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BIOTECHNICA '87 Hannover

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This is a summary of the third international exhibition and congress for biotechnology, held in September 1987 at Hannover, West Germany. Topics of the presentations reviewed are polypeptides in therapy, enzymatic and microbial transformation, and fundamental of bioprocess engineering. Subtopics include:

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BIOTECHNOLOGY CONGRESS: BIOTECHNICA '87 HANNOVER

1 INTRODUCTION

The third international exhibition and congress for biotechnology was held, as were the first and second, in Hannover, West Germany, from 22 through 24 September 1987. More than 4000 participants from a total of 29 countries attended Biotechnica '87. Of these participants, 25 percent came from non-European countries. The industrial sector, universities, and research institutes were strongly represented with the majority from industrial organizations. Two-thirds of the participants play a key or influential role in purchase decisions. Interest was focused on scientific and technological innovations in biotechnological products and processes as well as on laboratory and plant engineering.

More than 400 exhibitors showed their products in a recently enlarged exhibition area. The 1987 exhibitors, as compared with those at the 1986 congress exhibition, were mainly from European countries. This is indicative of the increasing role of BIOTECHNICA in integrating European biotechnological research and industry. For the first time countries like France, the UK, Spain, and Hungary (and also Australia) participated with national stands. In addition, countries such as the US, the German Democratic Republic (East Germany), and the Netherlands also had larger exhibition stands than at the 1986 congress.

Biotechnology research institutes and enterprises seeking cooperation partners for technological developments and services formed an impressive sector of the exhibition. The largest groups were equipment and process engineering companies, which traditionally are intermediaries in the transfer of research results into application. They play a key role in industrial know-how for biotechnology. In the scientific field, the application-oriented international congress focused on three topics: (1) polypeptides in medical therapy, (2) enzymatic and microbial transformation, and (3) processes involving genetically modified cells. Summaries of the presentations in the scientific section are presented in this report. There is no plan to publish the proceedings of this biotechnology congress.

The Opening Plenary Lecture

E. Katchalski-Katzir (Department of Biotechnology, Tel Aviv University, Tel Aviv and the Weizmann Institute of Science, Rehovot, Israel) presented the opening plenary lecture of the congress. In his excellent lecture, Katchalski-Katzir, an internationally recognized scientist as well as a former president of Israel, gave a historical background of the development of biotechnology and discussed the impact of the basic sciences on modern biotechnology. Because his talk emphasized the importance of the role played by the basic sciences, I will summarize it.

He said that the remarkable discoveries in molecular biology, which can be attributed in turn to fundamental findings in biochemistry, biophysics and genetics, have given rise to prodigious developments in the various areas of modern biotechnology. Biotechnology today has thus been established as a discipline combining the findings of molecular biology with those of modern chemical, pharmaceutical, and agricultural technology.

The living world is characterized both by enormous diversity and by striking uniformity. Life processes going on in the cell are surprisingly alike throughout the plant and animal kingdoms. Moreover, the cells of prokaryotes and of eukaryotes contain remarkably similar consituents, notably the proteins which are responsible for the cell's chemical and physicochemical activities and the nucleic acids in which the cell's hereditary properties are encoded. Elucidation of the molecular structure and function and the mode of action of different proteins continues to absorb the interest of scientists involved in basic research.

Particularly noteworthy is the progress attained in the elucidation of the three-dimensional structure and mode of action of enzymes (such as lysozyme,

chymotrypsin, hexokinase, and citrate synthetase); monoclonal antibodies (such as those prepared against lysozyme, neuraminidase, and other antigens), membrane proteins (such as receptors for insulin, gamma amino butyric acid [GABA]), and the beta-adrenergic stimulants; the ion-gates; and photoreceptors (such as bacteriorhodopsin).

Information has also been acquired on new structures of DNA (such as the Z-DNA whose conformation is markedly different from that of the well-known A- and B-DNA) and on conformational alterations which occur in DNA and seem to play an important role in determining gene regulation and transcription. The recent determination by x-ray and computer graphic techniques of the three-dimensional structure of repressors (such as the Trp-repressor and the lambda repressor) and of regulatory proteins as well as the prediction of their specific interactions with DNA, sheds new light on the protein regulatory mechanisms involved in gene expression.

Some of the techniques derived from findings in molecular biology have opened the way to further explorations in molecular biology and have made new inroads in biotechnology. Technologies which have already become classical include:

- The solid-phase synthesis of oligopeptides and oligonucleotides
- New techniques (such as chromatography, high-performance liquid chromatography and affinity chromatography) for the isolation and purification of materials derived from living organisms
- Novel methods of tissue culture culture from plants and animals
- The various cell-fusion techniques and the formation of hybridoma cells
- Preparation of immobilized enzymes and cells
- Recombinant DNA technology and gene transfer in plants and animals.

Many of these techniques have been successfully applied in industry, agriculture and health-care.

Important new developments in basic research which are of relevance for biotechnology include:

- The devising of a technique for site-directed mutagenesis
- The design of new vectors and new methods of gene transfer into higher plants and animals
- Clarification of the mechanisms involved in electron transfer within a single protein and between proteins
- Computer-assisted modeling of proteins and nucleic acids
- Improved theoretical analysis of the intra- and intermolecular forces existing within proteins and nucleic acids, and predictions of conformational fluctuations and changes in these biopolymers under well-specified conditions.

The above developments can be expected, according to Katchalski-Katzir, to lead in a number of new directions. One can envisage, for example, exciting new insights into biocatalysis, with spin-offs in the production of enzymes and enzyme reactors having prespecified characteristics. They might also lead to an increasing number of transgenic plants (i.e., plants resistant to various herbicides and pests) and animals having desired traits. The possible development of biochips as an alternative to the standard semiconductor chips is being considered in industry; if successful, this could lead to far-reaching progress in bioelectronics. Computer display and modeling of the structural information revealed by protein and nucleic acid crystallography should facilitate the design and development of new proteins and new drugs. Finally, the growing amount of information acquired by molecular biologists on the life processes in prokaryotes and eukaryotes will unquestionably be of great practical interest to scientists working in biotechnology.

Katchalski-Katzir stated that modern biotechnologists can be proud of their achievements attained to date in the chemical industry (for example, the use of enzyme reactors in the production of

fructose-enriched syrup and of 6-amino penicillanic acid), in the pharmaceutical industry (for example, the production of growth hormones, Factor VIII, interleukins I and II, interferons, vaccines and receptors), and in agriculture (for example, plant tissue culturing, and the design of genetically modified plants and animals). He thinks that new insights and achievements in the basic life sciences will inevitably promote further progress in the biotechnological production of new species and new products of value.

2 POLYPEPTIDES IN THERAPY

Production and Isolation of Peptide Hormones

New methods for the production and isolation of peptide hormones including the genetic engineering of the insulin-like growth factor-I (IGF-I) were discussed by S. Josephson (KabiGen AB, Stockholm, Sweden). Many of the studies were carried out in collaboration with scientists at Sweden's Royal Institute of Technology and the Karolinska Institute, Stockholm, Sweden. The somatomedians or IGF's consist of the homologous peptides IGF-I and IGF-II as well as their variants. The IGF's show conformational similarity to insulin, proinsulin, and relaxin and consist of A- and B-domains homologous to those of insulin. The IGF's have diverse biological functions. IGF-I appears to be a major regulating hormone of postnatal growth, but the role of IGF-II is still unclear. Most tissues appear to be targets for IGF-I action, as indicated by the presence of receptors on a wide variety of organs and cells. The research on somatomedians has so far been hampered by the shortage of material, since only a few milligrams of IGF-I can be purified from a ton of human plasma. With the use of recombinant DNA technology it is now possible to obtain pure IGF-I in amounts sufficient not only for basic research purposes, but also for studies relating to specific clinical applications such as wound healing and growth of cartilage and bone.

