Overproduction of Microbial Metabolites

Strain Improvement and Process Control Strategies

Zdenko Vaněk
Zdeněk Hošťálek

Butterworths

Overproduction of Microbial Metabolites

Strain Improvement and Process Control Strategies

Edited by

Zdenko Vaněk
Institute of Microbiology
Czechoslovakian Academy of Sciences
Prague, Czechoslovakia

Zdeněk Hošťálek
Institute of Microbiology
Czechoslovakian Academy of Sciences
Prague, Czechoslovakia

Butterworths
Boston London Durban Singapore Sydney Toronto Wellington

Library of Congress Cataloging in Publication Data
Main entry under title:

Overproduction of microbial metabolites.

(Biotechnology ; 7)
Includes bibliographical references and indexes.
1. Microbial metabolism—Regulation.
2. Microbiology—Cultures and culture media.
3. Microbial genetics. 4. Microbial metabolites.
5. Microbiological synthesis. 6. Industrial microbiology. I. Vaněk, Z. (Zdenko) II. Hošťálek, Zdenek.
III. Series.
QR88.094 1986 576'.1133 85–31341
ISBN 0–409–90026–5

Butterworth Publishers
80 Montvale Avenue
Stoneham, MA 02180

10 9 8 7 6 5 4 3 2 1

Printed in the United States of America

CONTRIBUTORS

Teruhiko Beppu
Department of Agricultural Chemistry
Faculty of Agriculture
University of Tokyo
Tokyo, Japan

Margita Blumauerová
Institute of Microbiology
Czechoslovakian Academy of Sciences
Prague, Czechoslovakia

Christopher T. Calam
Biology Department
Liverpool Polytechnic
Liverpool, England

Richard Dales
Faculty of Engineering and Science
Polytechnic of Central London
London, England

Richard P. Elander
Industrial Division
Bristol-Myers Company
Syracuse, New York

Karl Esser
Department of General Botanics
Ruhr University
Bochum, Federal Republic of Germany

Armin Fiechter
Department of Biotechnology
Swiss Federal Institute of Technology
Zurich, Switzerland

Geoffrey Holt
Faculty of Engineering and Science
Polytechnic of Central London
London, England

Ralf Hütter
Institute of Microbiology
Swiss Federal Institute of Technology
Zurich, Switzerland

Joseph Lein
Panlabs Inc.
Deer Harbor, Washington

Friedhelm Meinhardt
Department of General Botanics
Ruhr University
Bochum, Federal Republic of Germany

Peter Niederberger
Institute of Microbiology
Swiss Federal Institute of Technology
Zurich, Switzerland

Robert D. Nolan
Pharmaceutical Division
Imperial Chemical Industries
Macclesfield, England

Gunter Saunders
Faculty of Engineering and Science
Polytechnic of Central London
London, England

Zdenko Vaněk
Institute of Microbiology
Czechoslovakian Academy of Sciences
Prague, Czechoslovakia

Vladimír Vojtíšek
Institute of Microbiology
Czechoslovakian Academy of Sciences
Prague, Czechoslovakia

Jaroslav Votruba
Institute of Microbiology
Czechoslovakian Academy of Sciences
Prague, Czechoslovakia

John N. Vournakis
Molecular Genetics Center
Dartmouth College
Hanover, New Hampshire

PREFACE

Many fine books on natural substances have appeared over the years—books full of chemical structures. If these substances exhibit some biological activity, then there are chapters on the detailed mechanisms of their action, including examples of actual or presumed applications. However, in all these publications the vast amount of work that must go into creating a high-production strain from a low-production, wild microbial specimen and into turning a biologically interesting compound into a competitive product usually remains concealed.

The last 40 years of turbulent development and change in the pharmaceutical industry are contemplated in the general and synthesis chapters of this volume. The book should put into focus the fact that the overproduction of microbial metabolites is a complex process that can be successfully achieved through a combination of physiological and genetic knowledge of metabolic control and through mastering the fermentation process with engineering know-how, including automation and mathematical modeling. At the center of attention remains the living cell, with its limitations and capabilities, as is well documented by the chapter entitled "Selection and Enrichment of Active Strains of Microorganisms and Their Use for Production of Immobilized Cell Biocatalysts." It is up to the reader to decide to what extent the intellectual charge of the chapter on "The Panlabs Penicillin Strain Improvement Program" has a more general validity that is applicable to the obtaining of high-production strains forming substances other than penicillins.

