T. Yoshida · R. D. Tanner (Editors)

Bioproducts and Bioprocesses 2

Third Conference to Promote Japan/U.S. Joint Projects and Cooperation in Biotechnology, Honolulu, Hawaii

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Preface

Introduction

During the week of January 6–10, 1991, the Third U.S.–Japan Conference on Biotechnology was held at the Asian-Pacific Conference Center at the University of Hawaii in Honolulu. This book is a compilation of the papers and posters presented at the Conference. The Conference was sponsored, in part, by the National Science Foundation and U.S. pharmaceutical companies including Ortho Pharmaceutical, Merck, Genentech, SmithKline Beecham and ABEC. Its purpose was to promote information exchange between Japanese and U.S. researchers, primarily academics, in biotechnology and to seek ways to carry out collaborative research in biotechnology.

The honorary chairmen of the Conference were Professor H. Okada and me. The formal program was organized by Professors J. Bailey and T. Yoshida. Twelve invited formal presentations were given from each side. In addition, both sides were invited to bring along five observers to the Conference who were encouraged to prepare poster presentations on their research. Paper abstracts plus bibliographies were exchanged prior to the Conference in order to promote maximum technical interaction between the participants.

The presentations were selected and equally divided among four preselected topic areas. These were 1) applied genetic engineering, 2) biocatalysis, 3) bioprocess engineering, and 4) cell culture. Six papers were presented in each topical area. During lunch and coffee breaks, the participants had an opportunity to discuss their work in one-to-one situations. In general, the Japanese academic research work was strongly applications oriented while the U.S. work appeared driven by support and direction through funding sources from the National Science Foundation. There appeared to be a strong desire on both sides to do collaborative research; however, it was found that the avenues of collaboration had to be driven by individual contacts. Clearly, the more basic the research work, the easier it appeared to collaborate.

The following is a brief summary of the papers presented in each topical area.

Applied Genetic Engineering

Although the title seems internally redundant, i.e., applied and engineering, it really represented an effort on the part of the program organizers to look at research concerned with the application of recombinant DNA techniques to produce useful products or to achieve more efficient processes. For example, the first paper in this area looked at the use of secretion-signal for mucor rennin to achieve secretion of pro-urokinase and human growth hormone in a recombinant yeast and then to use this knowledge to genetically engineer an improved rennin. The next paper discussed the use of genetic engineering techniques to improve the carbon conversion and energy metabolism in cells. In a third paper, the author discussed ways to genetically engineer the production of a tuna growth hormone in *E. coli.* The next paper focused on the usefulness of amplifying homologous genes in a bacterium in order to improve the fermenting characteristics of the solvent producing *Cl. aectobacterium.* The fifth paper was concerned with the cloning of the thermal stable alcohol dehydrogenase gene from *Bacillus stearothermophilus* NCA 1503 into a *Bacillus subtilus* as a way of shifting the pH optimum of the mutant enzyme from 7.8 to 9.0. The final paper in this series was concerned with methods to improve and/or optimize a recombinant fermentation process.

Biocatalysis

In this series of papers, such topics were discussed as the use of site directed mutagenesis to explore the structure and function of enzymes, the role of enzymes in chiral synthesis, and the mechanism of enzyme activity and function in organic solvents. In the chiral synthesis paper, nearly 100 examples of the use of enzymes in the commercial production of chemicals were reported. One process was described for producing up to 40 000 ton/year of acrylonitrile by enzymatic catalysis. Another paper discussed enzyme behavior in unusual environments such as super critical CO_2. A final paper discussed strategies for designing enzyme-like catalysis including the possibility of producing catalytic antibodies.

Bioprocess Engineering

This session provided a truly "mixed" bag. Topics covered included on-line diagnostic systems for controlling a fed batch fermentation, development of an expert system for a phenylalanine fermentation, development of micro-biosensors for biomedical research, sensors for bioprocess monitoring and control plus development of purification systems for fermentation products recovery. In the latter, paper systems included studies of 2-D gel electrophoresis and gradient

elution chromatography. Of particular interest was the paper on micro-biosensors which discussed the philosophy of designing micro-biosensors and the various potential applications.