The IGF-I gene was chemically synthesized on a DNA synthesizer developed at KabiGen and inserted into the production plasmid pZZ. This plasmid contains a promoter and a signal sequence from the *Staphylococcus aureus* protein A gene and the "ZZ-DNA fragment," which was also synthetically made. The ZZ fragment is structurally related to the protein A gene. The ZZ protein has similar IgG-binding properties to the *S. aureus* protein A. The IGF-I gene is inserted in phase after the ZZ gene and the construction is introduced into *E. coli* by transformation. As described by Josephson, this system has the following interesting features: (1) the protein A promoter, together with a signal sequence, contributes to a high production level as well as secretion of the fusion product ZZ-IGF-I into the culture medium in *E. coli* and (2) the growth medium can be applied to an affinity gel (IgG-Sepharose column) which binds the ZZ-IGF-I fusion protein. This method effects a 90-percent purification of the fusion product in one step.

The affinity-purified fusion protein is cleaved by a chemical method. A second IgG-Sepharose column step separates the cleaved ZZ part from the protein (IGF-I). Finally, the IGF-I is further purified using ion-exchange chromatography followed by size-exclusion chromatography. This method has been used for purification of IGF-I on a 100-mg scale. The pure IGF-I has been compared with native human IGF-I isolated from plasma. The recombinant IGF-I compares favorably with the native material in a radio receptor assay, and is currently being tested in animal systems to measure its potential as a growth stimulator. KabiGen, in collaboration with the Royal Institute of Technology, Alfa Laval, and Pharmacia has scaled up this production and purification system to 1000 liters. The production system is currently being used for the production of other peptide hormones--i.e., human secretin, vasoactive intestinal peptide, brain-IGF, and IGF-II.

Another use for KabiGen's production system has recently been found: it can be used as a general method to obtain specific antibodies both against peptide hormones (i.e., IGF-I, IGF-II and

secretin) and short peptides. Josephson thinks that the very good antibody response with the ZZ system might be due to the stimulation of the immune system with the ZZ part of the fusion protein. He stated that the above aspects present an excellent way of preparing new vaccines since: (1) the production level of the fusion protein is very high (800 to 1500 mg/l) and (2) the immune response is very strong. KabiGen has also developed two new peptide-based animal vaccines which are currently being tested. KabiGen has patent applications on all the methods described above.

Human Interleukin from Recombinant Mammalian Cell Lines

H. Hauser (Institute for Biotechnology Research, Braunschweig, West Germany) reported on human interleukin from recombinant mammalian cell lines. Interleukins comprise a class of polypeptides regulating growth and differentiation of lymphocytes and other cells active in immune responses. The synthesis of these substances as well as the exerted biological effects are part of the complex network of differentiation and function of the immune system. Interleukins are secreted by defined classes of producer cells upon intrinsic signals. Interleukin functions on the target cells are mediated by interaction with specific membrane receptors. The most extensively studied interleukin is IL-2, also called T-cell growth factor (TCGF). It is produced by T-lymphocytes upon antigenic or mitogenic stimulation. In order to be biologically active, IL-2 must interact with its membrane receptor, the IL-2-receptor. Il-2 was recognized for its ability to promote the proliferation of activated T-lymphocytes--a prerequisite for cytolytic action. Moreover, it plays a role in differentiation, modulates the expression of cell surface markers, and affects the release of gamma interferon in certain subcellular subsets.

The production of large quantities in pure form of IL-2 is a prerequisite for biochemical and functional studies as well as for clinical trials. Immunodeficiencies, organ transplantation, and cancer therapy are the main areas of clinical interest. The complementary DNA (cDNA) gene of IL-2 has been cloned. In an attempt to characterize IL-2 upon production in different mammalian cell types, Hauser and his coworkers have genetically engineered the gene to be efficiently expressed. For this purpose, vectors with various viral and cellular promoters were constructed. In addition, retroviral vectors, based on the murine myeloproliferative sarcoma virus (MPSV), were developed, allowing transfer of foreign genes in a variety of different rodent cell types. To improve expression of these constructs, different parts of the structural IL-2 gene with and without intronic DNA sequences were integrated into these vectors and introduced into different cell types. Selected recombinant hamster CHO, BHK, and mouse L-cells constitutively secreted IL-2 at a level of 1 microgram per 10^6 cells in 24 hours. IL-2 was purified from serum-free cell culture supernatants in a two-step procedure. For comparison, extraction of natural IL-2 from stimulated peripheral blood lymphocytes and from a tumor T-cell line (Jurkat) was carried out, and the biological activity and biochemical properties of IL-2 from these sources were analyzed. No significant differences were found with respect to the biological activity and to the protein moiety. However, differences were found in the percentage of secreted IL-2 molecules containing a carbohydrate side chain and in the content of sugars in this single glycosyl moiety.

Recombinant cell lines producing IL-2 were grown in fermenters under various conditions including serum-free media, thus allowing the purification of secreted IL-2 with low protein contamination.

In order to understand the interaction of receptor with ligand, the IL-2 receptor gene (Tac) was expressed in mammalian cell lines. According to Hauser, site-specific mutagenesis in the genes of IL-2 and Tac will allow the testing of mutants with respect to protein-protein recognition. In combination with structural data of these compounds this could

lead to the modeling of low molecular weight substances.

Atrial Natriuretic Factors

Methods for the production and isolation of atrial natriuretic factors (ANF) and their clinical application were reported by K.D. Döhler (Bissendorf GmbH, Wedemark, West Germany) in collaboration with W.G. Forssmann (Anatomical Institute, University of Heidelberg, West Germany). A number of clinically important disease states are characterized by abnormal fluid volume retention. Congestive heart failure, cirrhosis of the liver, and the nephrotic syndrome each lead to excessive fluid accumulation in the body. An increase in extracellular fluid volume is also thought to contribute to the development of hypertension. Hypertension, or chronically elevated blood pressure, is one of the major causes of illness and death worldwide. In many instances, the retention of excessive fluid and hypertension are the result of an impaired capacity of the kidneys to excrete sodium (natriuresis) and water (diuresis).

The mammalian heart--in particular, the atrial myocardium--possesses cells which contain numerous membrane-bound storage granules. These characteristic secretory granules resemble those of peptide hormone-producing cells. It has been shown that crude tissue extracts of atrial myocardium produce a rapid and potent natriuretic response when injected intravenously into nondiuretic rats. In recent years efforts have been devoted to the purification and chemical characterization of cardiac hormones in rats and other mammals. Numerous later publications, using cDNA and genome studies resulted in the demonstration of a family of cardiac hormones, peptidic in nature, with interesting species homologies in human, porcine, bovine, rat, and mouse. Forssmann and his group discovered that the hitherto described short-chain peptides are part of a precursor molecule composed of 126 amino acids. From a prehormone of 151 amino acids a signal peptide of 25 amino acids is cleaved, resulting in the storage form of ANF (amino acids 1 to 126). It seems that under normal physiological conditions only a 28 amino acid C-terminal portion (ANF 99 to 126) of the prohormone is released into the blood stream. ANF 99 to 126 retains full vasoactive, natriuretic, and diuretic activity.

