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INDUSTRIAL ENZYMES – DEVELOPMENTS IN PRODUCTION AND APPLICATION

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ABSTRACT

The utilization of gene technology and of new production technologies have made industrial enzymes with improved properties or better cost performance available. This has in turn opened important new areas of enzyme applications. The benefits to the customers are considerable: cost savings in the application process, improved product quality, and in most cases also a significantly reduced impact on the environment.

Gene technology offers several benefits to the enzyme industry. This technology enables the use of safe, well-documented host organisms easy to cultivate, the microbial production of enzymes of animal and plant origin, the realization of enhanced efficiency and high product purity, and also the production of enzymes with improved stability and activity.

Developments in production technology include advanced control methods, the use of expert systems, and the application of large-scale crystallization.

As case stories the development of a lipase and of a cellulase is described. The effect on environment of enzyme application and production is discussed.

KEYWORDS: Enzyme application, enzyme production, gene technology, expert system, continuous cultivation, lipase, cellulase, environmental impact.

INTRODUCTION

Enzymes have been produced in large industrial scale for several decades. And the basic technologies have been developed to sophistication: screening of microorganisms from nature, strain development by mutation to obtain high yield and purity, cultivation of the selected organism on a medium and under conditions that induce enzyme formation, and finally clarification, concentration and stabilization of the broth to yield the enzyme preparation.

Similarly, applications of enzymes in many industries are already well developed. Figure 1 shows the distribution of sales of industrial enzymes on industries. The most important market sector is still the use of proteases for household detergents. Another large user of enzymes is the starch industry.

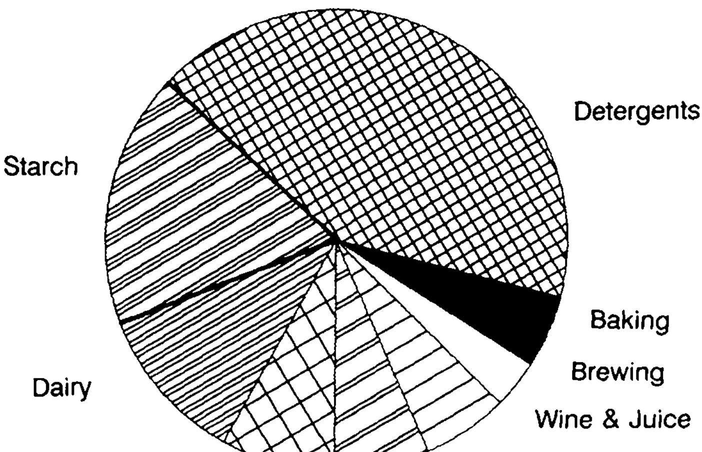

Figure 1. Distribution of Industrial Enzyme Sales on Industries. Total 4030 Mill DKK.

However, despite well-established production technology and well-established areas of application, the enzyme industry is now experiencing a revolution (1). Modern biotechnology enables the transfer of genes between organisms and also the modification of the structural gene. Production processes are hereby facilitated, and the properties of the enzyme may be tailored to the application.

This development is supported by a simultaneous strong development in the process technologies. The fast introduction of methods for the gene-spliced organisms is one important task. Independently, new process control methods ensure a better utilization of raw materials and capacity. Product recovery is being developed to produce crystal-pure enzymes in tonnage quantities.

The significant progress in genetics and in process technology enables the enzyme industry to offer products with improved properties and often at reduced costs. Enzymes will in many important areas of processing become cost-efficient and will replace existing cumbersome and polluting methods. Our work environment and the environment at large will gain by the introduction of enzymatic processing.

Let me first briefly review important benefits offered by genetic engineering, and then discuss the progress in process technology. Finally, I wish to illustrate the development by examples from different areas of enzyme application.

