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Commercial Enzymes Production by Recombinant DNA Technology: A Conceptual Works

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Abstract: Until the advent of genetic engineering, enzyme producers were limited in their ability to produce innovative products for the marketplace. They were constrained to isolate enzymes from organisms approved for the industrial use. The desired characteristics could be enhanced only using classical in lit agenesis techniques. When these methods failed, no alternatives were available. Commercialization depended on incremental yield improvements gained by continuous programs of strain development. The ability to use recombinant DNA (rDNA) techniques has removed many of these barriers. Enzyme producers recognized early the potential for commercialization new products using genetic manipulation. They worked with a wide variety of single-celled organisms that were simpler and thus, easier to understand then the higher orders of plants and animals. The organisms already were well characterized for growth and expression rates. Short life cycles allowed rapid testing. These systems were ideal for genetic manipulation using rDNA techniques. Genetic engineering, combined with an understanding of biocatalysts to predict alterations for enzyme improvements, is revolutionizing the production and use of enzymes in the marketplace. Offering a recombinant produced product represents the culmination of a long and complex effort on the part of a multitude of disciplines: molecular and microbiology, X-ray crystallography, enzymology, protein and organic chemistry, biochemistry, fermentation and formulation engineering, assay chemistry and technical service/applications, marketing, sales. Because of the variety of disciplines required, a critical mass is needed to innovate products successfully and them to market. The continued proliferation of novel enzyme products requires development of core technologies so complex and expensive that they can be justified only rDNA technology must consider regulatory issues, ownership protection and consumer acceptance.

Key words: rDNA, enzyme, gene, RNA, protein

INTRODUCTION

Industrialization of biotechnological processes has led to the rapid use of enzymes in staggering quantities in food processing, animal and poultry feed, detergents, leather and tanning, textile, pharmaceutical, sugar and starch, oils and fats, beverages and several other industries. Enzymes have become an important and indispensable tool in many industries in recent years all over the world, they are natural catalysts and boost the chemical reaction several fold. The application of enzymes had been dominated in three major areas: food, pharmaceutical and feed. Enzymes have been extensively exploited commercially in the food, detergent, pharmaceutical and baking and textile industries.

Specialty enzymes used in new drugs, antibiotics, diagnostics and analytical techniques account for only 10% of the market, in 1989, the demand of industrial enzymes was US$ 650 million which rose-up in 1993 to US $1 billion and it is growing markedly due to proliferation of bio-based industries all over the world. The important world producers of enzymes are: NOVO, Nordisk, GENECOR International, GIST Brocades and Pfizer etc. In India, the numbers of enzyme producing industries are increasing substantially. In the whole world, the enzyme consumption rate is increasing day by day. It is believed that world wide use of enzymes will be increased[1]. This is because enzymes are often products of single genes and modern methods of recombinant DNA technology can be used to enhance their yields and modify their specificities in suitable microbial hosts. To reduce the expense of producing and using enzymes, bacterial stain development, enzyme and cell immobilization and stability enhancement are being

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explored. The race to improve production yields and quality while lowering costs is placing an increasing pressure on industry researchers to be up to date in respect of novel cost-effective applications of industrial enzymes. Environmentally sound applications for enzymes in areas such as food processing, fine and specialty chemicals, pharmaceuticals, textiles, wood pulp and paper and detergents has created a creating a critical demand for new technologies. Today, scientists are creating modified enzymes that have properties superior to those of nature enzymes. These genetically modified enzymes are created in three ways[1].

- Genetic manipulation of the organism to select a variant of the natural enzyme;
- *In vitro* mutagenesis of the gene and its expression in a suitable host;
- Production of a completely new enzyme by creating a catalytic antibody.

Historical perspective: In the late 1800s, discoveries in biology, chemistry and enzymology culminated in what is now recognized as the beginning of the biotechnology age. In 1865, Gregor Mendel proposed the basic rules of heredity, that traits were controlled by elements we call genes. Garrod, who suggested that genes work by controlling the synthesis of specific enzymes[2]. As analytical procedures improved, progress was made in defining the actual structure of proteins. Early in the 20th century, German chemist Emil Fischer established that proteins were polymers of nitrogen-containing organic molecules called amino acids. Proteins could contain up to 20 different amino acids in a highly varied and irregular order, but any given type of protein maintained a unique amino acid sequence.

Since all enzymes were shown to be proteins, the key question became how genes participated in the synthesis of enzymes. Although it was known that genes possessed a unique molecular constituent, deoxyribonucleic acid (DNA), there was no way to show that DNA carried genetic information. James and Francis sorted out the molecular structure of DNA[3,4]. More importantly, they recognized and proposed its function: the storage and replication of genetic data in the sequence of the base pairs. The tools were close at hand for manipulating genes in organisms, producing proteins of interest and altering them for different characteristics. Methodologies were soon developed to implement the postulated ideas. Knowing the primary structure and the three-dimensional conformation, proposed a precise chemical mechanism for the hydrolysis of a substrate by an enzyme (lysozyme)[5]. With succeeding work on other enzymes, this effort indicated that the chemical mechanisms of enzyme-catalyzed reactions could be understood.

