ENZYME PRODUCTION

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Summary

Commercial enzyme production has grown during the past century in volume and number of products in response to expanding markets and increasing demand for novel biocatalysts. Microorganisms constitute the major source of enzymes, but several enzymes are also obtained from renewable animal and plant sources. Traditional enzyme production relied on the natural hosts as raw materials, however genetic engineering has now given a choice for producing sufficient quantities of enzymes in selected production hosts including microorganisms and transgenic plants.

Production of a new microbial enzyme starts with screening of microorganisms for desirable activity using appropriate selection procedures. The harsh environment to which several enzymes are subjected during process applications has given impetus to screening of extremophiles for enzymes having desirable features of activity and stability (see also *Biotechnological Exploration of Extremophiles in the Genomic and metagenomic era*). The level of enzyme activity produced by an organism from a natural environment is often low and needs to be elevated for industrial production. Increase in enzyme levels is often achieved by mutation of the organism. An alternative strategy that has gained favour is production of the enzyme in a recombinant organism of choice whose growth conditions are well optimized and whose GRAS status is established. Random or site-directed mutagenesis with the purpose of engineering the activity and stability properties of an enzyme prior to its production is becoming a common practice. The microorganisms used for enzyme production are grown in fermenters using an optimized growth medium. Both solid state- and submerged fermentation are applied commercially, however the latter is preferred in many countries because of a better handle on aseptic conditions and process control. The enzymes produced by the microorganism may be intracellular or secreted into the extracellular medium.

Isolation and purification, i.e. downstream processing of enzyme from the raw material constitutes the subsequent key stage in the production process. The desired level of purification depends on the ultimate application of the enzyme product. The industrial bulk enzymes are relatively crude formulations while speciality enzymes undergo a thorough purification to yield a homogeneous product. A traditional downstream processing scheme involves stages of clarification for separation of the enzyme from the solids comprising the raw material, concentration to reduce the process volumes, and

purification to separate it from other soluble contaminants. In case of the intracellular enzymes, disruption of cells or tissue for release of the product is among the primary separation steps. There is a choice of different separation techniques for each stage. Chromatography is the major technique for high-resolution purification of enzymes. Some separation techniques allow integration of the downstream processing stages required for purification thus reducing the number of steps and hence the production costs. The enzyme is finally formulated as a liquid or solid product. In either case, stabilizing additives are added for rendering long shelf life to the product. Some enzymes are immobilized to solid supports or enzyme crystals are cross-linked to render them insoluble and stable for repeated or long term use in a process application. Large scale production of enzymes has to comply with the standards set by International Organization of Standardization for ensuring quality and production efficiency, and also environmental management control, whenever applicable.

1. Introduction

The production of enzymes is central to the modern biotechnology industry. The traditional industrial enzymes continue to have expanding markets, and the recognition of potential to use biocatalysis in various industrial sectors for new applications generates demand for enzymes with novel activities and/or improved stability (see also *Microbial enzymes for food processing*). Man has utilized enzymes throughout the ages either in the form of vegetables rich in enzymes, or as microorganisms used for a variety of purposes, for instance in brewing, baking, and in cheese production. However, it was only in the 19th century that the various biological conversions were ascribed to the action of enzymes. This was initiated by the isolation of an enzyme complex from malt by Payen and Persoz in 1833, termed ‘diastase’ that converts gelatinized starch into sugars, primarily maltose. The history of modern enzyme production really began in 1874 when the Danish chemist Christian Hansen first produced rennet by extracting it from dried calves´ stomachs with saline solution. Apparently, this was the first enzyme preparation of relatively high purity used for industrial purposes. During the early part of the last century, in the Far East, an age-old tradition involving the use of mould fungi called *koji* (see also *Food fermentation and processing*) in the production of certain foodstuffs and flavour additives based on soya protein and fermented beverages, formed the basis on which the Japanese scientist Takamine developed a fermentation process for the industrial production of fungal amylase. The process included the culture of *Aspergillus oryzae* on moist rice or wheat bran, and the product was called 'Takadiastase' which is still used as a digestive aid. The value of the industrial enzymes market was estimated to $ 2 billion, and has increased at an average annual rate of 3-5 percent during the past decade. A number of companies are competing in the industrial enzymes business, Novozymes dominating with 45 percent of sales, followed by Danisco that holds a 20 percent share of the market.

The industrial or bulk enzymes include proteases, amylases, lipases, etc. which are required in large volumes, but have an inherently low unit value so that they demand significantly lower manufacturing costs. On the other end of the scale is the therapeutics sector with products such as urokinase, which are produced in lower volumes and at inherently greater manufacturing cost. In between these two lie the diagnostic enzymes. Table 1 lists some of the companies, which are producers of enzymes belonging to the

different categories.