The book often provides different views of the same complex of problems, occasionally arriving at different conclusions. This is perhaps an admirable and stimulating feature.

CONTENTS

PART

I

Physiology

CHAPTER

1

Physiology and Pathophysiology of Secondary Metabolite Production

Zdenko Vaněk
Margita Blumauerová

I. INTRODUCTION

In a review of the physiology of actinomycetes published in 1953 by Perlman, 543 references were presented. The author nonetheless complained that the physiology of these organisms had not yet been studied in detail, that practically nothing was known about mechanisms of the synthesis of interesting and important compounds (antibiotics) by actinomycetes, and that studies of the function of these compounds in the lives of producing organisms had not even begun.

The review was written at the beginning of the 1950s, which were characterized by immense development of the antibiotic fermentation industry with its special tasks; namely, to introduce new antibiotics and to produce them in high yields in the shortest possible time by adjusting cultivation conditions or by using mutant strains. However, it was soon recognized that the sensitivity of actinomycetes to antibiotics they themselves produce must be included among the limiting factors and that actinomycetes possess mechanisms for the degradation or inactivation of the compounds produced. The difference between production and nonproduction cultures

was then assumed to lie only in the quanitity or intensity of these detoxification mechanisms. At that time, a deeper study of various physiological observations—for example, that different antibiotics are produced when actinomycetes are cultivated on agar versus a liquid medium and that the addition of certain compounds to the medium, or changes in the cultivation conditions, leads to changes in the type of compounds produced—could not yet be performed. Perlman's (1953) review fully realized the difference between the study of actinomycete physiology under natural conditions and under the conditions of industrial fermentation. He wrote: "Certainly the levels of antibiotic substance found in the soil or other natural habitats are far below the levels which might be inhibitory to the majority of microorganisms of the soil."

II. BEGINNING OF SUBMERGED CULTIVATION

Since the very beginning of the "golden" era of antibiotic compounds, pronounced attention has been devoted to methods for increasing their production. The main progress perhaps began when submerged fermentation of penicillin was introduced. In analyzing the dynamics of penicillin production it soon became clear that the rapid utilization of glucose facilitates a high growth rate of *Penicillium chrysogenum,* but, on the other hand, that these conditions are not suitable for penicillin production (Stefaniak et al., 1946; Johnson, 1952). The specific rate of antibiotic synthesis was higher at a lower specific growth rate. It was further found that penicillin production is favorably influenced by the presence in the fermentation medium of glucose, which facilitates rapid growth of the mycelium, and lactose, which is used up more slowly than glucose but which regulates the metabolic activity of *Penicillium chrysogenum* towards penicillin production. It was also shown that the slow addition of glucose influences antibiotic production as favorably as the addition of lactose (Soltero and Johnson, 1953, 1954).

The success of submerged (batch) fermentation of penicillin, together with the finding that it is advantageous to perform the fermentation as a two-step process, influenced considerably the development of the antibiotic fermentation industry. Similar relationships between the growth of production microorganisms and the synthesis of antibiotics were detected and even induced in the case of other antibiotics, such as streptomycin, tetracyclines, erythromycin, and nystatin, and of other compounds, such as alkaloids and gibberelins.

Later communications, in which penicillin production in continuous culture was studied, showed that the relationship between growth and antibiotic production is more complicated than was originally expected on the basis of the results obtained in submerged batch cultivation, since penicillin

can be produced even at relatively high growth rates (Pirt and Callow, 1960; Pirt and Righelato, 1967). These results, however, though theoretically quite important, were not commonly applied in practice.

Two most remarkable phases of the life cycle—the phase of exponential growth and the stationary phase—began to be understood as two incompatible phases that mutually compete for the key metabolic intermediates (Martin and Demain, 1978). The assumption of strictly separated systems of primary and secondary metabolism was thus formulated.