Currently available conventional preparations for the treatment of hypertension, renal failure, and various edematous states (heart failure, ascites, etc.) have important limitations and undesirable side effects. Thus, it would be of great medical and commercial value to produce and develop atrial natriuretic factors which can regulate blood pressure and kidney function by providing a complete but controlled range of physiological responses.

In recent years ANF 99-126 has been applied to human volunteers and to patients with various diseases such as severe heart failure, renal failure, chronic liver disease with ascites, hypertension, heart transplantation, and kidney transplantation. Synthetic ANF 99-126 was shown to reduce blood pressure and to stimulate natriuresis and diuresis and to have therapeutic effects on the above mentioned diseases.

Döhler and Forssmann's groups are engaged in collaborative efforts in the search, structure elucidation, synthesis, and pharmaceutical development of new peptides, including ANF's. The methods used by these groups include the extraction of peptides from human hemofiltrate by treatment with alginic acid. This technique can be used for batches of up to 1000 liters. Large-scale purification of the peptides is achieved in a single run by passing the extracted content of 10,000 to 100,000 liters through ion-exchange chromatography columns. Additional purification with purities of more than 95 percent is achieved by reverse-phase chromatography. Biological activities are tested in cell cultures, isolated organs, and *in vivo*. Structure elucidation is performed by gas-phase amino acid sequence analysis. Since most biologically active peptides are circulating in femtomole and picomole quantities, a complete sequence analysis can be

performed with a peptide quantity, obtained from 1000 liters of hemofiltrate. Synthesis of newly elucidated peptide structures with low molecular weight is performed by solid-phase technique. The principle of this technique is the stepwise attachment of amino acids to an insoluble polymer. Benzhydrylamine resin was chosen as a suitable insoluble polymer support for the synthesis. The methods of attachment of each amino acid residue on the polymer is comprised of deprotection by trifluoro-acetic acid, washing to remove excess acid, neutralization with organic base, washing to remove formed salts, coupling of the next amino acid using dicyclohexylcarboiimide and, finally, washing to remove excess reagent and by-products. Cleavage and concomitant deprotection of the peptide is achieved by hydrogen fluoride (HF) in the presence of anisole. After removal of HF and thorough drying under vacuum, the resin is immediately extracted with 2N acetic acid, and the extracts are freeze-dried. The crude product is purified by preparative reverse-phase high-pressure liquid chromatographic (HPLC) techniques. The freeze-dried product is treated with ion exchange resin (acetate form) and is again freeze-dried. Synthesis of peptide structures with high molecular weight (for example, ANF 1-126) is performed by recombinant DNA techniques. cDNA, coding for the peptide, is assembled by chemical synthesis. The synthetic DNA is built into a plasmid vector, which is incorporated into *E. coli* cells or into yeast cells. The peptide is then recovered and extracted from the cell culture.

Production of Proteins

The large-scale production of proteins by mammalian and hybridoma cells in a continuous bioreactor system was reported by K. Venkat (Verax Corporation, Lebanon, New Hampshire, US). Venkat described a fluidized bed bioreactor system for continuous culture with particular emphasis on scale-up and long-term aseptic operation. Cells are immobilized inside collagen-sponge beads (microspheres), which are strongly cross-linked to achieve long operational life. These microspheres have a mean diameter of 500 microns and are weighted with biocompatible metal particles to achieve a desired specific gravity. Wet specific gravities up to 3.0 are achieved by this technique, and the resulting beads are readily fluidized in a two-phase (liquid-solid) reactor. The microspheres are highly porous with up to 90 percent of their internal volume available for cell population. The characteristic pore size of the microspheres can be tailored to accommodate different cell sizes. Typically, pores of 20 to 40 microns are employed; these are interconnected, facilitating the entrance of the cells and the subsequent population of the entire matrix.

The microsphere production process, according to Venkat, results in a matrix morphology that has proven especially conducive to cell colonization and the attainment of high cell densities. Viable cell concentrations (inside the beads) of 2×10^8 cells/ml for hybridomas and 4×10^8 cells/ml for mammalian cells such as Chinese hamster ovary (CHO) cells are routinely achieved.

The fluidized bed reactor is designed to provide the mass transfer rates necessary to support the high cell densities attained inside the microspheres. The reactor can be oxygenated up to 25 mg-mole/liter of reactor volume/hour by recycle flow through an external, silicone-rubber-membrane gas exchanger. Flow velocity through the recycle flow is adjusted to achieve proper fluidization. At the proper fluidizing flow rates, the culture liquor separates easily from the slurry mixture of populated microspheres in a zone near the top of the reactor vessel. Typical solid fraction in the slurry is 40 percent. The vessel and recycle loop have been designed to specifically avoid gas-liquid interfaces which cause foaming.

Venkat stated that the fluidized bed system scales up without difficulty at constant depth; increases in reactor volume are obtained through expansion of bioreactor cross-sectional area. Twenty-fold increase in reactor size has been

achieved with good predictability, according to Venkat.

The bioreactor system lends itself well to full automation and computer control without human intervention except for routine maintenance on medium addition, but, more importantly full automation largely eliminates human error which, according to Venkat, is often the greatest contamination hazard to a culture.

Comparative performance data were presented for large-scale production of monoclonal antibodies (Mab) and mammalian cell-derived proteins for medical use. Typical volumetric productivities of 2.72 to 272 mg/liter reactor/hr for Mab's and 0.073 to 73 mg/liter reactor/hr for mammalian cell proteins, respectively, can be expected. Furthermore, Venkat said that by employing new and very strict asceptic technology, continuous production runs of up to 4000 hours have been carried out.

Mammalian cells immobilized in this culture system demonstrate higher cell-specific yields and productivity than the same cells in free-cell suspension or microcarrier culture. Proprietary medium improvements have led to a significant increase in cell productivity. Immobilized hybridomas demonstrate genetic stability over at least 200 divisions, whereas the same hybridomas in free-cell culture typically lose a large part, or all, of their productivity after about 40 divisions, according to Venkat. This characteristic is essential to a 4000-hour culture of cells that divide in 14 hours under fastest growth conditions. Also, cell growth is not generically essential to this system and so may be suppressed--often, with significant gain in cell-specific productivity.

A review of the design of key process and hardware engineering parameters to ensure reliable and optimum performance of the system was also presented by Venket. These included construction materials, selection of appropriate pumps, valve design, sterilization, and asceptic operations procedures.

Venket stated that economic modeling of this immobilized-cell, continuous-culture process, compared to batch and fed-batch cultures of hybridomas and attachment-dependent cells, demonstrates large potential reductions in the production cost. For unpurified Mab's, Venket predicts at least 50 percent. For proteins for medical use, such as Factor VIII, tissue plasminogen activator (tPA), interleukins and hormones, he predicts culture cost reductions of as much as 70 percent. However, Venket concluded by saying that while these economic models are sophisticated and are based on the best data available, only long-run and large-scale production experience will finally confirm the potential of this culture system.

3 ENZYMATIC AND MICROBIAL TRANSFORMATION

A review of this topic was presented by M.R. Kula (Institute for Enzymology, University of Dusseldorf, KFA Jülich, West Germany). The following paragraphs summarize the main points made by M.R. Kula and serve as an introduction to the presentations in this scientific section.

In the chemical and pharmaceutical industry great efforts, besides the development of new products, are presently directed to improving processing. Particular attention is being paid to increase the conversion of raw materials and to lower the amount of waste; to find alternate, energy saving technologies; and to avoid unspecific chemical catalysis by strong acids or alkali. In this context, enzymes (biological catalysts) find increasing applications. New production routes for chiral amino acids by biotransformation of chemical feedstocks include L-lysine from 2-amino caprolactame and L-cysteine from 2-amino-thiazoline-4-carboxylic acid.

