BIOTECHNOLOGY CHALLENGES FOR THE FLAVOR AND FOOD INDUSTRY

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Preface

In 1987 Quest International convened its 1st International Scientific Symposium on the timely topic of 'Healthy Eating: A Scientific Perspective'. This assembly at Woburn Abbey in Bedfordshire, England, initiated a series of symposia designed to explore current topics of great interest to the food industry on a scientific level. The success of the 1st symposium in the series was assured by bringing together an audience of senior technical staff from companies representing food industries in various parts of the world and the noted experts who were invited to present the latest information on the theme of healthy eating.

These Proceedings continue the series, providing an account of the 2nd Quest International Scientific Symposium on 'Biotechnology Challenges for the Flavor and Food Industry' held at Williamsburg, Virginia, USA in 1988. Historically, within the field of food ingredients, few technical endeavors have posed greater potential opportunities than biotechnology. However, equally important challenges face the workers in a number of related disciplines, and these individuals must communicate effectively and work in concert to develop these opportunities into useable technologies. Contributors to the 2nd Quest symposium represent many of the disciplines involved, and provide a broad perspective of the activities and excitement surrounding biotechnology today.

After an excellent introduction to the basics of biotechnology, current progress in microbiological fermentations, enzyme tailoring for specific applications, and plant biotechnology were discussed. Presentations included the application of genetic engineering techniques to food and flavors and the very important area of biochemical engineering which poses exciting challenges in such

areas as biocatalytic reactor design and operation, and in recovery of products. Excellent summaries of the status of biotechnology in the food and flavor industries of Europe, Japan, and the USA provided participants with a realistic perspective of progress to date. These discussions were placed into a real life context during a presentation on the USA Food and Drug Administration's regulatory requirements relating to the commercial introduction of a new substance, for human food use, produced by modern biotechnological methods.

The symposium provided a forum where ideas were freely exchanged between those less familiar with biotechnology and those at the cutting edge of biotechnology research. A consensus emerged which indicated that the food and flavor industries should actively participate in identifying and selecting specific opportunities for research and development so that resources can be concentrated and directed towards items and matters of commercial significance.

Additionally, there was a recognition that the food and allied industries must work with public policy and regulatory agencies to assure public confidence in these new products. This indeed will require an extra measure of communication and mutual understanding of diverse information. It is hoped that this volume will contribute to the intellectual processes which will lead to rational views and policies regarding biotechnologically-derived ingredients and foods. Through such we can look forward to a food supply with improved qualities of appearance, texture and flavor, and which is nutritious, safe and economical.

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Biotechnology in the Flavor and Food Industry—A Scientific Starting Point

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ABSTRACT

Biotechnology is of special interest to the flavor industry because it may enable the mass production of important flavoring materials at relatively low cost. Conceptually, the production of flavor compounds and other natural products using biotechnology seems straightforward. The previous difficulties associated with transferring genes from one organism to another are rapidly fading. The critical problem for implementing these new technologies is targeting and amplifying the enzymes required for biosynthesis of specific natural products. This chapter reviews recent scientific advances in the areas of cloning vectors, transformation methodology, protein engineering and enzyme technology that should have major impact on the bioengineering of natural products. The need for continued reliance upon traditional biochemical approaches for elucidation of metabolic pathways in plants and microbes is stressed.

Biotechnology, particularly genetic engineering, has been looked upon as a means of revolutionizing food production and quality. Of special interest to the flavor industry is the potential for producing

large quantities of any desired flavoring material or blend at relatively low cost.

Traditional breeding techniques and mutagenesis have been used to achieve increased yields in plants and microorganisms. However, since it is difficult to select for specific molecules, the extent to which breeding or mutagenesis may be used to enhance the level of any one compound is limited.

Conceptually, the production of flavor compounds and other natural products using biotechnological techniques seems straightforward. The strategy that seems most obvious is to amplify the rate limiting steps of appropriate biosynthetic pathways. These rate limiting steps are typically determined by one or more of the biosynthetic enzymes in the pathway. In theory, expression of each of these enzymes should be controllable by gene cloning techniques. In addition to amplification, it may be possible to eliminate points of negative regulation within pathways or competing metabolic pathways.

In reality, targeting and amplifying the enzymes intrinsic to the specialized metabolic pathways of natural product formation is no small task. Although the difficulties which stood in our way with regard to the transfer of genes from one organism to another are rapidly fading, the question of *which enzymes* are important targets for genetic manipulation remains. In other words, a detailed biochemical knowledge of flavor biogenesis pathways is essential for pinpointing the molecular targets for cloning and, in many cases, our knowledge of these metabolic pathways remains obscure.

