structure-stabilizing agents also confer protection against microbial contamination. Stabilization can be obtained by immobilization, but its potential goes beyond the field of enzyme product stabilization, because of its strong impact on enzyme process economics; therefore, enzyme immobilization will be thoroughly reviewed in section 4.1. A recent publication by Springer gives a complete and updated review on enzyme immobilization (Guisán 2006).

The last step in enzyme production is standardization and is a very relevant aspect since the producer must ensure an enzyme product of uniform quality to its customers. Enzymes are produced from biological systems so that variations among production batches are inevitable. Enzymes produced by extraction from natural products are expected to have significant variations from one batch to another. Microbial enzymes produced by fermentation under tightly controlled conditions are expected to have less variation, but differences in raw materials for media formulation may also produce significant variations. In the validation of a pilot process for the production of β-galactosidase from *K. marxianus* we observed variations of $\pm 10\%$ in volumetric activity among 80 production batches (Illanes et al. 1996). To solve this problem, enzymes are diluted with varying amounts of excipients to absorb such variations. Excipients can be inert materials or the same substances used for enzyme preservation or activity enhancement (Schoemaker et al. 2003; Liao et al. 2004). Enzyme products should have a product sheet and a certificate of analysis containing the mot relevant information for the end-user. Specific activity should be expressed in units of activity (desirably international units) per unit mass or unit volume of enzyme product. Enzyme producers usually have their own way of measuring activity that may be irrelevant for the intended use or sometimes not clearly reproducible. A relevant assay of enzyme activity should be developed by the end-user, but enough information on enzyme activity assay should be provided by the producer to check the quality of the enzyme. Storage stability is also relevant to the end-user and must be clearly specified by the producer, desirably in terms of half-life at specified storage conditions. Information of physical properties of the enzyme product like appearance, solubility, water content, swell factor (if solid) are usually provided. However, information on enzyme product composition and excipients is not frequently provided, despite its relevance to the end-user and the fact that in most industrial enzyme preparations the bulk of the product is represented by other proteins, stabilisers, preservatives, salts and inert diluents for standardization.

Any enzyme product released into the market must fulfil the requirements of quality and compatibility with its intended use and these should be granted to the corresponding regulatory agencies by the producer. Current standards consider the validation not only of the product, but also of the manufacturing process. Production should be made according to good manufacturing practices (GMP) and most enzyme producing companies have their corresponding ISO certificates. A leading

company in enzyme production has all its processes certified according to the ISO 9001:2000 standard.

Nomenclature

a	enzyme activity per unit mass of protein
a_0	enzyme activity per unit mass of protein in the intact cell
c	solute concentration
c_B	concentration of protein in the bottom phase
c_T	concentration of protein in the top phase
c_P	polymer concentration
E	enzyme activity recovered
K_P	partition coefficient for protein
K_S	salting-out constant
K'_S	organic solvent precipitation constant
K''_S	polymer precipitation constant
k_R	First order rate constant for protein release
k_D	First order rate constant of enzyme inactivation
m_i	molarity of ions
q_p	specific productivity
p	product molar concentration
p_R	released protein
p_T	total cell protein
PF	purification factor (ratio of outlet to inlet specific activity)
R	universal gas-law constant
S	protein solubility
S_0	protein solubility at infinite dielectric constant
S'_0	protein solubility at zero precipitant concentration
t	time
T	absolute temperature
V_T	volume of top phase
V_B	volume of bottom phase
X	cell concentration
Y	yield of recovery (ratio of outlet to inlet enzyme activity)
Y_T	yield of recovery at the top phase
Z_i	valence of ions
α	growth associated coefficient in Eq. 2.1
β	non-growth associated coefficient in Eq. 2.1
β_S	coefficient in Eq. 2.22
ε	dielectric constant
μ	specific cell growth rate
μ'	ionic strength
σ	surface tension
Γ	excess protein at interface

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