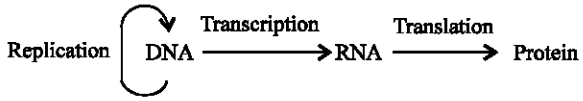
Simplified model (The process of protein or enzyme production in cell): Crick hypothesized a central dogma: that chromosomal DNA functions as the template for synthesis of ribonucleic acid (RNA) molecules (transcription), which move to the cytoplasm where they determine the arrangement of amino acids in proteins (translation).

The arrows indicate the direction for transfer of genetic information. The arrow encircling DNA shows that it is the template for its own replication. The arrow between DNA and RNA shows that RNA molecules are made on (transcribed off) DNA templates. Correspondingly, all protein sequences are dictated by (translated on) RNA templates. Thus, the genetic code, the relationship between nucleotide sequence and amino acid sequence, has been deciphered and appears to be structurally and functionally identical in all living organisms: microbes, fungi, plants and animals[4].

The DNA molecule consists of two molecular chains coiled in a helix and held together by hydrogen bonds (a weak noncovalent chemical bond). The subunits of each chain are nucleotides, which contain phosphoric acid and the sugar deoxyribose coupled to one of four nitrogenous bases: adenine (A), guanine (G), cytosine © and thymine (T). In the double helical DNA molecule, A always pairs with T and G always pairs with C in the opposite chain. Watson *et al.*[4] first postulated the significance of this base pairing: the sequence of one strand exactly defines that of its partner and allows self-replication.

A gene is a DNA segment that carries information for synthesis of a specific RNA molecule and therefore, usually of a specific protein (Fig.1). The gene defines the amino acid sequence in the form of triples of bases, called codons. The gene also contains various signal or control regions, called promoters and terminators, which are involved in the regulation and expression of the gene product (when and how often the RNA corresponding to the protein will be synthesized). Start and stop codons define the beginning and end of the amino acid sequence. The terminator region defines the end of the gene in the DNA molecule.

Protein production requires several steps. First, the double-stranded DNA unwinds and separates in the vicinity of the gene. Specific enzymes move along a single



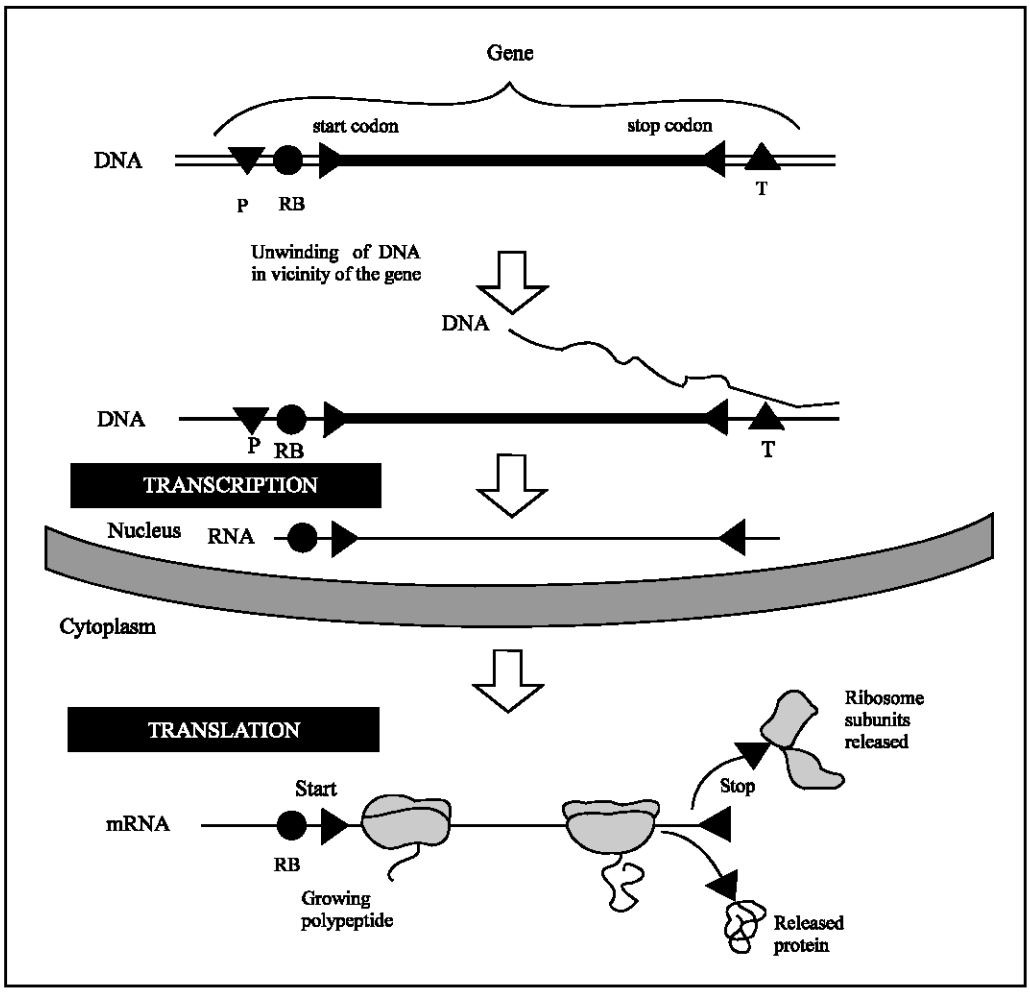


Fig. 1: Expression of a gene to produce protein. P, promoter; RB, ribosome binding site; T, terminator

strand, "reading" the bases. Using base pairing, they construct another nucleic acid molecule, RNA, as a complementary copy of the original sequence (a process called transcription).

