INDUSTRIAL USES OF ENZYMES

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Contents

1. Introduction
2. Sources of Enzymes
3. Enzyme Production
3.1. An Overview on Downstream Processing
3.1.1. Filtration
3.1.2. Centrifugation and Sedimentation
3.1.3. Flocculation and Coagulation
3.1.4. Cell Disruption
3.1.5. Extraction
3.1.6. Precipitation
3.1.7. Chromatography
3.1.8. Finishing Operations
3.1.9. Invertase Production: As a Case
4. Fundamentals on Enzyme Kinetic
4.1. Introduction
4.2. Specificity
4.3. Enzyme Activity
4.3.1. Quantification of the Enzyme Activity
4.3.2. Expression of the Enzyme Activity
4.3.3. Factors Affecting the Enzyme Activity
4.3.4. Briefing on Thermodynamic of the Enzyme catalysis
4.3.5. An Overview on Enzyme Immobilization
5. A Briefing on the Uses of Enzymes
5.1. Baking
5.2. Starch Conversion
5.3. Protein Modification with Enzymes
5.3.1. Introduction
5.3.2. Brewing
5.3.3. Dairy Industry
5.3.4. Miscellaneous uses of Proteolytic Enzymes
5.4. Enzymes in Fruit Juices
5.5. Miscellaneous
5.5.1. Detergents
5.5.2. Effluent and Waste Treatments
5.5.3. Flavor Production with Enzymes
5.5.4. Leather

5.5.5. Textiles
5.5.6. Pulp and Paper
5.5.7. Edible Oils
5.5.8. Enzymes in Animal Feeding
5.5.9. Enzymes as Analytical Tools
5.5.10. Enzymes as Medicines
5.5.11. Enzymatic Biotransformations
6. Conclusion
Glossary
Bibliography
Biographical Sketch

Summary

Enzymes are proteins capable to convert a specific compound (the substrate) into another (product) at a high reaction rate. They also are enantioselective catalysts, which enable them to be used either in the separation of enantiomers from a racemic mixture or in the synthesis of chiral compounds. Although the enzymes utilized today are also found in animals (chimosin, pepsin, trypsin and pancreatin) and plants (ficin, papain and bromelain), the bulk of them are of microbial origin (pectinases, glucoamylase, α-amylase, among others). As the microorganisms present high growing capability, huge amounts of enzymes can be produced in a short period of time as well as the strains can be genetically manipulated, in order to improve their enzyme production capability. Moreover, microbial enzymes are plentiful supplied, well standardized and marketed by several competing companies. Depending on the kind of process, enzymes can be employed in two forms, i.e., soluble (animal proteases and lipases in tannery, for example) or immobilized (isomerization of glucose to fructose by glucose isomerase, for example). Today, the enzymes are envisaged as the core kernel of biotechnology, because they are the main tools for the application of the basic biotechnological techniques (restriction enzymes for DNA-recombinant and cell fusion procedures) and processes (fermentation and cell culture). Moreover, they are largely used in analytical procedures, in human and animal therapy – as medicines or as target to drugs -, and in a variety of industrial processes (baking, brewing, starch conversion, protein modification, detergents, tannery, textiles, edible oils production, effluent and waste treatment, among several others).

1. Introduction

Enzymes are proteins having catalytic capability, i.e., they convert a compound – called substrate – into another at a high reaction rate. For example, the hydrolysis of sucrose into glucose and fructose catalyzed by invertase (a glycoprotein extracted mainly from yeasts) occurs almost instantaneously.