For the production of semisynthetic penicillins or cephalosporins, specific amino acids (or hydroxy acids) are needed to replace the original side chain on the beta lactam ring of the antibiotic, thereby rendering the resulting molecule more acid stable and less susceptible to cleavages by beta lactamases. Figure 1 shows the structure of Ampicillin as an

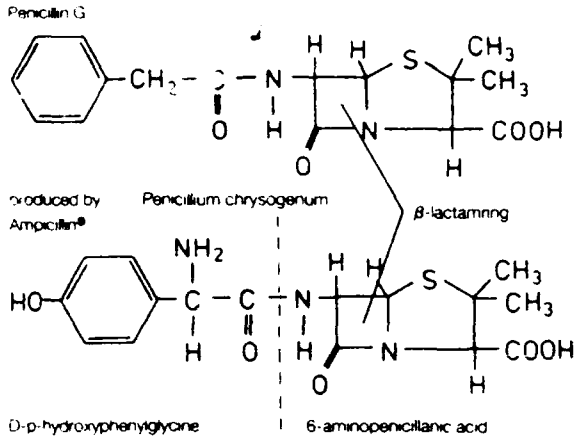

Figure 1. Structure of Ampicillin.

example. It contains D-p-hydroxyphenylglycine, a nonproteinaceous amino acid with the unusual D configuration at the chiral center. Several enzymatic routes have been described for the production of this compound. The largest manufacturer utilizes an immobilized microorganism, catalyzing the stereoselective cleavage of the hydantoin precursor into D-carbamoyl-p-hydroxyphenylglycine, which can be converted chemically to the desired product with retention of configuration. The important feature of this process is that an enzyme has been found sufficiently stable and active at slightly alkaline pH values, where the speed of the chemical isomerization of the hydantoin ring is fast enough to allow an almost complete conversion of the starting material into the chiral end product. The stereoselective cleavage by the hydantoinase acts like a sink to pull the isomerization and thereby replenishes the substrate for the enzyme. According to Kula, it is the combination of chemical and enzymatic concepts which makes this process so attractive and successful. It also teaches that chemicals not usually present in living cells nevertheless are targets for biotransformations.

In recent years, reactions previously considered pure organic chemistry have been investigated by biotechnologists--for example, epoxidation of olefins or hydrolysis of nitriles. For the latter reaction, enzymes offer unique possibilities in the regiospecific conversion of dinitriles or in the selective hydrolysis to amides. Improved understanding of microbial physiology and modern techniques in molecular genetics are being applied to develop such catalysts to their full potential. In addition, the bioreactor has to be optimized to make the best possible use of that potential for continuous processing. The first enzyme-catalyzed process to manufacture acrylamide from acrylonitrile is currently commercialized in Japan. Product concentrations of greater than 40 percent are obtained--a remarkable example that enzyme technology is not necessarily confined to dilute solutions with the inherent handling of large volumes and high cost for water separation.

To overcome problems with solubility in water, reactions in the presence of a water-immiscible solvent are being widely investigated. An important field for industry is the treatment of lipids and oils--for example, for the gentle hydrolytic cleavage of sensitive fatty acids, the transesterification for upgrading of palm oil fractions, etc. In such cases the lipid itself provides the organic phase. In other applications organic solvents are employed and the amount of water in the system may be reduced to a thin layer surrounding the protein surface. In biphasic reactors, the overall reaction scheme is complicated by the addition of new equilibrium and transport steps for which a data base has still to be generated.

Limitation in water activity is also sought to drive hydrolytic enzymes in the direction of synthetic reactions. Examples are the esterification of carboxylic acids or peptide bond formation. Proteases and peptidases are currently going through a boom of renewed interest in their properties, kinetics, and specificity for synthetic applications. One such example comes from the production of human insulin, still in growing demand for the treatment of severe diabetes. About one-third of these patients need a

substitution therapy corresponding to approximately 1.5-mg pure insulin per day, which leads to a predicted estimate of the annual world demand of approximately 5 tons. Proteases have been employed to convert pig insulin into human insulin which requires an exchange of the C-terminal alanine by threonine in the B-chain. A commercial process utilizes a transpeptidation reaction catalyzed by trypsin for this purpose.

By recombinant DNA technology human insulin can now be produced also in different hosts and from different gene constructs, which may differ mainly in the requirement of postsynthetic modifications to convert precursor into the active form, which contains two peptide chains held together by specific disulfide bonds. The conversion of fusion products is a new field of application of "old" enzymes under highly specialized reaction conditions and has wide implications also for products other than human insulin.

Another frontier of development in enzymatic conversions concerns coenzyme-dependent reactions which require an efficient simultaneous regeneration. Different approaches are under investigation. Isolated enzymes and a modified coenzyme may be employed in an enzyme membrane reactor for NADH-dependent reactions. Pilot plant studies have demonstrated the technical feasibility and stable long-term performance. Regeneration in this case is carried out by formate dehydrogenase. As an alternate route, the bioconversion of 2-ketoisocaproate into L-leucine with *Corynebacterium glutamicum* was investigated, utilizing a glutamate-dependent transaminase and the efficient ammonia fixation by this organism to regenerate glutamate. The necessary energy for the last step is derived from glucose. The steady progress in recent years and accumulated data base leads one to expect, according to Kula, that coenzyme-dependent reactions are just around the corner for large-scale utilization.

Over the last decades carbohydrates other than glucose, fructose, and lactose have not received much attention for biotransformations. Yet enzymes appear to be especially well suited, Kula said, to selectively modify single positions in such multifunctional molecules and these provide a whole array of new products and new synthetic routes for productions. New concepts in pharmacology are emerging from new insights into the role of glycoproteins, receptor structures, and biological recognition, creating a new demand in this field. Besides, simple carbohydrates are renewable resources and other uses than bioconversions into fuels are highly desirable. Investigations and progress in carbohydrate biotransformations have not yet reached a comparable intensity and scale as in the other topics discussed above. Kula stated, however, that the successful development of enzyme-catalyzed reactions in industrial processes provides encouragement as well as expectation.

Microbical and Enzymatic Processes

The topic of microbial and enzymatic processes for the production of biologically and chemically useful compounds was addressed by H. Yamada (Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan). Yamada and his group have recently been carrying out studies on the synthesis and transformations of various biologically and chemically useful coenzymes, amino acids, and amides, using microbial enzymes as catalysts. The basis for their investigations is that reactions catalyzed by enzymes and enzyme systems display far greater specificities than more conventional forms of organic reactions and, of all the reactions available, enzymatic synthesis and transformations have the greatest potential. Yamada presented two examples of enzymatic processes for the production of D-amino acids and amides. These are described in the following.

Synthesis of D-phenylalanine and Related D-Amino Acids. Dihydropyrimidinase is an enzyme that catalyzes the ring-opening reaction of dihydropyrimidines. A crystalline preparation of microbial dihydropyramidinase has been obtained by Yamada and his group and has been characterized in some detail. They have shown

that this enzyme catalyzes the hydrolysis of a variety of 5-monosubstituted D-hydantoins including not only those of naturally occurring amino acids, but also of some unnatural amino acids. For example, D-forms of 5-phenyl-, 5-hydroxyphenyl-, 5-chlorophenyl-, 5-methoxyphenyl-, and 5-thienyl-hydantoins are well hydrolyzed to the corresponding D-forms of the N-carbamoyl glycine derivatives. The L-isomers of hydantoins are spontaneously racemized under the mild alkaline conditions used for the enzymatic hydrolysis. Therefore, DL-hydantoins are completely converted to the corresponding D-forms of N-carbamoyl amino acids. These N-carbamoyl amino acids are quantitatively transformed to D-amino acids on treatment with $NaNO_2$ under acidic conditions.