RNA is chemically alike to DNA but contains the sugar ribose rather than deoxyribose and the base uracil (U) than thymine. Once RNA containing genetic information from DNA is constructed, it moves from the nucleus to the ribosomes located in the cell cytoplasm and functions as a template for protein synthesis (translation). When the RNA reaches the cytoplasm, it is called messenger RNA (mRNA). The ribosome's attach them at the ribosome binding sequence and synthesize the protein from individual amino acids available in the cytoplasm.

Classical mutagenesis: Since DNA contains all the information required for proper growth and maintenance of the cell, copying DNA must be essentially foolproof to insure correct reproduction and replication of the organism. Mutation is a change in DNA, a variation in the nucleotide sequence, which is passed to succeeding generations. This process happens continuously in nature at very low levels. In the laboratory, mutations can be induced by:

1. Errors in base pairing at transcription or translation.
2. Interference with the enzymes that synthesize or repair DNA.
3. Mutagens, chemicals that interfere with DNA function (an analog incorporated into DNA instead of a base).
4. Exposure to ultraviolet radiation that is absorbed by the DNA and damages it.
5. Direct chemical attack on the DNA[6].

A program of directed mutation plays an important role in enzyme production improvement. Such a program

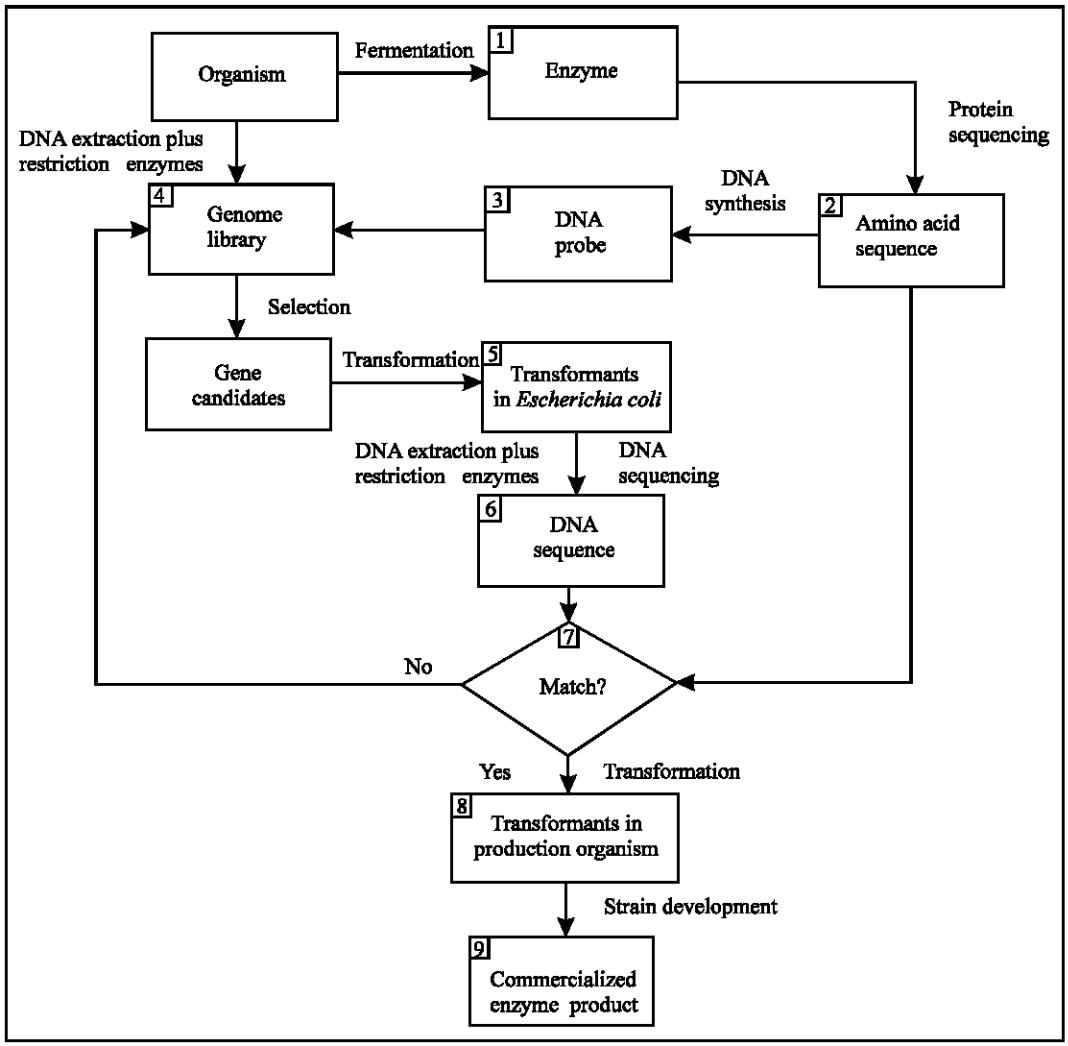


Fig. 2: Summary of steps to clone a gene into a new host[6]

is very effective and used extensively. Most production organisms were developed this way. Mutagenesis programs in the past have given a steady increase in productivity over time by selecting higher yielding mutants or preferential producers of a selected activity. The main disadvantage of classical mutagenesis is that it is random and time-consuming. Organisms can be manipulated and the selection process strictly adhered to and still the objective is not achieved. Also, a characteristic cannot be manipulated if it is not expressed in the organism at all. Classical methods combined with genetic engineering offer a more complete approach to enzyme improvements.