Cell Culture

Four of the six papers in this session focused on the culture of plant cells. Two of the papers looked at ways through genetic engineering to regulate the production of desired secondary metabolites. One paper was concerned with somatic embryogenesis as a method for the large scale production of transgenic plant varieties. Another emphasized the design and operation of bioreactor systems that would maximize volumetric productivity. This included individual strategies such as strain selection, medium development, cell immobilization, product secretion, *in situ* product removal and elicitation. The fourth paper discussed the design of a photo-bioreactor suitable for plant cell cultivation. The remaining two papers were concerned with the design and operation of bioreactors for the cultivation of animal cells. One of the presentations discussed an effort to increase the quantitative understanding of how binding and trafficking aspects of growth factor/receptor interactions influence mammalian cell proliferation.

There was general agreement among the participants that the periodic organization of these conferences promotes an understanding and greater appreciation of biotechnology activity in our respective countries. Through the informal contacts, opportunities were identified for postdoctoral and visiting scientist exchanges beyond those offered by governmental agencies. Also, these contacts catalyzed scientific friendships and enhanced the appreciation of each other's culture. Clearly, they are very valuable, particularly in view of the very low costs of the conferences. To my Japanese colleagues—dozo yoroshi-ku. To those of you who find these exchanges interesting, consider applying for observer status at the next one. To those companies that supported the Conference and to the NSF for the basic travel grant—thank you.

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Contents

1 Biochemical Engineering and Biotechnology

1.1 Biochemical Engineering and Biotechnology – An NSF Perspective

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Abstract

Engineering research related to the life sciences is becoming more important as an increasing number of products from genetic engineering and cell fusion technology reach the market place. Novel bioprocess engineering, both upstream and downstream, is also needed to provide a fundamental engineering basis for the economical manufacturing of substances of biological origin. Research linking the expertise of engineers and life scientists is crucial to providing such a fundamental basis, and requires individuals who are broadly competent in each of their fields and who are also willing to collaborate on research projects. This presentation intends to provide an overview of how the Engineering Directorate within the National Science Foundation plans to provide support for the engineering research needed to address the problems of the economic production of products obtained from the most recent advances in the life sciences.

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1.2 Use of Fluorometry for On-Line Monitoring and Control of Bioreactors – Microbial Cell Concentration and Activity, Plant Cell Metabolism, Mixing Time, and Gas Hold-up

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Contents

This paper discusses the potential for fluorometry in monitoring fermentations. Emphasis is on the application of multiple excitation fluorometry. This application includes monitoring of microbial concentrations and activities, monitoring metabolic response to perturbations of plant cell cultures, and determining mixing times and gas hold-up of real fermentations.

List of Symbols and Abbreviations

AFU	arbitrary fluorescence units
DO	dissolved oxygen
MEFS	multiple excitation fluorometric system
NFU	normalized fluorescence units
NADH	reduced nicotine adenine dinucleotide
C	fluorescence signal
C_0	initial fluorescence signal
C_f	final fluorescence signal
t_{exp}	time constant which is the time when signal reaches 63.212%, i.e. $(1 - e^{-1})$ of maximum response
t_{lag}	lag time which is defined as the time for first response

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1 Introduction

On-line monitoring and control of bioprocesses is one of the most challenging research areas in biochemical engineering. Sterilizable dissolved oxygen (DO) and pH probes have been available since the 1960s. No other biosensors developed since then have proved to be as good as the DO and pH probes. Dissolved oxygen, pH and temperature along with off-gas analysis are the only parameters which are monitored on-line in industrial fermentations. None of the present on-line measurement techniques involves intracellular parameters. This limitation has hampered the development of bioprocess control since our ability to control and perform on-line optimization of fermentation processes is limited by our ability to monitor what is happening in the fermentation.

In order to control a bioprocess, we need to be able to measure on-line substrate, product, and cell concentrations, as well as cellular activities within the fermentor in a non-invasive manner. Techniques currently exist to achieve this based upon enzyme, immunological and optical density probes, as well as using indirect measurements by material balancing around the bioreactor, specifically measuring oxygen uptake rates (OUR) and utilizing simple metabolic models to estimate cell growth, substrate uptake, and product formation rates. Unfortunately, none of these probes are practically robust devices. Enzyme and immunological probes cannot withstand in situ sterilization. Optical density probes are reliable only for non-mycelium cultures growing on clear, non-particle containing media, conditions that are generally uncommon in fermentation industry. The models used to estimate cell growth, substrate uptake, and product formation rates from OUR are based on conditions of defined media and single substrate limitations. As such, these models may not apply in many real situations.

