CONFERENCE PROCEEDINGS SERIES

Harnessing Biotechnology for the 21st Century

EDITED BY
Michael R. Ladisch
and
Arindam Bose

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Message from the Chair

BIOTECHNOLOGY HAS CHANGED DRAMATICALLY since our first symposium (formerly named Fermentation Technology Symposium), which was held in Japan in 1960. Today, molecular biology tools are used in health care, animal nutrition, chemical production, and agriculture. These tools were not even thought of in 1960. If we compare the technical presentations from the 1960 symposium to our program in 1992, we see few similarities between the two symposia.

The Organizing and Program Committees developed 11 symposia, which contain 36 sessions. A total of 185 invited papers will be presented, representing worldwide biotechnology researchers.

I am pleased to inform you that a conference proceedings has been prepared and will be available during and after this symposium. The proceedings was edited by Michael R. Ladisch of Purdue University and by Arindam Bose of Pfizer Central Research.

I hope that you enjoy the oral presentations at the Ninth International Biotechnology Symposium and that you will obtain a copy of the symposium proceedings.

DANIEL I. C. WANG
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June 1, 1992

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Preface

THE INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY sponsors a conference on biotechnology every four years. The intent of these conferences has been to provide a snapshot of the underlying science and the enabling technology that have allowed commercialization of biotechnology. The proceedings of the earlier conferences of this series have become valuable reference volumes for academicians, practicing scientists, and engineers in industry. We hope this volume will continue to fill that need.

The generally accepted definition of biotechnology is that it "includes any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses" (U.S. Congress, Office of Technology Assessment; *Commercial Biotechnology: An International Analysis;* OTA–BA–218, 1984). This definition encompasses both the "old biotechnology" (for example, production of organic acids and antibiotics by fermentation) as well as the "new biotechnology" (i.e., processes employing microorganisms, plants, or animals that have been modified by recombinant DNA or by other modern genetic manipulation techniques). Biotechnology has been contributing to human well-being for thousands of years—beginning with the use of microorganisms to ferment foods during the dawn of civilization to the wonder drugs (antibiotics, anthelmintics, and cholesterol-lowering agents) of the present era. The principal impact of the new biotechnology has so far been in the pharmaceuticals arena. However, introduction of new products and services in the areas of agriculture, food additives, energy production, and waste treatment are imminent. The Program Committee of the Ninth International Biotechnology Symposium strove to attain a balance between the coverage of the old and the new biotechnology when assembling the technical program for this conference.

The schedule of oral presentations at this symposium consists of 185 invited papers. Care was taken to ensure that approximately half of the presenters were from outside North America. Each invited lecturer was requested to submit a manuscript for inclusion in this proceedings volume. The submitted papers have been organized into 11 symposia, each of which is subdivided into two or more sessions.

The first three symposia include topics in molecular genetics, biochemistry, and microbiology and provide the scientific basis for the manipulation of organisms constituting commercially significant biotechnology production systems. The fourth symposium covers biocatalysis, including cutting-edge issues such as catalytic antibodies and the use of enzymes in novel organic synthesis tasks.

Biochemical engineering topics (microbial and animal cell bioreactors, product

recovery, and monitoring and control of bioprocesses) are the subject of the next three symposia. For the new protein biopharmaceuticals, the cost of product recovery and formulation can often exceed 80% of total bulk drug production cost versus generally less than 50% of total cost for fermentation-derived antibiotics. Thus, a considerable incentive to develop new separation processes for those biopharmaceuticals exists. Most of the papers in Symposium VI address aspects of protein processing, including folding or refolding and formulation.

Within the next decade, the new biotechnology is likely to have a significant commercial impact on agriculture, treatment of wastes, and production of liquid fuels from renewable resources. The sessions on bioremediation, impacts of transgenic plants, and biomass utilization contain contributions from many leading researchers in those fields. Finally, to illustrate the potential of biotechnology in promoting economic development, descriptions of relevant projects in several developing countries are included in Symposium XI.

Any biotechnology development project, by its very nature, is an interdisciplinary effort with life scientists, engineers, and regulatory professionals working closely together. That diversity is also reflected in these proceedings. Specific sections of this book may be of interest primarily to certain groups of professionals. However, we shall consider our efforts in editing this volume worthwhile if a reader finds something useful in papers that are outside the area of his or her expertise.

We thank the numerous corporations, government agencies, and individuals (listed elsewhere in this volume) whose generous financial support made the organization of this conference possible. Special recognition is owed to our Distinguished Corporate Sponsors; their offers of financial support early in the conference planning cycle allowed us to attract prominent speakers from outside North America. We thank Norma Leuck for her assistance in ensuring the timely completion of this volume. We are also grateful to Dianne Ruddy, Latisha Best, and Michelle Hicks of the American Chemical Society's Meetings Department for handling the administrative chores and local arrangements for the conference. Finally, our heartfelt thanks are due to Cheryl Shanks and the staff of the American Chemical Society's Books Department for making this volume possible.

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Expressing, Processing, and Secretion in Eukaryotes: Session B
C. F. Goochee and W. Fiers: *Co-Chairs*

Expression in Transgenic Systems: Session C
W. H. Velander and R. Strijker: *Co-Chairs*

OPTIMIZATION OF HETEROLOGOUS GENE EXPRESSION USING GRAM NEGATIVE BACTERIA

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The last 3-4 years has seen considerable growth in the use of a wide range of gram negative bacteria (e.g. Salmonella Typhimurium, Pseudomonas putida, and Zymomonas mobilis) for the expression of heterologous gene products. However, Escherichia coli has been and continues to be the work horse of the microbial gene expression field. No other system has been developed (thus far) that can not only express virtually any gene product at levels sufficient for detailed biochemical analysis or product development but also express undefined coding sequences (open reading frames) in amounts sufficient to determine the identity of the gene product and to characterize its function. I will describe advances made over the last few years in our ability to optimize the expression of heterologous gene products in E. coli with respect to both quantity and quality of the proteins being produced with the understanding that many of these same approaches can be applied to heterologous gene expression in other gram negative bacteria.

