EXTRACTIVE BIOCONVERSIONS

edited by
Bo Mattiasson
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Series Introduction

Bioprocess technology encompasses all of the basic and applied sciences as well as the engineering required to fully exploit living systems and bring their products to the marketplace. The technology that develops is eventually expressed in various methodologies and types of equipment and instruments built up along a bioprocess stream. Typically in commercial production, the stream begins at the bioreactor, which can be a classical fermentor, a cell culture perfusion system, or an enzyme bioreactor. Then comes separation of the product from the living systems and/or their components followed by an appropriate number of purification steps. The stream ends with bioproduct finishing, formulation, and packaging. A given bioprocess stream may have some tributaries or outlets and may be overlaid with a variety of monitoring devices and control systems. As with any stream, it will both shape and be shaped with time. Documenting the evolutionary shaping of bioprocess technology is the purpose of this series.

Now that several products from recombinant DNA and cell fusion techniques are on the market, the new era of bioprocess technology is well established and validated. Books of this series represent developments in various segments of bioprocessing that have paralleled progress in the life sciences. For obvious proprietary reasons, some developments in industry, although validated, may be published only later, if at all. Therefore, our continuing series will follow the growth of this field as it is available from both academia and industry.

W. Courtney McGregor

Preface

Biotechnological processes were long regarded as a sequence of different unit operations. The goal was then to optimize each individual step as far as possible. However, since biology in itself contains many levels of regulation, this was not the optimal way to carry out processes in all cases.

By integration, some of the unit operations are carried out simultaneously or intermittently in a coupled way. Since product inhibition and product instability are two major limitations of biotechnological processes, it was quite natural to look for synergies by integrating bioconversion and downstream processing.

This volume highlights these new developments. In doing so, we deliberately excluded the area of immobilized biocatalysts, although processes based on such technology may fall within the area of extractive bioconversions. Today, there are extensive reviews and books covering this area, although in most cases the emphasis is not on integration.

Many separation technologies are described, some in separate chapters, some as examples under a wider topic. New, interesting separation techniques that are just about to be accepted in biotechnological work have been included in our effort not only to describe the present status, but also to illustrate future paths. Examples of this are extractive bioconversions in supercritical solutions and the use of ion-pair extraction for separation.

Concerning applications, most experience is available from work with microorganisms. Therefore, this volume focuses on this topic. However, the principles

of integration may also be applied to other fields of biotechnology (a separate volume on hybridoma cell cultivation has been published in this series).

Many combinations of processes and separation steps may be constructed. In this volume we draw attention to the many possibilities that extractive bioconversions offer to biochemical engineering, by inviting leading scientists from different disciplines to describe their techniques, results obtained, and forecasts for future development.

The chapters therefore differ widely: some are theoretical, others more descriptive. It is our hope that this volume will stimulate more thinking and more work along the lines of process integration.

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Objectives for Extractive Bioconversion

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I. DEFINITION

The term "extractive bioconversion" is synonymous with in situ product recovery. Both terms deal with the concept of increasing the productivity or performance of biotechnological processes by the continuous removal of a product from the site of its production.

II. INTRODUCTION

Biotechnological processes employ a combination of biology and technology to construct an efficient process. The biological part of such a system takes advantage of the diversity of conditions under which specialized microorganisms operate successfully. The technological part, of course, involves optimizing the process by creating favorable operating conditions and eliminating, as far as possible, some of the constraints exerted by the biology of the organism of choice.

Several levels of metabolic control have developed throughout evolution. Reversibility of enzymes, feedback allosteric control, gene regulation, permeability of cell membranes, and other features all help to control the metabolic flows in proper pathways under controlled conditions. One characteristic of these control levels is that they are sensitive to enhanced concentrations of certain metabolites. In other words, when the concentration of a metabolite increases, a whole series of control mechanisms are set into operation.

III. NEED FOR IMPROVEMENTS IN PROCESS DESIGN

A typical biotechnological process is characterized by rather low productivity in comparison to what is normal for chemical synthetic reactions. Furthermore, the product stream is dilute, which leads to high costs in the subsequent isolation and purification of the product.

From the technological point of view a maximal product concentration is desirable, whereas biology may have many mechanisms that counteract such increases. Bioprocess technology aimed at optimizing productivity therefore addresses the task of increasing productivity by means of technological solutions that enable circumvention of the biological limitations.