Where is the scientific community directing its attention? To use genetic engineering for production of flavors one needs the following information: (1) the compound's structure; (2) its position within a metabolic pathway; (3) the identity of each enzyme within that pathway; and (4) a knowledge of each enzyme's regulation. The enzymes are of particular importance since they usually constitute the rate limiting steps and are the direct targets of genetic engineering. Once an enzyme is targeted, we can begin to apply the appropriate tools of biotechnology, from which there are many to choose. The usual strategy is to generate a genetic (cDNA) or immunological probe (antibody) and to clone the gene into a viral,

bacterial or eukaryotic vector. The gene may then be inserted into a chosen host organism (transformation), where, under appropriate conditions, it is expressed. The expectation, of course, is that this procedure results in increased output of the desired material.

The evolution of genetic engineering has been dependent upon the steady appearance of new and quite elegant biochemical, genetic and immunological tools that are making our task an easier one. Our goals, lofty as they are, therefore, are continually more realistic and technologically attainable.

Other chapters in this book cover specific applications of this broad technology and the ramifications for the flavour industry. For an indication of the ways in which biotechnology may be applied to the flavor industry, the IFT Expert Panel on Food Safety and Nutrition has prepared a Scientific Status Summary.[1] This contribution outlines some very recent developments in the areas of molecular biology and enzymology. Each is at the cutting edge of biotechnology and will undoubtedly contribute to our quest for biotechnologically derived food ingredients.

GENETIC TECHNOLOGIES

It has been approximately a decade since the first genetic engineering experiments were successfully conducted. We are now beginning to see commercialization of genetically engineered pharmaceuticals such as α-interferon, tissue plasminogen activator, various hematopoietic growth factors and of food-grade enzymes such as chymosin and lactase.

The process of gene cloning, the backbone of genetic engineering, basically consists of: (1) targeting the gene of interest; (2) constructing a genomic library; (3) inserting the gene into a host (transformation); and (4) obtaining stable expression of the protein This process has become almost routine in *Escherichia coli* and yeast. In other host organisms, such as plants, and in systems where multistep conversions are involved, certain complexities exist that must be overcome for concepts to become commercial reality.

Where has progress been made? Quite simply, on virtually all fronts, as this essay is intended to document.

Cloning Vectors

No longer must one specialize in molecular biology to clone a gene. However, one does need a probe (cDNA or antibodies) to screen the contents of these genomic libraries. Once this is achieved, anyone may take advantage of the unprecedented wave of standardization that has taken place. The recent appearance of 'cloning kits' with detailed instructions have eliminated the need to 'start from scratch'. Genomic libraries from a wide range of organisms and tissue types as well as cloning vectors now are no farther than a phone call away.

An example of one of the more popular vectors that has emerged as a tool for cloning genes from plants and other eukaryotes is λgt11.[2] Lambda is a virus that infects *E. coli*. To construct a cDNA library, cDNA is ligated onto the λgt11 vector and *E. coli* is infected. The advantage of this system is that colonies are screened for positive clones with nucleic acid probes or with antibodies directed specifically against the protein of interest. This probe is now being used quite successfully with a range of plant,[3,4] microbial[5-7] and mammalian [8-10] systems.

Transformation Methodology

In theory, once a gene is identified, it may be inserted into the chromosome of almost any species. The process of introducing a gene into a host cell is known as transformation. The proper expression or function of a transferred gene is also of great concern. Procedures for transformation and expression in some simple bacteria such as *E. coli* or *Bacillus* and unicellular fungi such as yeast are now well established. However, as the complexity of an organism increases, gene transfer becomes a much greater technical challenge. Plants and filamentous fungi are more difficult to transform than bacteria or yeast. This is due in part to an increase in the number of chromosomes and the amount of DNA, and to more highly regulated mechanisms of transcription and translation. Nonetheless, the development of methods for integration of genes into plant tissue and their stable expression has been intensively pursued. Many plants have now been successfully transformed.[11]

Until recently, the main transformation system for plants was the

DNA-transferring bacterium *Agrobacterium tumefaciens*. Although *A. tumefaciens* successfully transforms dicotyledenous plants with the Ti plasmid,[12,13] it does not work well with cereal grains, which are monocots. The past 2 years have seen tremendous strides made towards overcoming this problem by the development of novel ways to transform plant cells with foreign DNA. For example, Dr Westcott discusses the use of 'hairy root' technology to transform monocots in Chapter 4.