BENEFITS OF GENETIC ENGINEERING

Previously the basis for production of industrial enzymes was a naturally occuring microorganism producing a native enzyme with useful properties. Exploitation of the diversity of nature through efficient and laborious screening of the microsphere in various environments has yielded new products. Productivity of the natural microorganism has been boosted - in some cases up to a thousandfold - by mutations.

The genetic engineering techniques enabling the transfer of genes between species and the modification of genes offer many benefits. Benefits which are readily utilized for the production of industrial enzymes.

1. Enzymes naturally occuring in other organisms may now be produced in large-scale fermentation processes. Enzymes of animal or plant origin may be produced independently of the supply of animal and plant tissue.

   Chymosin, the active protease in calf rennin, is already being produced by fermentation.

2. Enzymes found in minute concentrations in exotic microorganisms often difficult to grow may be produced by selecting host microorganisms which are easy to cultivate on cheap raw materials and producing a broth from which the enzyme is easy to purify.

   As an example, anaerobic microorganisms offer a huge untapped resource of very diverse enzymes not available today because of the difficulties in anaerob cultivation.

3. Productivity may be boosted by selecting an efficient gene construction. Examples are the use of multiple copies per cell of the structural gene, or the use of a strong promoter or an efficient signal sequence.

4. Genetic engineering enables us to select host organisms and cultivation conditions that are safe to the manufacturing personnel, to the user of the product, and to the environment at large. Often an enzyme with interesting properties is found in an organism which is insufficiently characterized or is related to a toxin-producing or to an opportunistic pathogenic organism. Such uncertainty can now be avoided by transfer of the enzyme gene to a safe host.

5. Genetic engineering opens new avenues to enzymes with improved stability, activity or specificity. Through changes in the sequence of the structural gene, one or more amino acids in the enzyme molecule may be substituted by other amino acids thus changing the charges and the structure of the enzyme.

   By changing two amino acids in our best-selling detergent protease, Savinase, a new enzyme with greatly improved stability in the presence of bleaching agents was created. It is now marketed under the name Durazyme (2).

PROGRESS IN PROCESS TECHNOLOGY

In the past decade the greatest contribution of process technology to enzyme production has been the development of control methods. The most important factor in the optimization of an enzyme fermentation is the yield in relation to the nutrient consumed. Control strategies for nutrient feeding have, therefore, been the focal point of process development.

Process control of the fermentation is, however, also important in order to secure a broth with good and reliable properties for purification of the enzyme. More recently, advanced control methods have also been introduced to the down-stream processing steps in order to optimize process economy and secure high, uniform product quality.

Process control systems are today based on direct digital control. A typical system for a fermentation plant is shown in Figure 2. The hierarchical system ensures a high reliability through easily exchangeable modules of the lower levels and back-up units at the plant control level.

Enzyme fermentation utilizes to a high extent alternative process modes:

fed-batch

repeated fed-batch, and

continuous processes

In the 1970's our company succeeded in developing a fully continuous fermentation process for the enzyme glucose isomerase (3). As far as is known, this was the first large-scale instance of continuous enzyme production.

This process offers considerable advantages:

* Increased productivity. In the glucose isomerase process the productivity increased very significantly because the same yield per unit of volume could be obtained at a mean residence time considerably shorter than the cycle time for the batch process.

* Suitable limitation of nutrients. Excess of easily metabolized nutrients inhibits the synthesis of many enzymes. Optimal production of glucose isomerase requires dual limitation of glucose and oxygen. This is easily maintained in the continuous process.