As a result of these fundamental examinations of the production of D-amino acids, Yamada and coworkers have developed a new process for the production of D-p-hydroxyphenylglycine. This amino acid is an important component of semisynthetic penicillins and cephalosporins and has so far been synthesized chemically as a racemic mixture, which was then optically resolved through rather complicated processes. In Yamada's process, the starting substrate, DL-5-(p-hydroxyphenyl) hydantoin is synthesized through a newly established amidoalkylation of phenol with glyoxylic acid and urea under acidic conditions. The hydantoin is then hydrolyzed asymmetrically by the action of microbial cells with high enzyme activity. This step is followed by the decarbamoylation with $NaNO_2$. The present process may be the most economical process to date for large-scale production of D-p-hydroxyphenylglycine, according to Yamada. D-phenylglycine and D-valine can be produced in a similar manner. The production plant for these amino acids has been built at the Jurong Industrial Estate of Singapore by Kaneka Singapore Co.

Production of Acrylamide and Several Chemically Useful Amides. Acrylamide and methylacrylamide are produced industrially as monomers for synthetic fibers, flocculant reagents, etc. Yamada has proposed a new process using a microbial enzyme--nitrile hydratase--as a catalyst for the hydration of the nitriles. The new enzyme, nitrile hydratase, was highly purified and characterized in some detail by Yamada and his group. They proved that the enzyme catalyzes the hydration of various aliphatic nitriles including acrylonitrile and methacrylonitrile. Screening of microorganisms with high enzymatic activity revealed that *Pseudomonas chlororaphis* B23 showed the highest enzymatic activity when grown with isobutyronitrile as a major nitrogen source. Although the microorganism produces an amidase together with the hydratase during growth, the amidase is inactive towards acrylamide and methylacrylamide. Therefore, theoretically, stoichiometric conversion of the nitriles to the amides is possible even when the hydratase contaminated with amidase is used as the catalyst, according to Yamada. Practically, the reaction is carried out by exposing the nitriles directly with cells of the microorganism at low temperatures (usually 5-10°C).

Under suitable conditions, more than 400 g/l of acrylamide can be produced in a molar yield of nearly 100 percent, as stated by Yamada. In a similar manner, methacrylamide (200 g/l), acetamide (150 g/l), propioamide (560 g/l), n-butyramide (160 g/l and crotonamide (200 g/l) can be produced.

After optimization of the culture conditions for *Pseudomonas* nitrile hydratase production, the enzyme activity in the grown cells was 900 times higher than that previously reported. This process is simple, clean, and rapid; requires no special equipment involving energy; and yields a highly pure product. Yamada stated that the Kaneka Singapore Company will begin the commercial production of acrylamide using the immobilized cells of *Pseudomonas chlororaphis* B23 sometime in 1987.

Recently, a crystalline nitrile hydratase from *Pseudomonas chlororaphis* B23 has been prepared and characterized in some detail by Yamada and his group. The enzyme has a molecular weight of about 100 kilodaltons and consists of four

subunits identical in molecular weight. The enzyme contains approximately 4 gram atom of iron (III)/mole enzyme. The role of iron (III) in the enzymatic hydration of nitriles is currently under investigation by Yamada and his group.

Enzymatic Conversion of Insulin

The enzymatic conversion of porcine insulin and biosynthetic insulin precursors into human insulin was reported by E. Rasmussen (Novo Industri A/S, Bagsvaerd, Denmark). The conversion of porcine insulin to a human insulin ester as used by Novo in the production of human insulin is a transpeptidation reaction in which porcine insulin reacts with trypsin in a predominantly organic medium to produce a human insulin ester in a one-step reaction. The reaction mechanism is as follows: first, porcine insulin and trypsin form a Michaelis-Menten association complex, which subsequently by elimination of alanine forms an acyl-enzyme compound. This acyl-enzyme compound, which in the presence of water would hydrolyze to trypsin and des-B30-insulin, reacts in the presence of an amine to form a new Michaelis-Menten complex bewteen an amide of des-B30-insulin and trypsin. If a threonine ester is chosen as the amine, this amide of des-B30-(porcine) insulin is, in fact, a human insulin ester. If the amine component is present in a large excess the net result of the reaction is that porcine insulin is converted to a human insulin ester.

Rasmussen stated that in order to enable this reaction to proceed, the following two conditions must be fulfilled:

1. The water activity must be low. Otherwise the net result will be a simple tryptic hydrolysis of porcine insulin. Another effect of the low water activity--which is achieved by dissolving the reactants in a mixture of water and a water-miscible organic solvent--is that practically no reaction takes place at the other basic amino acid of insulin, i.e., the arginine at B22.

2. The amino component must not be in a zwitterionic state, i.e., the amine is not protonated. This means that the threonine must be present as an ester (or an amide). An example of the reaction conditions is given in Table 1. After the necessary purification, the ester group of the human insulin ester is then cleaved off and the resulting human insulin is subsequently purified further to meet specifications for human use.

Table 1
Reaction conditions for the conversion of porcine insulin into human insulin.

Component	Concentration
Threonine ester	1 M
Acetic acid	2.5 M
Water	20% (w/v)
N,N-Dimethylacetamide	≈60%
Porcine insulin	2-8 mM
Trypsin, porcine	0.01-0.2 mM
Temperature	12°C
Reaction time	24 h

Rasmussen then discussed one of the special problems encountered in the production of insulin by recombinant DNA (rDNA) technology--i.e., how to produce a two-chain molecule containing three disulfide bridges. He then presented the solutions developed at NOVO: single-chain insulin precursors, which, during the production in the manipulated microorganism (yeast), are folded correctly with simultaneous formation of the correct disulfide bridges and after isolation can be converted to human insulin by simple enzymatic reactions.

Two types of insulin precursors have been produced at Novo by rDNA technology. The first type has the amino acid sequence B(1-29-N-X-A (1-21), where B (1-29) denotes residues (1-29) of the B-chain of human insulin; A (1-21) denotes the A-chain; and N are n nonbasic amino acids. The peptide N-X can also be omitted. Examples of this type of precursor are:

- B (1-29)-A-(1-21)
- B (1-29)-Ser-Lys-A(1-21)
- B (1-29)-Ala-Ala-Lys-A-(1-21).

These precursors can be converted to human insulin ester by the same transpeptidation reaction as described for the conversion of porcine insulin (after the necessary adjustment of the reaction conditions, Markusson et al. 1987). The further purification and final conversion to human insulin follows a scheme similar to that used in the conversion of porcine insulin (loc. cit.).

The second type of insulin precursor has the structure B(1-30)-X_1-X_2-A(1-21), where B(1-30) denotes the B-chain of human insulin, A(1-21) the A-chain, and X_1 and X_2 two basic amino acids (alike or different). An example of this type is B(1-30)-Lys-Arg-A(1-21). The conversion of this type of precursor proceeds in two steps. First, the bond between Arg and A(1-21) is cleaved by trypsin, and subsequently the two basic residues, Arg and Lys, are removed by the action of carboxypeptidase B (Thim et al., 1987). The purification follows the normal column purification scheme as used by Novo in the production of monocompetent insulins.