With protoplasts, individual plant cells whose cell wall has been digested away, it is now possible to efficiently introduce DNA by a process called electroporation. During electroporation electrical impulses of high field strength reversibly permeabilize the membrane which allows DNA to penetrate.[14–16] Although electroporation is straightforward, regeneration of plants from the protoplasts has been difficult. However, it seems that this problem is close to being solved as plants have now been regenerated from protoplasts of rice[17] and maize.[18] In addition to electroporation, micromanipulation or direct injection has been explored as a means for protoplast transformation.[19]

It is now even possible to directly transform the intact (i.e containing the cell wall) plant cell with DNA that is coated onto high-velocity microprojectiles.[19–21] Tungsten microprojectiles are injected into plant tissue using a particle gun and transformants are screened using standard methodology. Expression of foreign DNA has been reported in maize[20] and *Chlamydomonas*.[21]

Together, electroporation, microprojectile technology and protoplast regeneration show great promise for simplifying the application of genetic engineering techniques to plants and fungi. With these tools in place, we should now begin to see the focus of plant biotechnology shift from methods development to process development. Dr Lilly addresses process development in Chapter 5.

PROTEIN ENGINEERING AND THREE-DIMENSIONAL STRUCTURE DETERMINATION

'Protein engineering' has recently come into use as a technology for probing enzymatic reaction mechanisms and for improving the way

enzymes work.[22] For example, allowing them to operate at higher temperatures would greatly increase the efficiency of many enzyme-catalyzed reactions. Protein engineering may also allow enzymes to act on molecules slightly different in structure than their natural substrates or over a wider range of pH, and this may prove useful in ingredient production. This technology involves additions to or rearrangements of the internal bonds which contribute to an enzyme's three-dimensional structure. This is achieved by a reordering of the nucleotide sequence in such a way that one or maybe two amino acids are introduced to achieve the desired effect; this procedure is called site-directed mutagenesis.[23]

Currently, protein engineering has been applied mostly to the small group of enzymes whose three-dimensional structure is known.[24–26] Computerization and improved knowledge of crystallization portends a dramatic increase in the number of proteins whose structures will be solved in the near future. Structures recently obtained include interleukin-2,[27] the oncogene protein human c-H-ras p21[28] and a bacterial bifunctional enzyme that catalyzes two reactions.[29] In addition the science of crystallography was extended to obtain structures of an antibody–lysozyme complex[30] and of an integral membrane protein.[31] The three-dimensional structure of a trypsin mutant with its active site modified by site-specific mutagenesis has even been determined.[32]

ADVANCES IN ENZYME TECHNOLOGY

As new processes and ingredients continue to appear, growth in industrial applications of enzymes will accelerate. Recent advances have necessitated a fundamental change in the way in which we think about enzymes. Because enzymes by nature are environmentally labile, we may have been erroneously led to believe that there is a limit to the kinds of reactions which enzymes catalyze. Now, new and creative approaches, pioneered by Dr Klibanov, are demonstrating that enzymes can catalyze reactions under conditions previously thought to be too extreme, and that enzymes or enzyme mimics catalyze reactions that do not occur biologically.

Antibodies with Catalytic Function and Enzyme Mimics

Although the idea of developing catalysts that 'mimic' the action of enzymes has been around for some time, it is only in the last couple of years that real breakthroughs have been made. Two types of synthetic enzymes are under development, synthetic 'enzyme mimics'[33] and monoclonal antibodies that possess enzyme activities, the so-called 'abzymes'.[34,35] Both show great potential as catalysts for chemical transformations for which no known enzyme in nature exists. In addition, these early breakthroughs demonstrate the potential for producing 'super' catalysts that possess few of the many limitations faced by today's enzyme industry.[1]

Enzyme Reactions in Non-aqueous Media

New aspects of enzymology are constantly emerging. One area which has recently blossomed and that will see widespread and immediate growth is non-aqueous enzymology. The pioneering work of Klibanov has proven that many enzymes can be made to function in organic solvents (against 'conventional wisdom'), provided that the enzyme remains surrounded by a layer of water.[36–39] This has implications for organic synthesis,[36–39] analysis,[40] sweetener production[41] and even the design of temperature sensors.[42] It is now being demonstrated that super-critical carbon dioxide is also a good medium for enzymic conversions, especially in reactions where lipophilic substrates and products are involved.[43,44]

THE CHALLENGE IN FOODS

Rapid progress is being made towards fine-tuning many of the diverse techniques that are required for successful gene cloning and protein engineering. The question of immediate concern for the food industry becomes: How can we put this technology to good use? In some cases, particularly in single-enzyme systems such as the use of chymosin for cheese-making or β-galactosidase for low-lactose

dairy products, the answer is clear. But in more complex systems, those involving metabolic pathways or tissue culture, molecular targets for genetic engineering often remain obscure. Much of our emphasis will, therefore, continue to focus on basic research.

The pharmaceutical industry has used biotechnology to build many new profitable products and processes on the foundation of basic research which it has funded over the last 40 years. This commitment to basic research has, up to now, been lacking in the food industry. The challenge to the food industry, then, lies in its willingness and ability to conduct and fund research to further understand the molecular basis of food functionality—flavor, texture, color, nutritional value and appearance. This effort is a prerequisite to the successful application of modern biotechnology's tools to food systems.

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