Recombinant DNA technique for production of enzymes: In the late 1970s, a set of methodologies emerged that permitted isolation of discrete DNA segments that could then be inserted into living cells. The key to this method was the discovery of restriction and ligase enzymes to cut and join DNA. The ability to manipulate and recombine DNA allowed a more focused approach to mutagenesis and created the potential for the manipulation of industrial microorganisms to synthesize valuable new enzymes or food ingredients.

Gene cloning: Figure 2 summarizes the steps in cloning (producing an identical copy of a gene in a new host organism. The remainder of this section provides a more detailed description of those steps.

Step-I. Obtain an enzyme of interest with desired characteristics: Often enzymes with characteristics needed for a specific application are produced at low

levels, in obscure organisms unsuitable for modern fermentation processes, or in hosts unacceptable for food applications. Such enzymes are likely candidates for cloning into production organisms.

Step-II. Determine the amino acid sequence of the enzyme: Automatic analyzers are now available that perform the chemical analysis steps once done manually. Phenylisothiocyanate is used to react with the N terminal of the amide bond. Trifluoroacetic acid is added to cleave one amino acid residue at a time, releasing a cyclic compound. This isothiocyanate derivative is analyzed, usually by High Performance Liquid Chromatography (HPLC). The protein sequence then can be read by comparing the results against standards. Because of degradation of the protein in the system over time, less than 30 amino acids in a sequence can be analyzed in a single run. Overlapping sections of the enzyme protein must be analyzed so the sequence can be assembled. The process often takes weeks of effort; a degree of luck also helps accomplish the complete sequencing pofoano enzyme.

Step-III. Construct a DNA probe: A probe is a single strand of DNA that encodes a section of the desired gene. A probe is made by synthesizing a short segment of DNA using radio-labeled nucleotides. Since the probe will be used to find a specific gene in a pool of DNA, a sequence is chosen to match a unique section of the gene of interest. Sequences containing codons for methionine or tryptophan are often used because only one codon specifies each of these amino acids. Sometimes sequences can be guessed because of a species tendency to use some codons preferentially.

Step-IV. Screen a genome library: A genome library is a tool of DNA segments that is likely to contain the gene of interest. Using standard procedures, DNA is extracted from the organism that makes the desired enzyme. The DNA is cut using restriction enzymes. Segments too small to contain a full gene are discarded. The mix is separated by size using gel electrophoresis. A quantity of the DNA probe is added and the material is warmed. Under controlled heating, the double-stranded DNA will "melt" and disassociate. After cooling, it will come back together; however, the single-stranded probes will bind to the sections they match. If bands can be detected autoradiographically, they contain sequences similar to those of the gene. The band is cut out and the DNA it contains is replicated[7].

Step-V. Transformation: The process of genetic transfer of DNA is called transformation. DNA fragments are inserted into plasmids or phages, which are used because they are easy vectors with which to transfer foreign DNA into intact cells. The DNA of interest, inserted into the plasmid or phage, is mixed with organisms whose cell walls have been made receptive by chemical treatment. These organisms are usually special strains of *Escherichia coli* that have been characterized and modified carefully so that they can be transformed easily[7]. The DNA enters some of the organisms and is replicated with the host DNA. The transformation process is random and is not successful for every organism in the experiment. Therefore, an important step is screening the population for the organism that has taken up the new DNA from among the many who have not.

Transformed organisms are selected by adding markers to the gene before transformation. Markers are sections of DNA inserted with the desired gene to make selection and detection of successfully transformed organisms a straightforward process. Often the marker confers an ability to survive under specific growth conditions and must be used with specially modified hosts that lack the specific trait. The markers will be removed before transformation of the gene into a production organism. The transformed organisms, which contain the DNA of interest, are grown to provide a source of recombinant DNA for further testing and manipulation.

Step VI. Sequencing the chosen DNA: The ability to sequence DNA was one of the more important breakthroughs that made genetic engineering practical. Two methods for accurately establishing the order of the four bases A, G, C and T along a given DNA segment have been developed. DNA strands are separated (since only one strand needs to sequence) and labeled, at one end by enzymatically removing the phosphorus atom and replacing it with a radioisotope of phosphorus. The solution of single-stranded DNA is then divided into four pools.

In Maixam and Gilbert's method[7], each pool is treated with chemical reagents, for example, dimethysulfate or hydrazine, using conditions that selectively destroys one or two of the four nucleotides in each pool. These reactions result in four pools of radioactively tagged strands of all possible lengths, ending in the nucleotide destroyed. In practice, nucleotides destroyed are G, G+A, T+ C and C, each in a separate pool. Sanger's method makes use of the enzyme DNA polymerase to produce fragments ending in specific bases. The pieces of single-stranded DNA serve at templates for synthesis of complementary strands of DNA. The nucleotides necessary to form the complementary strand are added to each of pools along