The enzymes have been used along the centuries in industrial processes as tannery, brewing, bakery, dairy, etc. Depending on the kind of the process, the enzymes were employed as soluble catalysts – as the proteases and lipases from animal origin in tannery or gastric juice from the abomasum of the suckling calf in dairy – or through the use of whole microorganisms cells, mainly in fermentation processes like brewing and

bakery. In spite of the role of the microbial cells in the fermentation process was well established by Pasteur in 1876, the enzymatic mechanisms began to be understood only between the end of 19^{th} and the beginning of the 20^{th} centuries, when some enzymes were isolated and produced in industrial scale, mainly invertase and amylase (which hydrolyses the starch), and their kinetic behavior determined through appropriate math modeling. Indeed, the study on the enzymatic catalysis goes on beyond to the foreseeable future, insofar as molecules other than proteins (ribonucleic acids, structurally modified antibodies, synthetic peptides and chemically modified proteins) can present such capability. Besides, the development of the immobilization techniques – in which a catalyst is attached to an inert material aiming to carry out the reaction as a continuous process and to allow the recuperation of the catalyst after the reaction ended – enlarged the use of the enzymes, as will be discussed further.

Nowadays, the enzymes are considered the core kernel of the biotechnology, because they are the main tools for the application of the basic biotechnological techniques(the DNA-recombinant and cell fusion), the target of therapeutics drugs and the indispensable intermediate in all biotechnological processes (fermentation and cell culture). Besides, they have a fundamental role in maintaining the life of any kind of organism, because the occurrence of all cell metabolic pathways depend on the participation of at least one enzyme.

Today, enzymes are used in analytical procedures, in human and animal therapy as drugs or as target to drugs and in industrial processes.

2. Sources of Enzymes

The enzymes can be attained from animals (chimosin, pepsin, trypsin, for example), plants (ficin, papain and bromelain) and microorganisms (pectinases, glucoamylase, α-amylase, among others). **[**See also– *Enzyme Production***].** However, the nature of the source dictates the availability, the cost of source material, the ease of recovery and many other factors.

From the microorganisms, in principle, it can be obtained any kind of enzyme similar to those of animals and plants. The tendency is to use the microbial enzymes on an industrial scale, because of they are plentiful supplied, the producer can control all phases of the enzyme production, the commercial enzyme preparation is well standardized, the enzyme market has a lot of competing suppliers, the source, i.e., the microorganism, presents a high growing capability, so that huge amounts of enzyme can be produced in a short period of time as well as the microbial strain can be genetically manipulated - through the DNA recombinant technique, mainly - in order to improve its enzyme production capability. As a microorganism strain can often produce several different enzymes, the producer can operate the fermentation plant in order to shift the production to one enzyme in detriment of the others, so coping with the market fluctuations.

In particular for enzymes used in food processing, the supplier must inform the costumer if the microbial source was an engineered strain. This is due simply to the fact that the producer has to cope with rigid legal obligations. In that way, the fact that the

enzyme is produced by means of engineered microorganism may require a new approval for the enzyme, and possibly also any food prepared using it may then become a "novel food" and subject to completely different testing for approval. Another point of great interest for the enzyme user – still regarding the use of an engineered strain – refers to the eventual presence into the enzyme preparation of a different compositional spectrum of enzymes and side activities. This fact can affect negatively the costumer's process by introducing new by-enzymes in the reactor leading to unexpected characteristics into the final product. In short, the good supplier-costumer relationship is a critical point regarding the efficient employment of enzymes in industry.

3. Enzyme Production

As all commercially valuable enzymes are proteins, so they can be produced through processes using quite similar unit operations, i.e., extraction from an adequate source (animal, vegetal or microbial), filtration, centrifugation, precipitation, purification, drying, stabilization, standardization and packaging. In biotechnology, all unit operations comprised between filtration and packaging are generally said downstream processing.

The enzymes of animal and plant origin are produced through the maceration of tissues, organs, leaves and fruits – often residual materials from the cattle rising and agriculture activities - followed by the extraction with water or organic solvent. Differently, the microbial enzymes are attained from either procariotic (bacteria) or eucariotic (yeasts, fungi, mainly) cells cultured in a liquid or semi-solid medium, carried out in a special reactor called fermenter, being the process known as fermentation.