There are several ways to improve the overall behavior of a biotechnological process: the cells can be modified so that the product is excreted prior to reaching inhibiting concentrations, selective mutation of the cell can be employed to eliminate points of metabolic pathways sensitive to feedback inhibition, or the concentration of an enzyme of critical importance to the whole metabolic pathway can be amplified. This last point is as yet pure speculation; very little is known about what happens to the cellular physiology as a result of such changes.

Product elimination can be achieved by reacting the product in a subsequent step to transfer it to a form that does not influence the equilibrium (see Chapter 9), or it can be removed from the site of catalysis by permeabilization (see Chapter 11) or by selective elimination by membrane filtration processes (Chapters 2, 4, and 5), by extraction (Chapters 3, 6, and 7), by evaporization (Chapters 4 and 10), or by adsorption (Chapter 8).

Another approach to improving the productivity of a process is to increase the catalyst density in the reactor. This can be achieved by immobilization (1-5) and by catalyst recycling (Chapters 2 and 13).

In high-density catalytic processes the demand for efficient process control is high. Development is underway, but no real working system has yet been presented.

The present volume deals with various aspects of integration of bioconversions and the first steps in downstream processing, a technology that may lead to the development of a totally new breed of bioreactors.

In this chapter we discuss the different mechanisms by which the level of metabolites is controlled in nature and how they influence the biotechnological process; in addition, various technical solutions for the circumvention of these biological constraints are discussed. The rest of the volume explores various techniques as applied to the continuous removal of products during the process, a procedure called extractive bioconversion or in situ product recovery.

IV. INHIBITION PATTERNS

The metabolic control of metabolite levels may be exerted on different levels. The best known is reversibility in the enzyme-catalyzed process leading to product inhibition. Here, direct control occurs when an enrichment of products takes place. This type of control is especially important for reactions running close to equilibrium, for example isomerase-catalyzed reactions (6) and many enzymatic steps catalyzed by NAD (P)-dependent dehydrogenases (7). In feedback control, however, the product from one enzyme-catalyzed step interferes with another enzyme activity several steps earlier in the metabolic pathway. Well-known examples of this type of metabolic control are found in the synthesis of aromatic amino acids (8). Yet another type of control of catalytic activity is substrate inhibition. Here, the enzyme catalysis is affected by allosteric control as a result of more than one type of binding site for the substrate molecule on the enzyme. These types of metabolic control are schematically illustrated in Fig. 1.

The systems are exemplified by well-defined enzymes; this is because such systems are the best known. Of course one can foresee similar behavior for transport proteins carrying out the transport of substrate and product molecules across membrane barriers, for example.

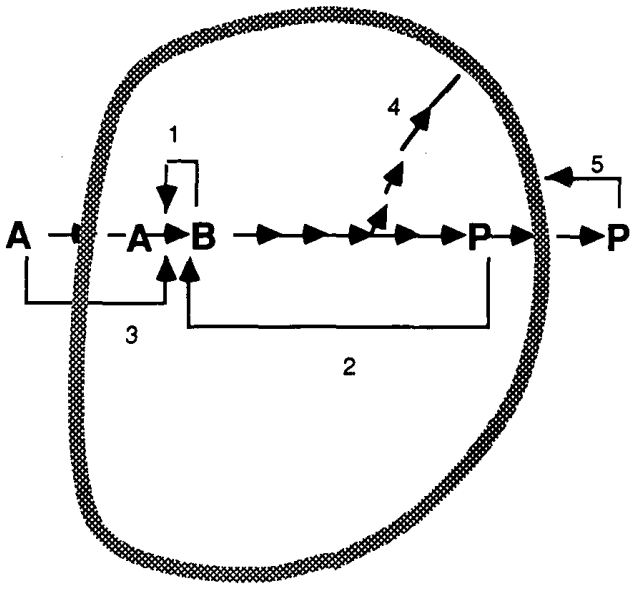

Figure 1 Schematic presentation of the different types of inhibition taking place in a bioconversion process: (1) product inhibition; (2) feedback inhibition; (3) substrate inhibition; (4 and 5) unspecific inhibition by by-products.