Rasmussen concluded that enzymatic conversion is of great importance in the production of human insulin today, and also in the years to come will play a central role in the production of human insulin by rDNA technology. Whether in the future it will be possible to produce insulin directly in microorganisms in sufficient yields remains to be seen, but one of Rasmussen's coworkers recently described direct production of insulin in small amounts in yeast (Thim et al., 1987).

Microbical Conversion

The microbial conversion of alpha keto isocaproic acid to L-leucine was discussed by J. Berke (Degussa AG, Hanau, West Germany). This research was carried out in collaboration with R. Wichman (KFA Jülich GmbH, Jülich, West Germany). L-amino acids can be produced stereospecifically from chemically produced precursors, as α-keto carbonic acids, by biocatalysis. Berke said that the use of whole microorganisms as biocatalysts, in contrast to isolated enzymes enables the use of complex multistep reaction pathways towards the product desired, whereas by use of isolated enzymes the cost of product recovery will be low because much fewer byproducts are formed.

Biomass, obtained by fermentation with a costly effort of time, energy, and nutrients, is an important byproduct which is usually discarded after separation. By biomass retention, an enhancement of productivity or conversion yield could be possible for continuous fermentation if the product-forming reaction steps are not linked strongly to cell growth. Ideally, according to Berke, all biomass can be used as a biocatalyst.

At high space/time yield of L-leucine formation, the growth rate of the microorganisms used by Berke and his group was low. Therefore, they investigated the applicability of biomass retention for chemostatic culture. After preliminary experiments with a fermentation volume of 4 liters, a scale-up was performed using a fermentation system with 100 liters of fermentation volume. A steam-sterilizable centrifugal separator was used for cell retention. At a concentration of 50 g/l of cell dry weight, a conversion of 98 percent was reached at a space/time yield of 38 g of L-leucine per liter per day. A high selectivity was achieved, according to Berke. The concentration of L-leucine could be increased up to the limit of solubility. Berke indicated that these results give hints to improvements of the experimental setup, media composition, start-up procedure, and retention time.

Enzymes as Catalysts

The topic of enzymes as practical catalysts in carbohydrate synthesis was addressed by C.H. Wong (Department of Chemistry, Texas A&M University, College Station). Carbohydrates constitute a class of important molecules which are the current targets for synthetic organic and medicinal chemists. Increasing evidence shows that this class of compounds plays a central role in immunology and other biochemical recognitions. Many cell-surface glycoproteins containing complex carbohydrate moieties carry specific oligosaccharide determinants

involved in specific receptor-ligand interactions. Chemical methods for the synthesis of complex carbohydrates are highly developed but are complicated by multiple protection and deprotection steps and by difficult problems in stereoselectivity.

In connection with the interest of Wong and his group in developing practical enzyme-catalyzed practical synthesis of organic molecules with biological significance, they have recently developed several enzymatic approaches to carbohydrates and their derivatives. The enzymes, aldolases, are used in asymmetric C-C formation for the synthesis of unusual and usual monosaccharides and for the preparation of isotopically labeled sugars. Lipases are used as catalysts to selectively deprotect acylated sugars and to acylate free sugars. Hexokinases coupled with an ATP regeneration are used for selective phosphorylation of fluorinated sugars. Alcohol dehydrogenases coupled with NAD(P)H regeneration are used for selective reduction of acyl furan derivatives and keto aldehyde acetals to alcohol derivatives which are precursors to L-sugars. Glysosyl transferases coupled with the regeneration of UDP-sugar and UTP are used for synthesis of oligosaccharides. According to Wong, these enzyme products can be further converted to different sugar derivatives of potential interest as pharmaceuticals.

4 FUNDAMENTALS OF BIOPROCESS ENGINEERING

An overview of this topic was presented by K.Schügerl (Institute of Chemical Engineering and Industrial Chemistry, University of Hanover, West Germany). He said that in general, microorganisms have well-controlled metabolic sequences, designed for optimal growth in their natural environment. The traditional mechanism for changing their properties for a commercial exploitation are mutation and selection. The low probability of natural mutation (1 to 10^8) can be increased from 1 to 10^5 by chemical or physical mutagenesis. However, this method is still fairly inefficient. Another way to change the genetics of the organisms is by recombination, by mating, or by protoplast fusion.

The genetic information is encoded in the nuclear DNA. Many bacteria and some yeasts also contain small DNA molecules (plasmids) which are inherited after cell division. These plasmids are often used as vehicles (vectors) for cloning DNA into the recipient organisms, which is called recombinant DNA technology or genetic engineering.

Genetic engineering gave new impetus to biotechnology since it transfers genes from one organism to another--i.e., one that is easier to cultivate. By uptake of the foreign genes, the microorganisms often are able to produce not only proteins necessary for their metabolism, but also other proteins which in nature are produced only by animal or human organisms and therefore are often barely accessible.

Well-known examples are human proteins such as insulin, interferon, somoatostatin, and growth hormone. Also enzymes such as penicillin acylase as well as virus envelope proteins (hepatitis B virus envelope protein) were produced in this way by recombinant *E. coli* strains; the vectors used are often derivatives of the plasmid pBR322. Recently some proteins (interferon, for example) have also been produced by recombinant *Bacillus subtilis* and yeasts.

However, some proteins cannot be produced by microorganisms. They are formed by genetically modified animal or human cells. Such proteins are, for example, blood plasma components which are usually produced from blood serum. One of the very important components is Factor VIII, a protein which plays an important role in blood coagulation and which is absent from the blood of hemophiliacs. Other proteins such as tissue plasminogen activator (tPA), to which urokinase belongs, are able to dissolve blood clots and are therefore used for the treatment of thrombosis in myocardial infarction. Other proteins which also can be produced by genetic engineering techniques are vaccines, hormones such as calcitonin, and growth factors (interleukins I and II). The *in vitro*

preparation of recombinant vectors for insertion and transformation are carried out by genetic companies. For example, Genetech Co., US, prepared genes used for the formation of human insulin in *E. coli* bacteria and for the formation of Factor VIII in animal cells (BHK [Baby Hamster Kidney]).

The question considered in this session, Schügerl said, is how these cells can be handled on a technical scale for industrial production. The two main problems are how to overcome (1) biological hazards and (2) plasmid instability--i.e., to maintain biological safety and genetic stability. Stability is defined in this context as the ability of transformed cells to keep the plasmid unchanged during the growth of microorganisms manifesting their phenotypic chacteristics.

Natural plasmids maintained in bacterial host cells are very stable, even in the absence of selection pressure. The plasmid maintenance is secured by the replication control genes and the partition control genes, both of which are present in the plasmid themselves. The functions expressed by the partition control genes secure an even distribution of plasmid molecules at the point of cell division. In the cloning vector construction from natural plasmids, only the replication control functions are usually selected. If cells are grown without being selected for the presence of the plasmid the consequence often is the lack of stability. Even with a high copy number, cloning vectors, which are usually stable, often become destabilized after insertion of DNA fragments.

By insertion of partition regions into bacterial plasmids, it is often possible to avoid a significant decrease in yields from cloned genes, even if the cells grow in the absence of selection pressure. However, plasmid instability can also be avoided by using controllable expression vectors.