The semi-solid culture – used in large scale by the first time in 1894 for the production of amylase from *Aspergillus oryzae* grown in humidified cooked rice mass for one week ("Koji Process") – occurs as follows: a) The semi-solid medium (corn bran, wheat bran, soy bran, rice, barley, for instance), is submitted to vapor jets for cooking and sterilization; b) The semi-solid medium is distributed uniformly throughout several trays, then they are inoculated with the microbial pure strain, in general a fungus; c) The fermentation is left to occur under aseptic conditions for 6 or 7 days. After that, the fermented semi-solid medium (FSSM) containing the desired enzyme can be treated by one out of two possible procedures. If a crude enzyme preparation is desired, the FSSM is directly submitted to drying, grinding and sieving followed by the standardization and packaging of the final product. Otherwise, the FSSM is submitted to the aqueous extraction, filtration, centrifugation, precipitation, purification, drying, stabilization, standardization and package of the final product. This process is quite good when the enzyme is excreted into the solid medium by the microorganism, such as pectinases, fungal amylase and cellulases.

The submerged culture is the preferred fermentation process for growing enzyme producing microorganisms. The microbial cells are maintained in suspension through constant agitation and under controlled growing conditions (pH, temperature, nutrients, dissolved oxygen concentration, among others). The medium is an aqueous solution of substances readily available in large quantities at low cost – raw material costs will be related closely to the value of the final product, mainly in the case of the enzymes,

which are mostly low volume and medium cost products -, such as starch hydrolysate, molasses, corn steep liquor, whey and many cereals. At the completion of the fermentation the enzyme may be present within the microorganism or excreted into the medium. When inside the cell, then the suspension is centrifuged or filtered and the supernatant or filtrate is discharged and the cell cake collected; otherwise the cell cake is discharged and the liquid phase is collected. Depending on the enzyme to be intra or extracellular, the fermentation must be carried out in a way that the cell growth is optimized or not, respectively.

3.1. An Overview on Downstream Processing

The downstream comprehends several unit operations which are aimed at concentration and purification of the enzyme, often present in a raw extract of disrupted cells. The main unit operations employed are filtration, centrifugation, sedimentation, flocculation, coagulation, cell disruption, extraction, ultrafiltration, precipitation, crystallization, chromatography, evaporation, drying and packaging.

The first task in formulating a downstream strategy is to define or acknowledge the required purity of the enzyme. The allowable ranges of impurity concentrations, and the specific impurities which may be tolerated, will be dictated by the end-use of the catalyst. All microbial enzyme products to be used in foods or medicines are required to meet stringent purity requirements with regard to toxicity. At present only a small number of microorganisms are used for enzyme production. Responsibility for the safety of an enzyme product remains with the manufacturer. In practice, a safe enzyme product should cause no allergy and be free from toxic substances and pathogenic microorganisms. It must be borne out that the enzyme recovery usually accounts for a large portion of the product cost and in some cases is the major manufacturing cost.

3.1.1. Filtration

The rate of passage of a liquid through a filter of unit area is dependent on the pressure difference applied, the resistance of the filter material, the viscosity of the liquid, and the resistance produced by cake already present. Thus the effectiveness of a filter will initially be high, but will fall as material accumulates and perhaps compresses. Filter aids, such as diatomaceous earth, retain finer particles and are valuable in enzyme isolation, but they tend to occlude liquor containing the enzyme and will damage downstream equipment if allowed to pass into the filtrate. Siliceous materials constitute a health hazard and must be treated with care, particularly when dry.

The commonest forms of industrial filter are the plate and frame press, and the rotary drum filter. The former consists of filter cloths trapped between corrugated plates; fluid passes in at one side of the cloth and out, via the corrugations, to a pipe serving all the units in the battery. To remove the solids the plates must be parted manually or semi-automatically and the cloths thoroughly cleaned. Fungal mycelia are readily removed in this manner, as are bacteria after flocculation and enzymes precipitated by ammonium sulphate.

In the rotary drum filter, vacuum is applied to the inside of a hollow drum rotating in a

trough containing the material to be filtered. Sediment accumulates on a filter cloth from which it may be removed by a multiplicity of methods.