III. SURFACE AND SUBMERGED CULTURES

It was somewhat forgotten that strains of penicillia used for the production of penicillin at the beginning of the 1940s produced penicillin only in surface cultures and that only the introduction of new strains (NRRL) in 1951 facilitated the submerged production of this compound. Also, in the case of streptomycin, freshly isolated strains of *S. griseus* have never produced more than 100–200 μg/ml of the cultivation medium (Schatz et al., 1944), and under stationary cultivation conditions streptomycin production has followed the growth curve of this microorganism. Under submerged growth conditions the course of the production and growth was biphasic (Waksman, 1967). Similarly, *Bacillus subtilis* cells cultivated in synthetic media produced bacitracin during their growth phases (Hendlin, 1949).

It would certainly be possible to present a number of other examples of different developmental phases where the production of secondary metabolites by different microorganisms differs both quantitatively and qualitatively when the cultivation proceeds in solid media as a surface or submerged process. For example, see the outstanding review by Kalakoutskii and Agre (1976) about the development and differentiation in actinomycetes, in which they tried to help "some of our industrial colleagues to realize that actinomycetes do not just grow and produce everything possible, but they also somehow develop."

Surface cultures are more suitable for studies of differentiation within colonies, serial mycelium formation, and so on. Because the growing hyphae are attached to the agar surface, their position might give some information on chemotactic responses and interactions. Although evidence of differentiation, both macro- and microscopic, is more clearly expressed in surface cultures, most physiological studies with actinomycetes have been conducted with submerged batch cultures.

Morphological and very probably physiological and biochemical manifestations of differentiation in submerged or surface cultures differ quite markedly. Spore formation has repeatedly been reported to occur in submerged cultures, the spores and sporebearing structures formed under these

conditions seeming to differ morphologically and physiologically from those of surface cultures. Surface and submerged cultures might also differ in the relative areas of cell contacts, which tend to be more extensive in surface cultures. It is certainly difficult, if not impossible, to deduce the physiological significance of a secondary metabolite or metabolites for a producing microorganism from experiments aimed at the improvement of industrial production of a certain excessive metabolite. Papers in which the biosynthesis of secondary metabolites was studied in unimproved microbial strains under natural conditions and conditions at least partially resembling the natural ones are actually very rare.

Papers concerning the biosynthesis of mycophenolic acid and other metabolites in *Penicillium brevicompactum* (Detroy et al., 1973; Detroy and Worden, 1979; Nulton and Campbell, 1977; Campbell et al., 1980; Doerfler et al., 1978) represent a significant contribution here. First it was found that mycophenolic acid is produced by submerged cultures even during balanced growth. It had originally been assumed that this metabolite is snythesized from the initial growth phases, immediately after spore germination. It was found, in fact, that synthesis started only after a 24-hour lag that had nothing in common with the exhaustion of nutrients. The production of ergosterol, which is considered a primary metabolite, started as late as six hours after the appearance of mycophenolic acid. This finding led to the assumption that cultivation of *P. brevicompactum* under submerged conditions impairs the developmental sequence of metabolic and regulatory processes that was optimized for growth on solid or semisolid substrates during millions of years of evolution.

When investigating the biosynthesis of mycophenolic acid on Czapek-Dox agar it was shown that mycophenolic acid is a specific product of the aerial mycelium. It was also found that a further metabolite, brevianamide A, is produced after mycophenolic acid under these conditions, primarily in metulae, sterigmata, and the upper parts of aerial hyphae carrying the above structures. Conidiophores of *P. brevicompactum* that have atop them a penicillus mature enough to produce brevianamide will rotate when illuminated by visible or ultraviolet light. If no brevianamide is present, no rotation is observed. Careful microscopic observation reveals that water appears to be pumped up the conidiophore on illumination-rotation. A dehydrated conidiophore does not rotate, but it does recover the ability on rehydration. There is the possibility that brevianamides are implicated in water translocation from the growth substrate up the conidiophore to the penicillus (Campbell et al., 1982).

In this connection the study by Lafond et al. (1978) of the production of mycotoxins (fumigatin and spinulosin) in *Aspergillus fumigatus* (Fresnius) is certainly of interest. In a liquid nonaerated medium of Raulin-Thom, fumigatin appears at the beginning of the growth phase and then immediately disappears from the cultivation medium. Spinulosin is produced only after fumigatin. Both compounds are actively degraded during growth.