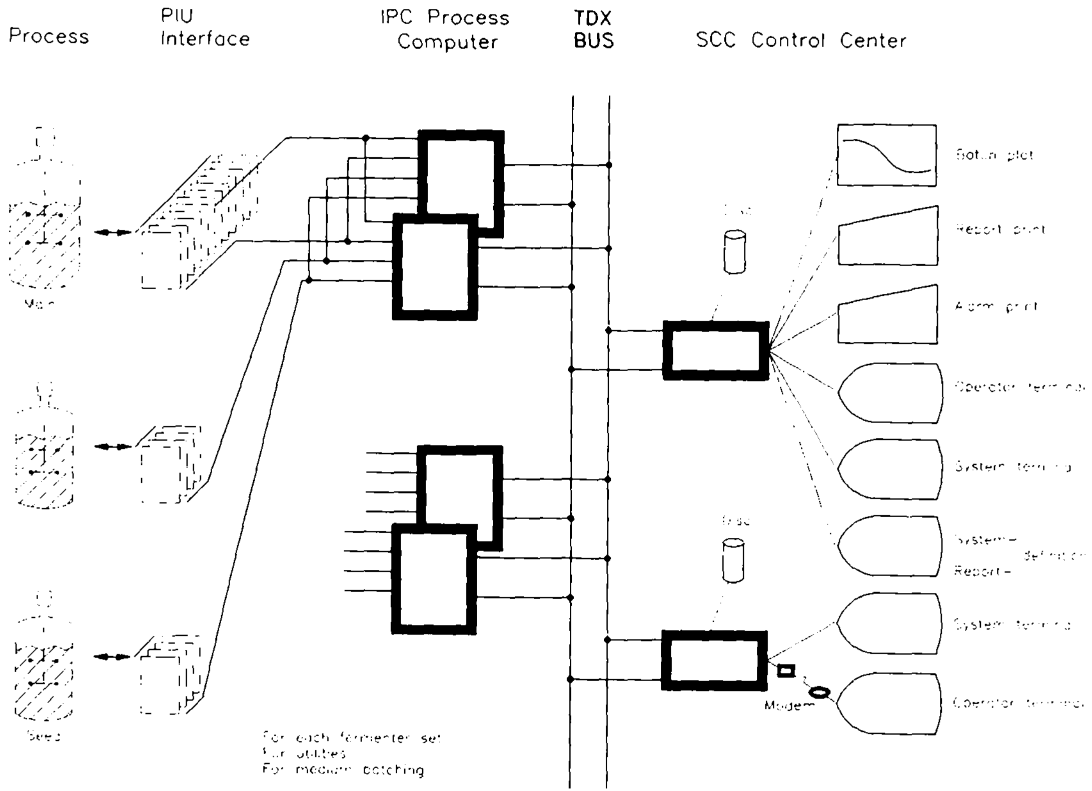

Figure 2. *Process control system for fermentation plant. At the control center level of the system, the tasks may be switched to the other system control center (SCC) within a few minutes. At the lower level, "hot" back-up computers with automatic switch-over function may be applied. In many cases this is not required because an IPC (Industrial Process Computer) is easy to exchange and reload.*

* Uniform product quality. The constant milieu in the fermenter leads to a uniform composition of the broth.

* Even and low load on utilities such as steam, air and cooling water supply.

These favourable results obtained in the glucose isomerase production has encouraged us to use continuous fermentation for other products.

In the evaluation of alternative process modes, the factor which really counts is the yield based on nutrient consumption. A significant productivity gain doesn't outweight a slightly reduced efficiency in nutrient use.

Today the most important task in process development is to develop control methods and process modes for the genetic engineered microorganisms. The stability and the productivity of the new strains pose new challenges to fermentation control.

Another current development is the introduction of expert systems for the supervision of fermentation processes.

When the concept of expert systems was first introduced to me, I found the theory interesting, but it was difficult for me to see applications in the fermentation plant in the immediate future.

Cooney (4) demonstrated in 1988 that an expert system based on a set of few simple rules could detect and identify many irregularities during a fermentation run.

Right in these days we are introducing an expert system in our production plant to supervise the fermentation of one of our major products. The system named BEOLOGIC (5) is a SW product developed by the Danish company Bang & Olufsen. The inference engine runs on a standard PC or an IBM PS/2 which is directly connected to the process control system. This rule-based system was selected because it is very general, fast, and compact. Engineers from the B&O company have assisted in developing our application. Process data and trends are generated by the existing process control system. The set of rules for the particular process and equipment is developed by process engineers in our department.