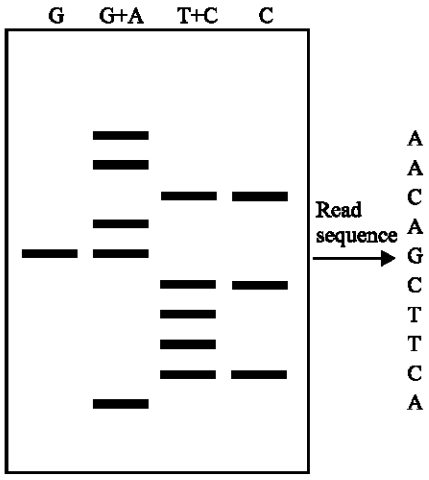


Fig. 3: Reading the sequence of a DNA fragment

with the polymerase enzyme. In addition, a small amount nucleotide analog, a radioactive 2′, 3′-dideoxynucleotide, containing one of them the four base types is added to each pool. The concentration is controlled so only one base will be incorporated per strand. The polymerase builds the complementary strand along the DNA fragments until one of the base logs is inserted in the chain. Further strand elongation is blocked; since the analog interferes with placement of the next nucleotide. The result is four pools of radioactive complementary strands of DNA of all possible lengths, ending in a specific base.

Gel electrophoresis[7] is used in both methods to separate each pool by length, with a size resolution of a single base. The gels are slabs of polyacrylamide with sample applied at one end. An electric potential causes the fragments to move through the gel in a track with the smallest fragment moving most quickly. The gel is then used to expose X-ray photographic film.

The resulting autoradiograph shows four tracks of bands. Since the gel his resolution of one base and all possible fragment lengths exist in the lout pools, the DNA sequence can be read directly from the gel (Fig. 3). Because of the ease of the enzymatic method and the ready availability of dideoxynucleotide reagents, the Sanger's method is becoming the method choice for sequencing DNA.

Step VII. Verify the DNA has the right sequence to make the enzyme: The DNA sequence is compared with the known protein sequence of the desired enzyme. If the sequence does not match, a new genome library must be screened. The DNA fragment is examined to determine if the entire gene is present. Start and stop codons allow recognition of whether the gene is intact. With some additional effort, the gene can be isolated using restriction enzymes to cut and trim it until only the coding sequence remains.

Step VIII. Transform the gene into an appropriate host: A toxin-free organism with a demonstrated high production capacity for foreign gene products is chosen. The highly produced native genes are deleted, while the transcriptional, translational and secretory control elements are retained. The gene of interest is transferred into the chosen production organism. Transformed organisms are selected (Step 5) and cultured. The expressed enzyme is purified and analyzed using general protein chemistry methods to insure that it is identical to the native enzyme.

Step IX. Improve the yield to levels acceptable for commercialization: Living is possessing intricate control systems to insure efficient use of material and energy resources. Often, cell regulatory elements limit the amount of enzyme produced, making commercialization economically unfeasible. However, changes in the control systems can be used to boost yield[8]. This area is of great Importance to enzyme producers and will be discussed in greater detail in a later section.

Complementary DNA: Another option for obtaining DNA suitable for enzyme production is the construction of complementary or copy DNA (cDNA). Messenger RNA is a complementary copy of a DNA sequence. When an organism is actively making a protein, reasonable amounts of mRNA are available in the cytoplasm and can be recovered. The mRNA can be used as a template to make a single strand of DNA using reverse transcriptase enzymes. Polymerase enzymes add the complementary strand, yielding a DNA fragment that encodes the desired gene. This DNA is not complete because it does not have introns or transcription signals promotor but often cDNA is the alternative available for generating a gene.

Site-directed mutagenesis: The commercial advantages of transferring a gene from one organism to a more productive one are obvious. The techniques can be used to manipulate a segment of DNA of any length (even a codon, to change one amino acid in a protein sequence), although the benefit of small changes was not immediately obvious. The results of substituting a single amino acid for the glycine at the 166 position (in a peptide chain of 275 amino acids) in subtilisin from *Bacillus amyloliquefaciens*[9].The 166 position is non part of the catalytic site, but is implicated in substrate binding.

Enzyme design: Organisms evolve functionally optimal enzyme systems for their environment because doing so confers a survival advantage. However, the optimal enzyme system for chemical or food processes may require characteristics not generally found in nature. The greatest opportunity for improvement, therefore, lies in engineering those properties that have not been selected naturally.

Structure-function relationships: Subtilisins are among the most highly studied and well-understood enzymes. They are secreted in large amounts by a variety of *Bacillus* species. The protein sequences from several commercially important species and their three-dimensional structures have been determined. The enzyme mechanism is known and the catalytic and substrate binding residues have been identified. Extensive kinetic and chemical modification and crystallographic studies have been performed. Subtilism is an ideal model for the application of protein engineering to better understand the theoretical basis for catalytic systems.

X-ray crystallography provides a valuable tool for improving our understanding of structure-function relationships. The crystal structure can be incorporated into a three-dimensional modeling system that enables enzymologists to study the active site, the binding regions, how substrates fit, the possible conformations of the enzyme and stearic hindrances of suggested changes. By understanding the relationship between catalytic properties and structure, improvements in function eventually may be predicted for suggested changes in the structure[10].

Over 400 site-specific structural changes have been made in subtilism to verify computer-modeled predictions of improvements to enzyme properties. Some results were predictable; others still need explanation. Protein engineering has altered almost every property of the enzyme: catalytic site, substrate specificity, pH/rate profile and stability to oxidative, thermal and alkaline inactivation.