When a common paper or cloth filter is substituted by a membrane capable to separate from a suspension or solution, respectively, particles (including molecular aggregates) or molecules (or macromolecules) with mean diameter smaller than 5μ, the unit operation would be named microfiltration, ultrafiltration, nanofiltration or reverse osmosis (a particular kind of ultrafiltration, in which the membrane allows the passage of solvent molecules only). The microfiltration membrane has a mean pore diameter between 0.05 and 5μ, meanwhile the ultra, nano and reverse osmosis membranes have pores running through it of average diameter 500-5000 Å. Thereby, these membranes are capable for separating substances having a molecular weight between 500 and 300.000. Very small molecules will pass through the membrane and large ones will be retained at the filter surface. Intermediate-sized molecules will be retained within the structure of the membrane and will eventually block the pores. For this reason microporous filters have been superseded by diffusive membranes. Indeed, the diffusive membranes are formed by thousands layers of special polymers toughly superposed, through which the molecules with a certain mol weight can trespass each layer when submitted to an electric field - applied across the membrane - or a concentration, pressure or temperature gradient. In other words, the ultrafiltration is basically a molecular diffusion throughout the membrane. Thus the transport of a molecule across the membrane requires kinetic energy and occurs more readily at high temperatures. A readily permeable membrane would be readily hydrated and composed of a polymer with a strong specific affinity for its solvent. Conversely, a relatively unhydrated, rigid membrane would have low permeability, particularly when there was reduced affinity between the membrane polymer and the solvent.

In the most general sense a synthetic membrane is a barrier which separates two phases and restricts the transport of various chemical species in a rather specific manner. A membrane can be homogeneous or heterogeneous, symmetric or asymmetric in structure, it may be neutral, may carry positive or negative charges, or may be bipolar. Its thickness may vary between less than 100 nm to more than a centimeter. The electrical resistance may vary from several megaohms to a fraction of an ohm. The term "membrane" includes a great variety of materials and structures and a membrane can often be better described in terms of what it does rather than what it is. All materials functioning as membranes have one characteristic property in common: they restrict the passage of various chemical species in a very specific manner. Asymmetric membranes are surface filters and retain all rejected materials at the surface where they can be removed by shear forces applied by the feed solution moving parallel to the membrane surface. The so called "membrane polarization" is a phenomenon arising as a build-up of rejected solute at the membrane surface. This impedes solvent flux so that eventually it may no longer respond to an increase in hydraulic pressure. Thus the design of ultrafiltration apparatus is aimed at minimizing the build-up of such polarization layers, in small scale apparatus by stirring, and in larger equipment by maintaining high fluid flow rates over the membrane surface. They are used primarily in pressure driven membrane processes, such as reverse osmosis, ultrafiltration or gas separation. In other words, applications of these processes to enzyme isolation – or to another kind of biomolecules - at the pilot scale and industrial level are centered upon concentration.

As the micro and ultrafiltration membranes have been developed and improved, an apparatus known as membrane reactor (MR) has also been developed. This device can be used in enzyme continuous processes as an alternative to the conventional reactors (mainly, the continuous stirred tank reactor [CSTR], the packed- or fluidized-bed reactor). As a general rule, the conventional reactors operate with the enzyme linked to an insoluble inert support, whereas the membrane reactor can operate with the enzyme free in solution or linked to the membrane (the enzyme is confined on the surface or within the membrane). The MR can be shaped either as a CSTR coupled with a semipermeable membrane or as a hollow-fiber reactor, i.e., a tank without stirring filled with a sheaf of straight-lined hollow-fiber tubes of semipermeable membrane. However, the membrane/biocatalyst arrangement can involve or not an interaction between them. If they are linked, the membrane acts as catalysis and separation surface simultaneously; otherwise, it functions only as a separation surface. When the enzyme is in the soluble form, the membrane reactor can involve the recycle of the enzyme (the CSTR and the ultrafiltration membrane module are connected in series, generally called bimodule-MR) or not (the ultrafiltration membrane is adapted to the bottom of the CSTR as in a stirred ultrafiltration cell, generally called unimodule-MR). The main operational features of the MR are the homogeneous catalysis, the high activity per unit of volume, the absence of conformational and difusional effects and – if needed – the MR can be operated under aseptic conditions as well as with multienzymatic systems. Those features are gradually shifting the industry preference from the traditional immobilized enzyme reactors (packed- or fluidized bed reactor and CSTR) to the MR. This tendency is borne out by the use of the membrane reactor in a dozen processes, resulting in a large variety of products (gluconic acid, glucose, fructose, cyclodextrins, cathecol, among others).