The real value of the use of expert systems for the supervision of fermentation processes is only realized if the systems are connected and updated in real time.

We expect the expert system to be as capable as the most experienced and alert process operator:

to detect sensor or transmitter failure

to detect unusual development of the microbial culture

to determine the time and level of nutrient additions, draw-offs and harvest

to recommend to the operator further investigations, and

to advise the operator on corrective interventions

The expert system will draw upon the ability to combine many different data, to utilize the redundancy and exploit the experience built into the set of rules. The knowledge base may continuously be expanded to improve the system.

If our expectations are met, the system will later be utilized for more processes and for a larger segment of the plant.

EXAMPLES OF NEW APPLICATIONS

New biotechnology has brought about breakthroughs for enzymes in many areas of application (1). Let me just mention three such areas:

Lipases

The growing environmental concerns have also affected the household laundry process. There is a worldwide trend towards lower washing temperatures in order to save energy. This has become a major challenge for the development of detergent enzymes.

A particular problem is the removal of fat stains which tend to remain on the fabric during washing at low temperatures. Experiments demonstrated that the hydrolysis products of the fat were easily removed under normal alkaline washing conditions.

Hydrolysis could be achieved by a microbial lipase. The real difficulty was to find a lipase which was active under alkaline condition and which resisted bleaching agents and protease in the detergent composition.

After screening of a large number of microorganisms, a good candidate - i.e. an enzyme with the desired effect under the conditions of a real laundry - was identified. This enzyme we named Lipolase.

The next problem we faced was to produce the enzyme in an economical process. All efforts to improve yield by strain mutation and fermentation development were fruitless with this fungus.

We decided to develop an efficient production strain by transferring the Lipolase-gene to a host organism which was suitable for industrial production. Such a host was the fungus, *Aspergillus oryzae*, which is used traditionally in Japan for enzyme production.

The *Aspergillus oryzae* host transformed with a plasmid carrying the Lipolase gene was tested in a large-scale fermentation, and the enzyme purified by a simple downstream process. A very satisfactory yield was achieved, and the result was beautiful crystals (6).

The recombinant DNA technology brought forward this efficient production technique for a suitable naturally occuring lipase. By extending the technology through protein engineering techniques even more efficient lipases may be developed.

The 3-dimensional structure of Lipolase has been determined. We believe to have identified the catalytic center of the structure. The catalytic triad is situated on the bottom of a groove which is covered with a kind of lid. We believe this lid is important for the binding of the lipase to its substrate. We see many opportunities to develop more efficient lipases by modification of the catalytic center and of the lid.

In the field of detergent proteases recombinant DNA technology is now used both to develop economically feasable production methods for proteases newly isolated by screening and also to develop efficient modified versions of known proteases.

In addition to the economical advantages of the new enzyme preparations, it is also important to be able to offer a very pure and well defined material in order not to expose the consumer unnecessarily to inactive proteins.

The new detergent enzymes ensures an efficient laundry at temperatures 30-40°C below what was required using a non-enzymatic detergent. The energy requirements at different temperatures is shown in the slide. The energy consumption for enzyme production is less than 1% of the energy usage for the laundry. Table 1 shows the energy saved by reducing the temperature one level (7).

Table 1. Energy Consumption per Wash in a Household Washing Machine (3 kg clothes).

Wash Temperature	**40°C**	**60°C**	**95°C**
Energy Consumption	800 wh	1200 wh	2400 wh
Enzyme Consumption	0.5 g	0.5 g	0.5 g
Energy for Enzyme Production	6.8 wh	6.8 wh	6.8 wh
% of Energy Consumption	0.9 %	0.6 %	0.3 %

Celluzyme

In addition to the traditional function of a detergent, i.e. to clean the laundry, new functions may be added. And also here enzymes present a good solution. One such example is our Celluzyme product which has the effect that it makes cotton fabric softer and the colours brighter.