Amano Pharmaceutical Co.	**Nagoya, Japan**
BASF	Ludwigshafen, Germany
Biocon India	Bangalore, India
Biozyme Laboratories	South Wales, UK
Danisco	Copenhagen, Denmark
DSM	Delft, The Netherlands
Finnzymes	Espoo, Finland
Novozymes	Bagsvaerd, Denmark
Genzyme	Cambridge, MA, USA
Gist Brocades	Delft, The Netherlands
New England Biolabs	Beverley, MA, USA
Prodigene	College Station, TX, USA
Rhone-Poulenc	Cedex, France
Roche Molecular Biochemicals	Indianapolis, IN
Worthington Biochemical Corporation	Lakewood, NJ, USA

Table 1: Some of the enzyme manufacturing companies

The technology for producing and using commercially important enzyme products combines the disciplines of microbiology, genetics, biochemistry and engineering, which have developed and matured through time both singly and in an interactive manner. Demands for new enzymes arise from the development of new processes or from the unsatisfactory performance of known enzymes in established processes. The revolution in gene technology over the last two decades has had a big impact on enzyme industry. Genetic engineering techniques have enabled enzyme manufacturers to produce sufficient quantities of almost any enzyme no matter what the source, while protein engineering allows the properties of the enzymes to be adjusted prior to production. This chapter provides an overview of enzyme production processes starting from raw material to the finished product, and gives an insight of the various alternative technologies available for different stages of production.

2. Enzyme Source

The primary consideration in the production of any enzyme relates to the choice of source. In most cases, the desired activity can be obtained from several sources. Traditionally, however, the choice of source has been more restricted for some enzymes. For example, the enzyme rennet was until recently obtained from the stomach of suckling calves; the corresponding microbial enzyme led to an off-flavor in the cheese produced. Today, recombinant DNA technology is used to produce the calf enzyme in microorganisms.

Microorganisms represent an attractive source of enzymes as they can be cultured in large quantities in a relatively short period by established methods of fermentation (see also *Microbial Cell Cultivation*). However, the level of production of a particular enzyme varies in different microorganisms, and moreover the enzymes often differ in

composition and properties. One usually finds that the closely related organisms have enzymes with nearly similar properties, while unrelated organisms have enzyme systems that differ widely. The most critical feature of the organisms for producing industrially significant enzymes is their GRAS (generally regarded as safe) status, which implies that they must be non-toxic, non-pathogenic and generally should not produce antibiotics.

The GRAS listed microorganisms include fewer than 50 bacteria and fungi. Examples are the bacteria including *Bacillus subtilis*, *B. licheniformis*, and various other bacilli, lactobacilli, *Streptomyces* species, the yeast *Saccharomyces cerevisiae*, and the filamentous fungi belonging to the genera *Aspergillus*, *Mucor*, *Rhizopus*, etc. In case of *Bacillus*, mutants are selected that can no longer form spores.

Since *Aspergillus* cultures are frequently inoculated with conidia, enzyme production using these fungi relies on good spore formation. Most of the bulk enzymes (hydrolases) are secreted by the microorganisms directly into the culture medium, while some enzymes e.g. penicillin acylase and glucose isomerase are intracellular. For some applications, it may not be necessary to isolate the enzymes but the microbial cells themselves are used as enzyme source.

The organism is preferred which gives high yields of enzyme in shortest possible fermentation time. The production strains used in industry are normally modified by genetic manipulation to have high levels of production.

A common trend in the industry today is that the gene coding for the enzyme with desired characteristics is transferred into one of the selected microbial production strains which have all the required features of safety and high expression levels and for which the growth medium has been optimised, hence avoiding the need for optimization of individual enzyme producing strains.

A further short cut in the search for the right enzyme has been made in eliminating the step of screening, isolation and cultivation of microorganisms which may either be present in low number or produce low levels of the activity. Instead, DNA is directly isolated from an environmental sample and the possession of the desired activity is located using an appropriate gene probe. The gene is cloned and expressed in the desired production organism (see also *Methods in Gene Engineering*).

Despite the advantages of microorganisms as enzyme source, some enzymes are still economically produced from plant and animal sources. This is possible because of sufficiently high amounts of these enzymes in such sources and also as a means to convert inexpensive, renewable material like agricultural and slaughter waste into value added products (sees also *Environmental Biotechnology*).

Examples of the enzymes isolated from plants include several proteases such as papain, ficin and bromelain, and peroxidase. As mentioned above, rennet has been among the most industrially significant enzymes obtained from animal tissue. The other enzymes obtained from animal sources e.g. proteases like trypsin, chymotrypsin and urokinase, lactate dehydrogenase, lysozyme, etc., have diverse applications in industry, analysis,

and therapy.

In recent years, protein production in transgenic animals (see also *Transgenic animals*) and –plants (see also *Transgenic plants*) has attracted attention. Focus on transgenic animals (e.g. sheep, cattle) has been for the production of therapeutic proteins. The expression of the foreign gene is targeted to the mammary gland so that the protein is secreted directly into the milk.

Although both pharmaceutical and industrial proteins have been expressed in transgenic plants, they are suggested to be ideal bioreactors for production of the latter category of proteins. Production of bulk enzymes like α-amylase, xylanase, phytase, etc. combines the advantages of low production costs of plant biomass with the minimal purification requirements for such products.

The Dutch company, Gist Brocades has taken the lead in industrial scale plant based production system for phytase, an enzyme used as a feed additive in livestock farming for the purpose of breaking down the antinutritional factor, phytin into myoinositol and phosphate. This production is done with an idea of not having to extract the enzyme from the plant but rather to supplement the feed directly and thereby avoid the need to add the enzyme exogeneously.

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Biographical Sketch

Rajni Hatti-Kaul is Associate Professor at Department of Biotechnology, Lund University, Sweden. She has published more than 70 scientific papers and edited one book. Her research interests are in the fields of enzyme and microbial technology, bioseparations, and protein stabilization. Her work encompasses also biotechnology education and research in developing countries. She received a Ph.D. in Biochemistry in 1984 from University of Bombay, India.