Fluorometry is the technique which has great potential to be used as a bioreactor monitoring device. The reason is that there are some measurable fluorophores in growing cells such as NADH, tryptophan, pyridoxine and riboflavin. Because these fluorophores involve key vitamins, coenzymes and aromatic amino acids, their concentration changes may reflect the changes in cell concentration and cell metabolic state as well as environmental conditions. By measuring the fluorescence signals of whole broth through non-invasive optical fiber systems, one can obtain continuously on-line estimates of several fermentation parameters during a fermentation. Many of them are intracellular parameters. Fluorometry has very high sensitivity and good specificity. There is virtually no time lag in the measurements. This technique provides a means of monitoring what is going on inside the cells, rather than just the environment around them the way traditional biosensors do.

Reduced nicotinamide adenine dinucleotide (NADH) is the first cellular fluorophore which has been used to monitor bioreactors. There are two commercially available fluorescence probes (BioChem Technology and Ignold) for culture fluorescence measurements. Both of them are based on NADH fluorescence. Most of the results in previous fluorescence studies were obtained

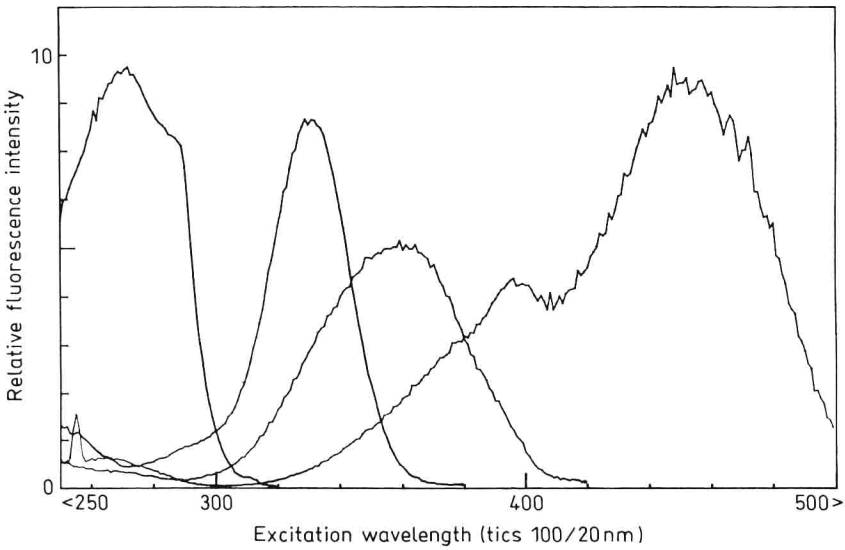

Fig. 1. Excitation spectra of the four major cellular fluorophores—excitation wavelengths vs relative fluorescence intensity. The spectra from left to right correspond to the fluorophors of tryptophan, pyridoxine, NADH and riboflavin respectively

using these NADH probes. However, using only NADH fluorescence for the monitoring of fermentations presents several problems. For example, the quantum efficiency of NADH is very low and NADH fluorescence is extremely sensitive to cellular metabolic state and environmental conditions. Since the optical filters used in the commercial NADH probes permit a relatively wide range of light wavelengths to excite the culture, the emission signal contains not only the NADH fluorescence but that of several other cellular fluorophores. Hence, it is not explicit as to what particular fluorophores are measured by commercial NADH probes. Additional useful information can be obtained by monitoring other cellular fluorophores than just NADH fluorescence [1, 2].

Based on the reasoning outlined above, we decided to build a new type of fluorometric probe titled—multiple excitation fluorometric system (MEFS)—and to monitor four major cellular fluorophores—tryptophan, pyridoxine, NADH, and riboflavin—in whole broth cultures. The reasons for selecting these particular four fluorophores are several-fold. Firstly, and most importantly, these fluorophores are key metabolic components. Secondly, they are optimally or near optimally excited by Hg arc lamp spectral lines of 289, 313, 334, 365, and 404 nm (Figs 1 and 2). Thirdly, these four fluorophores, when excited at selected wavelengths, fluoresce in separate and distinct regions with little or no overlap (Fig. 3). For example, the fluorescence emission spectra of NADH and riboflavin overlap if both of them are excited at the wavelength of 365 nm. However, NADH does not fluoresce when excited at 404 nm while riboflavin strongly