Two basic systems are available for developing controllable expression vectors: either a temperature-sensitive repressor/promoter combination or one that was derived from a metabolically controlled gene. The latter type could either be effected by the addition of a nutrient for the growth medium (as in the case of lactose for the lac promoter) or by removal of a nutrient (as with trytophan and trp promoter/operator region). However, control of metabolic promoters on multicopy plasmids has not proved to be very successful. Therefore, metabolically controlled promoters generally were superceded by temperature-controlled systems. Schügerl then discussed two types of these bacteria as follows:

1. Temperature-induced gene expression. The gene expression is controlled by the interaction between temperature-sensitive-repressor (cl 587) and -promoter. By increasing the temperature above 42°C, the repressor is deactivated and the gene is expressed; the product is formed in large amounts, which strongly affects the viability of the bacteria. The cell attempts to decompose the desired product. To avoid this, the product is formed as a fusion protein, from which the desired product is obtained by fission.

The production is carried out in two stages: in the first stage, the cells are cultivated at low temperatures (at about 30°C) and in the second stage, the temperature is increased, the gene is expressed, and the product is formed in large amounts. Since the cell cannot decompose the fusion proteins, it deactivates them by making them insoluble and decomposes them in the cell as "inclusion bodies." The separation of inclusion bodies of the fusion proteins from the cell-inherent proteins is easy. The "inclusion bodies" are separated by centrifugation, renatured, cleaved, and isolated.

2. Temperature-induced plasmid replication. The copy numbers are low at a low temperature (at about 30°C). Therefore, the stability of the plasmid is high. The production again occurs as a two-stage reaction. The cells are cultivated in the first stage at a low temperature, and the fusion protein is formed in the second stage at 42°C, where the repressor is deactivated, the plasmid replication is

started, and the genes are expressed. Their separation, renaturation, and cleavage occurs in a similar manner to the downstream processing of fusion proteins produced by temperature-sensitive gene expression.

These techniques lead to a very high transcription and to a large amount of fusion proteins, which may constitute 30 percent of the total protein content of the bacteria. Such a high level of expression would yield plasmid instability without promoter control. The dynamic behavior of these systems has not yet been investigated systematically according to Schügerl, though the optimum process control is strongly influenced by it.

Bioreactors for Animal Cell Cultures

The topic of bioreactors for animal cell cultures was discussed by A.J. Sinskey (Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge). He indicated that the need for mammalian cell culture biorectors is expanding. The primary reason is the need for the production of therapeutic proteins including monoclonal antibodies (Mabs). *In vitro* cultivation of mammalian cells allows for standard production processes of such proteins and in many cases is the only acceptable technology, according to Sinskey.

Although many bioreactors are available, Sinskey thinks that an objective characterization of biorector performance is lacking. Bioreactors can be chosen on the basis of objective criteria including scalability in terms of surface to volume ratios, oxygen transfer, and shear properties. He said that bioreactors employing high-density techniques--i.e., hollow fiber reactors, encapsulated systems, or matrix support--are more difficult to evaluate and characterize.

Currently, the lack of mathematical descriptions of mammalian cell culture kinetics limits the ability to optimally design and control animal cell bioreactors, according to Sinskey. As a model to overcoming such limitations, Sinskey discussed a strategy for developing mathematical equations relating both the growth rate and the specific antibody productivity of the hybridoma cell line, CR-1606, to its environmental state. Sinskey stated that three steps were important in this development: (1) reduction of the state variable set via statistical experimental design, (2) functional description of the initial metabolic rates with respect to the reduced variable set, and (3) mechanistic and experimental characterization of the deviations from the initial rate equations that occur at states other than the initial state. An equation was subsequently developed relating the initial growth rate to serum, ammonium, lactate, glutamine, and cell concentrations, with serum and ammonium exerting the greatest influence. The equation is a superposition of a noncompetitive inhibition relation in ammonium and lactate, with Monod equations in serum and glutamine. The inhibition constants were inversely proportional to the ammonium and lactate levels, while the Monod constant for the serum level was proportional to the -0.2 power of the cell concentration.

The instability of the growth-promoting activity of serum was also demonstrated. This instability may be responsible for the rapid decline in the growth rate that was observed in low serum cultures, according to Sinskey. The addition of thiols, or the elimination of cystine, a disulfide, from the media was shown to stabilize the growth-promoting activity of serum, thus suggesting an important role for the oxidation/reduction state of the culture. Thiols were also shown to stimulate growth in low serum medium at low initial cell levels. This stimulatory effect of thiols was reduced as the initial cell concentration was increased. Sinskey considers that these results suggest that the spontaneous oxidation of active thiols in serum is responsible for the instability of serum activity, as well as for the declining growth rates in low serum cultures. Sinskey hypothesized that these thiols may actually be dithiols, such as lipoic acid.

The utility of the mathematical relations developed for a fed-batch bioreactor was demonstrated by using Kelley's Transformation to determine the optimal

control policy of a fed-batch culture of CRL-1606 hybridomas. Experimental implementation of an optimal control policy determined thus resulted in cell and antibody levels twice those which are typically obtained in batch cultures. The resulting growth rate, cell, glutamine, ammonium, and antibody profiles were reasonably predicted from a numerical fed-batch simulation only if serum instability was included. According to Sinskey, the results strongly indicate that both ammonium accumulation and serum instability limit the productivity of fed-batch mammalian cultures.

Evaluation of Gene Expression and Recombinant Fermentation Processes

The evaluation of gene expression efficiency and performance of recombinant fermentation processes was discussed by D.D.Y. Ryu (Department of Chemical Engineering, University of California, Davis). Genetic stability of certain recombinant microorganisms is one of the most important problems in scaling up and commercialization of the recombinant fermentation processes, according to Ryu. It has been reported that the presence of certain plasmids in high copy number has a deleterious effect on the host cell, especially in the case of high expression plasmids.

Several modes of bioreactor operation have been proposed by Ryu and his group to maximize the concentration of cloned gene product and the productivity of a bioreactor system for the unstable recombinant cell cultures. For the purpose of stabilizing the recombinant cells, several methods such as insertion of the genes involved in the partitioning of plasmids (*par*, *cer*, etc.), introduction of internal and external selection pressure, and genetic modification of the host-cell genotype have been suggested and evaluated.

One strategy to deal with the recombinant cells which are unstable due to an increased productivity of cloned gene is to separate the growth stage from the production stage by controlling the levels of cloned gene expression by using a genetic switch. According to Ryu, a two-stage fermentation system in combination with a temperature-sensitive gene switching system offers the possibility of minimizing the instability problem of high expression recombinants in continuous production of cloned gene product. The use of a thermoinducible promoter-operator enabled Ryu and his group to separate the growth and production stages by simply adjusting the culture temperature. They were then able to grow the cells under the repressed state with very little or no expression of the cloned gene product in the first stage and thereby minimize the expression-related instability problem. When expression is desired, it can easily be turned on by a temperature shift in the second production stage.

Thus, Ryu said that the two-stage system makes it very convenient to study the fermentation variables under both the repressed and derepressed (or expressed) conditions by operating the first stage at a lower temperature and the second stage at a slightly higher temperature. Since the recombinant cells can be maintained stable even under high expression condition, the two-stage continuous bioreactor system also permits the study of the effects of fermentation parameters on gene expression of recombinant organisms under better controlled conditions than is possible in a single-stage condition or batch culture system.

Ryu discussed the following points:

- The development of a genetically structured mathematical model for product formation in a two-stage continuous bioreactor
- Application of such a predictive kinetic model to evaluation of performance of recombinant fermentation
- Examination of the dynamics of heterogeneous cell population in a two-stage continuous culture system
- Development of a general methodology for evaluation of the gene expression efficiency for gene product of new recombinants constructed having a certain "host cell-gene" system

- Determination of kinetic parameters closely related to the plasmid instability.