The cotton fiber has a smooth surface when it is new. When the fabric has been used and washed a number of times, the fiber will look more like in Figure 3. You see that the so-called micro-fibrils have been formed. The cotton fabric loose it softness because these micro-fibrils interlock. The fabric becomes harch and unpleasant to touch right after washing. If the fabric is coloured, the micro-fibrils will give it a greyish appearance so well known from old T-shirts.

We have found an enzyme, Celluzyme, which will remove the micro-fibrils and leave the cotton fiber as smooth as when it was new. Figure 4 shows the effect on a fiber from the fabric shown in the previous slide after washing in a detergent fortified with Celluzyme.

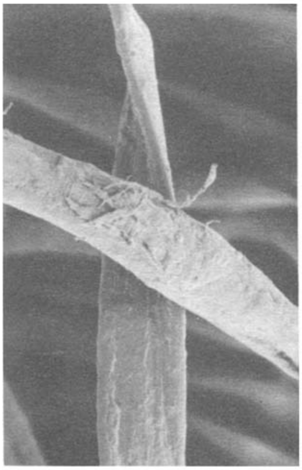

Figure 3.
Before treatment with Celluzyme.

Figure 4.
After treatment with Celluzyme.

The most striking effect of this Celluzyme treatment is seen when you wash an old, coloured garment with the characteristic greyish appearance. The cellulase-treated side of the garment will clearly distinguish itself from the part washed in normal detergent. It has regained an almost "new" appearance.

CGTase

Also the starch processing industry will experience a remarkable development in the application of new and better enzymes.

A field of renewed interest is cyclodextrins. These cyclic carbohydrates consisting of 6,7 or 8 glucose units in the ring can be made from starch by treatment with the enzyme cyclodextrin glycosyl transferase.

The traditional enzyme derived from *Bacillus macerans* requires liquefied starch as substrate. The amylase used for the liquefaction must be completely inactivated before CGTase is added. We identified a very heat-stable CGTase in a strain of the thermophilic and anaerobic bacterium, *Thermoanaerobacter*. The strain is difficult to grow in industrial scale, and the enzyme yields are far too low.

We, therefore, transferred the CGTase gene from the *Thermoanaerobacter* strain to a *Bacillus* host where the enzyme is expressed in acceptable yields.

The properties of the *macerans* enzyme compares well with the *Thermoanaerobacter* enzyme. The new enzyme is active up to 95°C. Preliquefaction can be avoided, and reaction time reduced considerably. Furthermore, we have experienced that the yield of cyclodextrin is up to twice as high as with the *macerans* enzyme.

ENZYME PRODUCTION AND ENVIRONMENTAL IMPACT

The enzyme industry is in an excellent position to contribute to a cleaner environment. First of all, enzyme technology offers industries and consumers an opportunity to replace processes using aggressive chemicals with mild, non-toxic enzyme processes. Secondly, the biotechnological processes used to produce enzymes have a minimal impact on the environment.

There are many incentives for industry to give high attention to the protection of the environment. Consumers in many nations are highly aware of the impact of products and processes, and they use their buying behavior to express strong wishes for a safe working milieu and a clean nature. Authorities and green-wave pressure groups put strict demands on manufacturing processes and products.

Most of the raw materials used for the manufacture of industrial enzymes are normal agricultural products suitable for food and feed. No highly toxic or aggressive chemical is used. Mild process conditions are used compared to the manufacture of chemicals, and no toxins are produced. So the working environment in the plant is safe.

The effluents from the production plant are non-toxic, but do contain nitrogen, phosphorous and organic matter. So they may promote a strong biochemical oxygen demand.