Random mutagenesis: Enzymes are large and complicated catalysts. The three-dimensional structures of fewer than 100 (of~10,000) have been determined to date. Painstaking study, prediction and expression as described for subtilism is not always feasible. Researchers with limited resources perform random mutagenesis on a selected gene followed by expression and testing. When little is know about the enzyme structure, numerous mutagenized enzymes are generated rapidly and screened for indication of change in the characteristics.

Successful enzyme engineering depends on the ability to screen for mutants. Development of a screen for rapid and accurate selection is central to the process of mutagenesis. Temperature stable mutants are among the first mutants commercialized from wild-type enzymes, because testing the enzymes at higher temperatures was an easy screen. Therefore, screening for commercializable candidates requires extensive testing, which is time and resource consuming.

Engineering for production improvement: Current enzyme production encompasses fermentation, recovery, purification and formulation technologies. An in-depth understanding of the fermentation parameters for each organism is required to maximize growth and enzyme production. Submerged-culture batch fermentation predominates, but fed-batch and continuous fermentation are taking hold rapidly. A different set of conditions generally is required for efficient recovery and purification of each enzyme. As a result, recovery processes frequently are designed as dedicated trains, reducing plant utilization and increasing capital investment. Use of recombinant techniques has begun to influence how enzymes are manufactured. Innovations in rDNA techniques and protein engineering allow producers to design a strategy for future production strains: the development of well-defined and well-understood bacterial and fungal hosts for overproduction of foreign gene products. The ability to express new proteins in "workhorse" organisms using common induction systems allows use of fermentation processes similar to existing schemes and rapid scale-up of new products. Such consistencies will expedite transition from the laboratory to the marketplace. Use of rDNA techniques allows dramatic changes in fermentation process design and economics[11].

Several other molecular biology options exist to boost productivity. Yields can be increased by inserting multiple genes copies with a combination of promoters to achieve optimal secretion. Nonactive sections of an enzyme can be deleted to reduce the biological burden to produce it in the cell. Sporulation controls can be modified to make an organism produce longer than it would in its natural cycle. Synthesis of extra protein represents a waste of valuable energy and intermediary metabolites for the host. In hyper producing organisms, the gene product may constitute a large portion of the total cellular protein.

Alterations can be made in the protein products themselves to improve production. Yields of a protease that autolyzes during fermentation and recovery can be improved by shifting the pH optimum of the enzyme away from the pH used during production. Changes in enzyme stability to temperature or certain compounds (oxidants) could allow wider selection of fermenter growth

conditions. Amino acid substitutions that alter the surface charge may allow more efficient extraction, for example, by enhancing ultra-filtration membrane efficiency. Production strategies that incorporate improved enzyme characteristics for end-user requirements and increased yield for improved economics and that better utilize recovery and purification equipment, will be a major new focus for rDNA technology application[5].

Process conditions and equipment: Since microbial enzymes are relatively low-volume products, it has been difficult to justify the development and construction of specialized equipment for submerged fermentation. Equipment and techniques are most often adapted from antibiotics fermentations. Tall cylindrical fermenter of stainless steel with capacities of 10-100 tons and furnished with strong mechanical agitators and air spargers are typical. The advantage of this traditional setup is flexibility. It is easy to switch between products. As in many other fermentation processes, control of an enzyme fermentation is hampered by the fact that neither growth nor product formation may be determined rapidly enough to be of value for control. Primary physical variables, such as temperature, airflow and pressure recontrolled within narrow limits. Other measurable variables, such as pH, oxygen tension, or oxygen consumption, are often also applied in process control. Foaming is normally controlled by automatic oil addition.

Recovery and finishing: Methods for the recovery of enzymes for use commercially are simple unit operations such as centrifugation, filtration, vacuum evaporation and precipitation of proteins. The complications in the processes arise from the character of the fermented broth. The broth has a variable, unspecified composition, a high content of colloidal material and often a high viscosity. The most significant development in recent years has taken place in the finishing processing for solid enzymes. Before commercial products were dusty powders, but today the majority of solid enzymes are supplied as dust less granulates or as immobilized enzymes[12].

An example of enzyme production by rDNA technology: In the biotechnology age, the ability to offer a new product to the market place successfully will require the combination of enzyme design and product information capabilities of enzyme producers and application information, requirements and acceptance of enzyme users and consumers. The example of the application of genetic engineering to solve an enzyme supply problem is illustrated by developments in the production of milk clotting enzymes for the manufacture of cheese[13]. Historically, the prevalent milk coagulant has been calf rennet, derived from the fourth stomach of sucking calves. The principle active protease in calf rennet is chymosin and pepsin. These two enzymes are closely related-both initiate the clotting process in milk by cleavage of casein. Chymosin is produced predominantly only in the first weeks after birth; thereafter, it is replaced increasingly by pepsin. Of the two enzymes, cheese makers because of its limited proteolysis, leading to minimal bitterness in aged cheese prefer chymosin.

High-quality calf rennet is in short supply because of a decline in the market demand for veal and an increase in cheese production. These conditions have led to unstable rennet prices. Alternative sources of milk-clotting enzymes have been offered commercially to meet market demands, including microbial coagulants derived from *Mucor miehei, Mucor pusillus* and *Endotkia arasitica.* However, unweaned calf rennet, specifically chymosin with very low levels of pepsin, is considered the preferred coagulant by the cheese industry worldwide.