A partial list of the key parameters of the model includes the plasmid segregation rate, growth ratio of plasmid-harboring cell to plasmid-free cell, specific growth rate of recombinant cell, plasmid content or gene concentration, the rates of transcription and translation, and others. In order to develop a general methodology for evaluation of the gene expression efficiency for gene product, theoretical and experimental studies were undertaken by Ryu and coworkers using recombinant *E. coli* K12ΔH1Δtrp/pPLc23 trpA1 as a "gene-host cell" model system in a two-stage continuous culture system.

The theoretical analysis of cell population dynamics showed that the recombinant cells could be maintained stable for a prolonged time in a two-stage continuous culture system. Fermentation performance of the recombinant *E. coli* cells in a two-stage continuous bioreactor system was examined experimentally, and the gene expression efficiency of the cloned gene product was determined based on the kinetic model proposed by Ryu. Based on their experimental results, the gene expression efficiency of the model system was found to be about two-fold more efficient (i.e., 41.8 mg TrpA protein/mg plasmid DNA) as compared with the average rate of protein biosynthesis by *E. coli* cells. The performance of two-stage recombinant fermentation was also simulated using the mathematical model developed. Ryu stated that general trends obtained from the model simulation agreed reasonably well with the currently available experimental data, although further refinements needed to be made. Ryu thinks that the methodology summarized above could be used for evaluations of the gene expression efficiency of other genetically engineered recombinants and of the performance of recombinant fermentation process for a given "host cell-gene" system.

Scale-Up and Manufacture of Drugs Produced by Recombinant Organisms

This subject was discussed by D.M. Fenton (AmGen Company, Oak Terrace, California). Fenton focused on important parameters which should be considered during research and development of new proteinaceous drug substances produced using recombinant DNA (rDNA) technology. He stated that it is imperative that the ultimate scale of product manufacture be considered in the early stages of molecular biology research. The ease of performing molecular manipulations should not determine the host or vector system used, according to Fenton. The choice of hosts, whether *E. coli*, yeast, bacillus, or mammalian cells should be based on the hosts's ability to produce an *in vivo* active protein and the feasibility of producing sufficient quantity of product in a cost effective manner. Fenton thinks that given the current state of the art, a prototrophic *E. coli* strain possessing extensive phage resistance and a stable inducible vector system should be used whenever possible. The use of hosts other than *E. coli* for intracellularly produced proteins is probably limited to products such as hepatitis B vaccine where some post-translational modification of the expressed protein is required or solubilization of inclusion bodies and correct refolding of the denatured protein is difficult. Although product secretion into the fermentation media offers several advantages for large-scale production, yeast and bacillus secretion systems have not yet attained production levels comparable with *E. coli* systems nor of glycosylation patterns comparable to mammalian cells, and their use at this time is probably limited, according to Fenton. If correct glycosylation is necessary for biological activity and mammalian cells must be used, a shear insensitive cell line capable of growth and product synthesis in low serum culture should be developed.

Following research maximizing expression levels in a molecular biology group, a project devoted to the production of a new drug substance usually moves into fermentation research.

The goal of fermentation research, according to Fenton, is to increase the scale of fermentation from flask to small fermentor and to increase fermentation productivity. Increased fermentor productivity involves, first of all, optimization of the expression of the recombinant product. Classical selection techniques are often employed to isolate highly expressing host strains. Protein expression is also often increased two- to ten-fold by manipulation of fermentation conditions. For example, work at AmGen has shown that by increasing the concentration of available amino acids and glucose during product synthesis in *E. coli* fermentation, the intracellular concentration of insulinlike growth factor can be increased from 3 mg/g dry cell weight to 30 mg/g dry cell weight. It has also been demonstrated that the intracellular accumulation of recombinant products in *E. coli* can be enhanced under conditions which limit cell growth. In the case of intracellular products, fermentation productivity can also be increased by increasing the number of cells per liter. High cell density fermentations are usually attained by controlled feeding strategies which limit the accumulation of toxic metabolites, according to Fenton. However, he stressed that particular attention must be paid to media components and fermentation physical parameters during the induction of the gene and subsequent product synthesis. Nutrient limitation during product expression can have an adverse effect on product integrity. Depletion of amino acid pools can result in amino acid substitutions in highly expressed products in *E. coli*.

In the US and in many European countries, it is a requirement that above a 10-liter scale the recombinant microbe must be inactivated before it is exposed to the environment. Thus, according to Fenton, methods are usually developed in fermentation research to inactivate the microbe in the fermentor in a manner which does not affect downstream product recovery. For example, extreme pH can be used to inactivate microbial cells. However, these conditions denature and precipitate intracellular proteins. These denatured proteins can severely complicate product purification. Therefore, Fenton suggests that product recovery is best served by inactivation procedures which affect lipid or cell wall components and do not precipitate or modify cellular proteins.

In protein purification research the goal is to quickly develop a purification procedure capable of producing milligram or gram quatities of protein so that toxicity and animal efficacy studies can begin, according to Fenton. Product purity and product characterization are of primary concern while manufacturing cost and process scalability are of secondary importance. Fenton said that a great deal of effort is devoted to protein characterization and the development of final product specifications. Effective communication between the protein chemist and the fermentation scientist is critical at this stage of development. Fermentation techniques maximizing product concentration can cause unwanted modifications of highly expressed protein. For example, in *E. coli* the percentage of methionyl, formyl methionyl, and desmethionyl protein can be altered by fermentation conditions. In mammalian cells the pattern of product gylcosylation can be changed. However, according to Fenton, although product characterization is important, the protein chemist must always remember the ultimate scale of manufacture. Techniques such as gel filtration and affinity chromatography should be avoided if the mature market size requires kilograms of product. Highly expressed intracellular proteins and extracellular proteins produced in media containing limited amounts of extraneous protein can usually be purified using scalable methods such as ion exchange and hydrophobic chromatography.

Fenton stated that if a product is pursued into human clinical trials, fermentation and purification process development is usually initiated. The goal of process development is to ensure that one is capable of producing projected market requirements in an existing or planned manufacturing facility. In addition, manufacturing cost considerations

become critical items. Low-cost fermentation media components must be evaluated, and methods must be developed for purification resin recycle. Processes which cannot be scaled due to physical limitation are corrected. For example, due to the high oxygen and heat transfer rates achievable in small fermentors, microbial cells can be grown at near their maximum growth rate to high cell densities. In larger fermentors, it becomes necessary to allow high densities to be reached.

The final task of the process development group, according to Fenton, is to work with the manufacturing group to ensure that sufficient quantities of product are available for clinical trials and market entry. The manufacturing group must successfully implement the protocols developed by research and process development with the added constraints of operating under government regulations.

5 CONCLUSION

BIOTECHNICA '87, the third international conference on biotechnology in Hannover, West Germany, was even more successful than the previous two conferences in terms of the number of participants and the increased number of exhibitors. BIOTECHNICA, as a technology fair with a highly diversified conference program, leads to cooperation at different levels--vertically from the generation to the application of biotechnology and horizontally between research institutes or enterpises working on the same subject. Via cooperation, the innovative potential of biotechnology can be exploited much faster than by establishing more individual research departments. This is the basic consideration of the BIOTECHNICA concept and its multiple communication levels.

This report of the scientific presentations shows that much progress is being made not only in basic research in biotechnology but also in the application of this research to products for the market. It is also evident that production poses many problems that must be resolved by the manufacturing companies but that progress has also been made in this area.

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