The major part of the spent dry matter from the enzyme production is collected as a sludge and subsequently spread on nearby farmland (8). The sludge consists of dead biomass, filter aid, nutrient surplus and insoluble debris. 500,000 m^3 of sludge containing 4-5% dry solids were reused in this way in 1990, including 800 ton of nitrogen and 285 ton of phosphorus. The sludge acts as an efficient slow-releasing nitrogen-phosphorous fertilizer. More than 90% of the nitrogen is bound in organic matter which means that the evaporation of ammonia is minimal. The distribution of sludge is controlled to ensure precise and uniform dosage.

Figure 5 shows how enzyme production fits into an ecological cycle with agriculture. The by-product from our industry is a good "raw material" for agriculture which in turn supplies many raw materials for enzyme production.

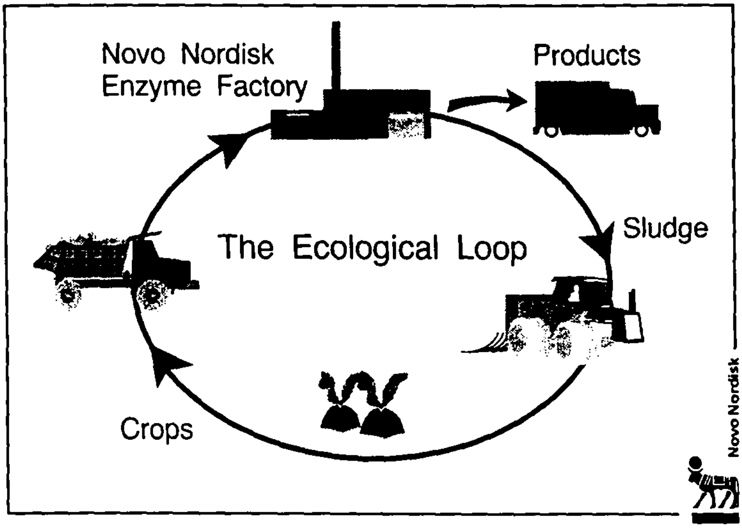

Figure 5. The "ecological loop" of enzyme production.

Remaining environmental problems in our production is the liquid effluent which contains additional nutricious material and also the vent gas from fermentation with its characteristic odour. Large pilot processes have been established for aerobic biological treatment of the liquid and for biological filtration of the off-gas. Full scale processes will start in 1-2 years.

Industrial enzymes play an active role in establishing more "green", more environmentally acceptable processes in many industries. Let me shortly mention a few examples:

In the pulp and paper industry, huge amounts of chlorine is used to bleach the pulp. Unpleasant reaction products are formed, and they are difficult to remove from the waste steam. Fortunately, the need for chlorine bleach can be reduced significantly by the use of the enzyme, xylanase, which facilitates the separation of lignin and cellulose fibers.

Through this process less than 10 g of enzyme protein will replace up to 30 kg of chlorine (9). The same brightness of the paper is obtained. The approximately 200,000 metric tons of chlorinated organic compounds discharged today can be reduced by nearly 50%. Chlorinated dioxins present in measurable amounts in product and effluent can be reduced dramatically to below detection limit.

Another example is from the leather industry where the hide traditionally is treated by a very unpleasant and polluting lime and sodium sulfide mixture which dissolves the hair. In a new process using proteases and other enzymes the hair loosens and can be filtered off and used for other purposes. The biological oxygen demand of the effluent is reduced dramatically. And the leather quality is improved (10).

CONCLUSION

I hope this presentation illustrates the rapid development which the enzyme industry undergoes in these years. Recombinant DNA technology and state-of-the-art process technology offer many opportunities.

The main developments are:

* New enzymes from "exotic" microorganisms provided by gene technology.

* Taylor-made enzymes with new properties through the application of protein engineering.

* Industrial enzymes of high purity. "Mono-component" enzymes to be used in smaller quantities.

* Higher yields and better utilization of raw materials.

* Enzymes manufactured as part of an ecological loop. Very low impact on the environment.

The advantages to the user of the new generation of enzymes are a cost-efficient process and a clean and safe solution.

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