Three manufacturers have announced commercial availability of recombinantly produced calf chymosin. Cloning and producing GE chymosin by controlled fermentation can provide a consistent supply of enzyme with properties analogous to those of the native chymosin. The proteins are identical, despite the fact that the enzyme is produced in three different microorganisms: the bacterium *Escherichia coli*, the yeast *Kluyveromyces marxinus* Var. *lactis* and the fungus *Aspergillus niger* Var. *awamori.* According to Heterologous Expression Systems for Calf Chymosin[14,15], the advantages and disadvantages of each expression system are given below:

Advantages

- Bacteria, especially *Escherichia coli*
- Well characterized production strain
- Yeast, such as *Saccharomyces cerevisiae*
- Chymosin is secreted as an active enzyme
- Safe production organism
- Filamentous fungi, such as *Aspergillus nieger* Var. *awamori*
- Chymosin is secreted as an active enzyme
- Safe production organism

Disadvantages

- Chymosin is deposited as insoluble refractile bodies and must be solubilized and renatured to be active
- Products from *E. coli* must undergo extensive purification prior to use in food
- Current published production levels are uneconomical
- Low frequency transformation system

Transformation: Expression and secretion of chymosin involved these steps.

Step-I. Development of a host strain with markers for transformation experiments: *Aspergillus niger* Var. *awamori* was chosen as a host because it is toxin free and a hyperproducer of glucoamylase. The organism was subjected to UV mutagenesis, which produced a mutation in the pryG gene, resulting in a requirement for uridine. Chemical mutagenesis of the same precursor produced a strain with arginine auxotrophy (inability to synthesize a needed growth factor) caused by a mutation in the argB gene. A double auxotrophic strain was selected from the progeny of a parasexual cross of the two single auxotrophic strains.

Step-II. Deletion of the host's protease, aspergillopepsin: A, which causes degradation of chy'mosin or produces bitter peptids in cheese To generate strains that were deficient in the production of aspergillopepsin A, a gene replacement strategy similar to that reported was employed[16]. Briefly, the aspergillopepsin DNA coding sequence was replaced by the Aspergillus nidulans airgB gene and used for transformation of the double auxotrophic strain. Of the transformants selected, four lacked the aspergillopepsin gene. Absence of aspergillopepsin activity was verified by several tests.

Step -III. Construction of a plasmid vector containing a selectable marker and an expression unit for prochymosin: A vector called pGAMpR was constructed (Fig. 4)[7] in which prochymosin cDNA sequence encoding the prochymosin-B isozyme were fused codon of the *A. niger* Var. *awamori* glucoamylase gene. The selectable marker Used in the Vector was the *Neurospora crassa* pyr4 gene, which corrects for uridine auxotrophy when its gene product (the enzyme orotidine-5'monophosphate decarboxylase) is expressed. The gene product of this construction is a fusion protein consisting of the *awamori* glucoamylase enzyme covalently coupled to prochymosin.

Step-IV. Transformation of the host strain with the expression vector and subsequent molecular analysis of the transformed strains: Aspergillopepsindeficje strains were transformed and a number of chymosin-producing strains identified and selected. The highest producing strains were analyzed and shown to contain the glucoamylase prochymosin expression unit integrated into the genome, possibly in multiple copies.

Step-V. Mutagenesis and screening of improved strains: The strains were subjected to further rounds of classical

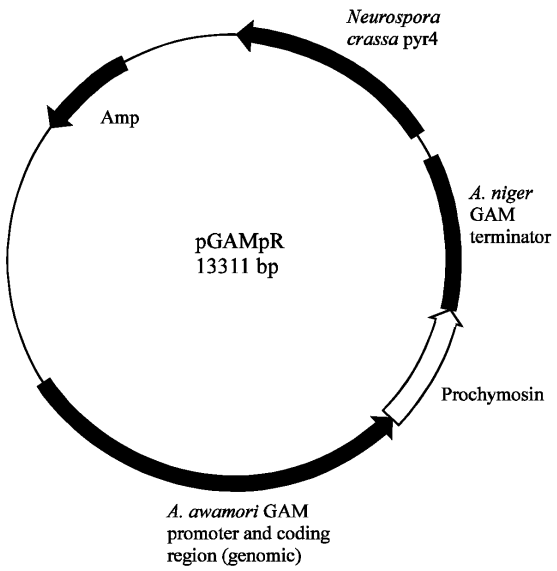


Fig. 4: Map of the glucoamylase-prochymosin fusion vector[17]