Literally hundreds of papers are published each year in which it has been reported that genes of prokaryotic or eukaryotic origin have been cloned and expressed in E. coli. The ability to express this myriad of genes has been aided by the development of numerous expression vector systems which use highly efficient, regulated, transcriptional regulatory signals to optimize production (for a review see Brosius, 1988). We, (Shatzman and Rosenberg, 1985) and others (e.g. Schauder and McCarthy, 1989) have shown that optimization of translational regulatory signals [ribosome binding sites (RBS)] is equally important to the optimization of heterologous gene expression. For example, in our laboratories, we have seen high levels of expression of various viral antigens using a phage λ P_L promoter coupled with translation initiation using the phage λ cII RBS. In contrast, little, if any, expression of these same antigens was obtained using a pTac promoter and lacZ RBS. In fact, this result had nothing to do with the promoter chosen to drive transcription as equal levels of gene specific message were obtained following induction of both vector systems. Instead, the differences in expression were due solely to translation initiation. When mutations were introduced into the lacZ RBS to make it identical to the cII RBS, equivalent levels of antigen expression were obtained from both promoter systems.

Over the last few years, the focus on the optimization of gene expression has gone beyond the study of regulatory signals to now include alterations in the coding sequence to be expressed. In addition, major efforts have now been made to understand factors which affect not only levels of production, but also the quality (solubility, homogeneity, folding) of the products themselves. It is these newer efforts which I will now focus on.

Optimization of gene expression via alterations in the coding sequence

Initial efforts to express a heterologous gene in E. coli can often lead to less than desirable results with respect to the quantity of protein produced. In some of these cases, even the use of several different transcriptional or translational regulatory signals does not improve the level of protein production. Several reports have been published which show that production of such gene products can be dramatically improved by making alterations within the gene coding sequence. For example, Seow et al. (1989) reported that expression of human tumor necrosis factor (TNF)-β could be

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increased from undetectable levels to 34% of total cell protein (TCP) by introducing silent mutations throughout the gene, which result in optimal codon utilization for E. coli, and also mutations in the 5' end of the gene, which minimize the development of secondary structure in that region of the message. Interestingly, expression levels were quite low (1-2% of TCP) if only the changes minimizing secondary structure were made, while no expression was detected when only the codon optimization changes were present. In contrast, others have shown that a more random approach to coding sequence alterations can result in vastly improved expression levels. Devlin et al. (1988) and Sathe et al. (1990) demonstrated that expression of human granulocyte stimulating factor could be increased from undetectable levels to almost 20% of total soluble protein by altering G and C residues in the 5' end of the gene to A and T resideus without affecting the protein sequence. These changes actually greatly decreased the presence of preferred codons in this region of the gene and did not create any significant changes in mRNA secondary structure. Reduction in the GC content of the 5' end of the bovine growth hormone gene has also yielded increased expression levels in E. coli (Hsiung and MacKellar, 1987).

While we have had many successes in our laboratories using this approach, random replacement of G-C base pairs with AT proved unsuccessful in optimizing the expression of human alpha-1-antitrypsin (A1AT). Only by using a process of mutagenesis and genetic selection were we able to find mutations in the 5' end of the A1AT coding sequence which allowed us to achieve expression of A1AT at 10% of TCP in contrast to <0.1% of TCP when the native coding sequence was used (Sutiphong et al. 1987).

Improved production of soluble and active proteins in E. coli

Typically, the first concern in heterologous gene expression is "how much can be made." However, after achieving high level expression, attention then must focus on the utility of the product for, unfortunately, most heterologous gene products expressed in E. coli are not produced in an active properly folded, or soluble form. Instead, most recombinant proteins accumulate as insoluble aggregates or inclusion bodies in the cytoplasm of the bacteria.

Considerable efforts have recently been made to understand what causes these proteins to be insoluble and to find ways of expressing these proteins in more soluble, active forms. Schein (1989) compiled a list of nine suggested reasons for the formation of inclusion bodies in E. coli which includes: excessively high rates of production, which do not allow sufficient time to achieve correct folding; high local concentrations of product leading to precipitation; and lack of post-translational modifying enzymes or foldases necessary to achieve the correct conformation.

Expression of subtilisin in E. coli using a high-expression vector under standard growth and induction conditions was recently shown to lead to production of an insoluble product from which very little properly folded and active subtilisin could be obtained (Takagi, et al, 1990). However, when cells were grown at 23°C instead of 37°C, an increase in active subtilisin of up to 14-fold was achieved. Further studies showed that a reduction in the concentration of inducer used in this expression system could increase levels of active product even with growth at 37°C. When both temperature and inducer concentrations were adjusted, active product accumulated at levels 16-fold above those found under standard production conditions. These studies suggest that the rate at which a protein is made can affect its ability to fold properly. Similar effects of culture temperature on product solubility have been observed for the production of human interferon α2 (Schein, et al, 1988). In these studies, overproduction of interferon at 37°C yielded only 5% soluble, active protein while growth at 28-30°C produced 73% soluble product. Thus, although total interferon production at 37°C was three times greater than at 28-30°C, the lower production temperature actually yielded a seven-fold increase in active product.

Work in our laboratories over the last 2 years have also shown that product solubility and