3.1.2. Centrifugation and Sedimentation

These unit operations are both based on density differences between insoluble particles and the surrounding fluid. Sedimentation relies on gravity and settling to achieve solid-liquid separation, and is generally performed in rectangular or circular flow tanks. Centrifugation, in turn, involves mechanical application of a centrifugal force to obtain a solid concentrate and clarified supernatant.

Centrifugation has become a widespread technique for the removal of solids, particularly under circumstances where filtration is ineffective - when, for instance, it is desired to remove a precipitated protein, which is frequently gelatinous or colloidal in nature – or unwanted.

There are several types of centrifuges for enzyme isolation, being broadly employed the tubular bowl centrifuge, multichamber centrifuge, disc bowl centrifuges, solid-bowl scroll centrifuge and perforate bowl basket centrifuge.

At this point, the ultracentrifugation unit operation must be remembered. The ultracentrifugation is based upon use of the ultracentrifuge, in which materials may be subjected to centrifugal forces many thousands of times stronger than the force of gravity. Ultracentrifuges have been constructed in which the rotor revolves 60,000 times per minute, giving centrifugal forces of the order of 500,000 times gravity.

Ultracentrifugation is valuable in determining the purity of colloids, and it has been applied especially to proteins. When solutions of proteins or other colloidal solutions are placed in glass or quartz cells in the rotor of the ultracentrifuge, they are subjected to a force depending on the angular velocity of the centrifuge and the distance of the colloidal particles from the axis of rotation. The rate at which the particles move is dependent upon this force and also upon the shape, size, and density of the particles, and upon the density and viscosity of the suspending medium. With homogeneous substances where the particles are all alike, the particles move under the centrifugal force as a sharp single boundary in the medium, whereas mixtures of particles of different kinds give multiple boundaries and fuzzy zones. For example, the ultracentrifugation is used in the separation and purification of virus proteins mixed with tissue proteins. The virus proteins, of very large molecular size, are centrifuged to the bottom of the cell at a centrifuge speed at which the smaller tissue proteins are left suspended in the medium. Also, ultracentrifugation is used in the separation of mitochondrial, microsomal, and nuclear fractions from disrupted tissue cells.

3.1.3. Flocculation and Coagulation

Coagulation is the result of direct adherence among very small particles present in a medium, due to the neutralization of the charge on the particles by adding polyvalent ions of opposite charge, thus permitting coalescence. Inorganic salts have long been used for this purpose while more recently organic polyelectrolytes have gained in popularity. Flocculation, in turn, refers to the formation of much more open aggregates in which the flocculating agent acts – a variety of natural polymers, such as gelatine, and a number of synthetic polymers (electrolytes or not) - as an extended bridge between particles. Inorganic ions cannot cause flocculation although they may be used to neutralize charge and assist flocculation. However, organic polyelectrolytes may cause simultaneous coagulation and flocculation. These techniques have been applied to whole cells, cell debris, and soluble proteins (enzymes included).

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Biographical Sketch

Prof. Dr. Michele Vitolo attained the degree in Pharmacy at the Sciences Pharmaceutical School of University of São Paulo (FCFUSP), Brazil. At the same University attained the Master degree in Biochemical and Pharmaceutical Technology and the PhD. in Biochemistry. Today, he is full Professor at FCFUSP, in which teaches the under-graduate course “Pharmaceutical Biotechnology” and the post-graduation courses “Industrial Uses of Enzymes” and “Application of Immobilized Enzymes and Cells”. Over the last three decades has published about 150 full papers and more than 200 abstracts and short communications in the proceedings of international congresses. Supervised more than 30 Thesis. Two patents on the application of enzymes in sugar industry were asked to the Brazilian Patent Office. Today, his research interest relates to the use of membrane reactor for carrying out enzyme catalyzed reactions.