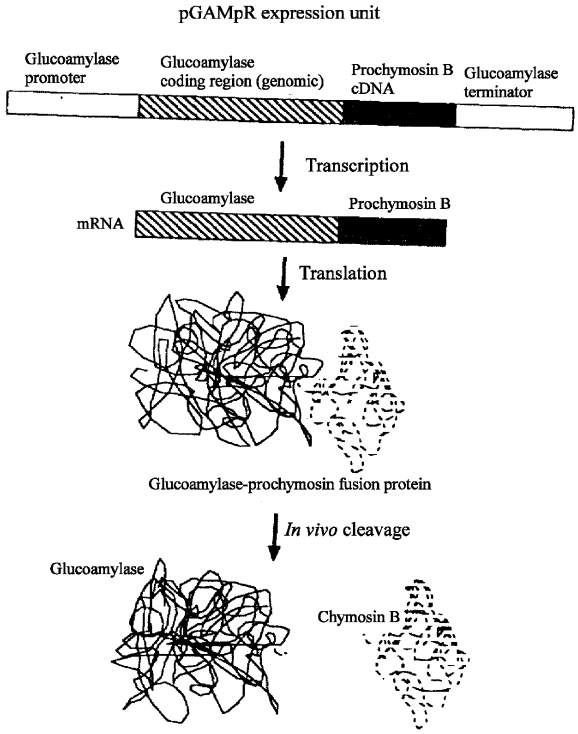


Fig. 5: Schematic model for the production of chymosin from the glucoamylase prochymosin expression unit[17]

mutagenesis, screening and selection for overproduction of chymosin. In addition, all possible production strains were tested carefully to insure that they produced no mycotoxins or antibiotics. The produced of the

transformed strain is chymosin B, an isozymes containing aspartic acid at position 290 instead of glycine as found in chymosin A. Chymosin B has a Lower specific activity than chymosin A but is reported to be more stable. Both isozymes are found in natural products.

The Fig. 5[17], shows the production of mature chymosin by the recombinant organism. Only mature chymosin and mature glucoamylase are observed in the fermentation medium. This suggests that the fusion protein undergoes an *in vivo* processing step, resulting in the cleavage of glucoamylase from prochymosin B and the subsequent self-activation of prochymosin to chymosin. The cleavage of the two enzymes can be effected either by prochymosin itself or by other endogenous *Awamori* proteases. Chymosin B was purified using standard biochemical methods. The enzyme was compared exhaustively to animal-derived chymosin using physical, immunological and functional tests. All results confirmed there was no significant difference between native and recombinant chymosin expressed in *A. niger* Var. *awamori*.

Fermentation and recovery: Production of chymosin is accomplished by submerged fermentation. On completion, an in situ cell inactivation protocol is used in which the cells are killed before the fermenter broth leaves the contained fermenter. The pH of the broth is lowered by acid addition while maintaining normal agitation and aeration. After 1h, viable cell counts are reduced to acceptable levels.

First, the broth is filtered to remove cells and other debris. Further processing is required because the harvested broth contains residual nutrients as well as endogenous enzymes produced by the organism. Two processing schemes were developed to recover high quality chymosin from the broth. The first consists of addition of sodium chloride followed by separation on a hydrophobic chromatography column. Chymosin binds to the resin while impurities pass through the column. The resin is washed and the chymosin eluted with a low ionic strength buffer. The product is then formulated, sterile filtered and packaged.

The second process involves chemical extraction using a two-phase liquid-extraction system. Selected conditions allow efficient extraction of the chymosin into one phase, leaving the impurities in the other. The phases are separated by centrifugation the chymosin-containing phase is passed over an ion exchange resin to bind the chymosin, which allows complete removal of the extraction phase chemicals. The resin is washed and the chymosin eluted. The product is then formulated, sterile filtered and packaged. The finished product meets an extensive list of specifications chosen to insure that a consistent, safe, high-quality product is produced.

Enzymatic processes proceed under milder conditions, give purer products and impact the environment to a lesser extent, yet still must compete against established chemical methods. A debt is owed to individuals who pioneered the discoveries leading to the commercial use of genetic engineering. Although the methods described in this chapter seem relatively simple, they have been developed painstakingly over a number of years. Once defined, however, they are straightforward and easily accomplished, requiring only care and attention to achieve results. Indeed, high school biology classes perform cloning experiments as part of their laboratory requirements. However, the directed use of these techniques to introduce commercially viable products has been a long struggle; practice is a combination of extensive training and understanding, careful persistent work, a large amount of both skill and art and some luck as well.

The successful Biotechnology Company must be proficient in all phases of enzyme engineering and design, process development and day-to-day production, marketing, distribution and administration. The technology required to exploit microbes also includes development of more efficient and creatively engineered fermentation facilities and product separation processes. These areas all must be handled in-house, adding costs to already strained research and development budgets. The industry is still young and can not support engineering, design and contraction farms similar to those available to well-established industries (for example, the petrochemical or food processing industries). For these reasons, the application of' biotechnology has not yet reached its full potential. Returns from research and development programs cannot be realized quickly. Therefore, target markets must be large to justify the expense involved. High value-added industries will be the primary markets for enzyme manufacturers, for example, the petrochemical, energy or environmental industries, in which historically favored processing requirements do not exist.

The use of enzymes for alternative processes or new products has been discussed at great length. They are characterized by relatively undifferentiated nonproprietary products, forced to compete on a price basis. The majorities of useful enzymes are hydrolytic in nature and are used as processing aids. Enzyme production and use often requires extensive capital expenditure. The advent of rDNA technology changes this outlook. Genes can be transferred into high-yielding organisms for more economical applications. Enzyme engineering can alter

substrate specificities and pH or temperature optima for improved efficiency. Enzymes for synthesis reactions are on the brink of commercialization.

However, biotechnology and Genetic Engineering (rDNA technology) is a highly skilled and specialized science. Insight is needed to identify opportunities in which novel enzymes might excel. Such insight requires knowledge of the benefits and willingness by scientists (Genetic engineer) to work jointly with enzyme suppliers to pioneer new applications. The opportunities are unlimited.

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