Besides effects on the molecular level, there are many examples in which enhanced levels of metabolites can drastically change the performance of a bioconversion process. When producing organic acids or alcohols severe product inhibition has been reported. These effects may to a large extent be ascribed to effects on membrane permeability properties caused by these products (9-11). Thus, the metabolite causes a more general influence on the cell and does not specifically affect the enzyme(s) essential for the biocatalysis leading to the formation of the product. Weak acids may, upon interfering with the membranes, cause a depletion of the pH gradient across the membrane. Since this gradient is believed to be responsible for the phosphorylation of ADP, such effects may cause severe disturbances in the energy supply to the cells and thus switch the metabolism in new directions. From model studies, it is known that when the phosphorylation of ADP in *Saccharomyces cerevisiae* is inhibited, an increased rate of glycolysis is observed (12-14).

It may sometimes be difficult to clearly differentiate between the different types of negative effects on the biocatalyst. The more simple the biocatalyst, the easier this differentiation becomes. When dealing with pure enzymes, free or immobilized, a fairly good theoretical background exists for both molecular properties per se and microenvironmental effects (15). The theoretical background for the knowledge of events on a cellular level is far less developed. Microbial physiology is an underdeveloped discipline, and much more attention must be directed to this issue before full potential can be reached by improvements in fermentation technology and process control. For immobilized cells, the work of identifying the rules of the game has just begun (16).

Another area that is poorly understood is that of plant cell biology. In many cases the vacuole has been regarded as a sink for many substances produced by the cell, products that have been difficult to excrete. Sometimes these stored molecules are attractive from the biotechnological point of view. It would be desirable to change the transport routes in the cell and cause excretion of the molecules to the surrounding medium instead. This problem has not been generally solved. However, in the area of cell permeabilization (see Chapter 11), it has now been shown that it is possible to change the permeability properties to such an extent that the vacuole can be emptied of its contents of storage molecules without ruining the cell metabolism. This has been interpreted in terms of a temporary permeabilization that ceases after treatment. Membrane properties are restored, and metabolism can continue as before. By the use of adsorbents in the medium some changes in the transport directions in the cell have been reported (17).

Besides the fact that the production rate may be reduced by a buildup of product concentrations, there are other reasons for running extractive fermentations. Some of these are discussed in the following sections.

V. PRODUCT STABILITY

When product is excreted into the medium, the often hostile conditions may efficiently reduce the yield of active substance if the product is not removed immediately. The bubbling of air or oxygen in aerobic reactions, the shear forces generated by vigorous stirring, and the presence of hydrolytic enzymes are three strong reasons for efficiently and rapidly removing the product from the medium. In this volume the elimination of sensitive antibiotics (cycloheximide) by the use of solid sorbents directly in the fermentor is discussed (18).

The production of extracellular proteins is not problematic when working with conventional strains: the proteases from one organism do not attack other exoenzymes from the same organism. However, when introducing foreign gene material into organisms and expressing new proteins, the old proteases become a severe threat to the new proteins. Thus far the solution has been to eliminate the genes for proteases as efficiently as possible without endangering the welfare of the cell.

Another appealing alternative is to trap the target molecule by a specific sorbent as soon as it is excreted or to render the proteases ineffective by applying protease inhibitors. These inhibitors may be added either free in solution or bound to solid sorbents. This latter approach is more general when a broad-spectrum protease inhibitor preparation is used. This has not been reported in practice yet to any large extent. However, there may be some activity along these lines in the area of mammalian cell culturing.

Yet another reason to eliminate a product from a culturing system is when the product is hazardous to the cell itself. Examples that have been frequently cited in literature are those of the genus *Clostridium*. These cells produce autolysins, that is, proteases that cause autolysis of the cells (19,20). To avoid this, the now-classic experiment by Hedén and Puziss, who cultivated *Clostridium tetanii* and developed a much more efficient cultivation system by continuously extracting the toxin by means of an aqueous two-phase system (21).

When the product is an intermediary metabolite in a metabolic pathway, it is recommended that it be removed before large concentrations build up. In such situations one often operates with mutants lacking the subsequent enzyme in the sequence, thereby making it possible to enrich the metabolite of interest. The mutant may be fully deficient in the subsequent enzyme activity, or the enzyme may be severely disturbed. In the latter case very high substrate concentrations may initiate catalytic activity as well as starting feedback inhibition processes. Therefore, it is highly desirable to remove the product from the site of production. This is performed by reducing the content in the broth, thereby making it more favorable to excrete the product from the cell. Another attractive alternative is to facilitate elimination from the cell interior by means of permeabilization (see Chapter 11).

When developing extractive bioconversion processes it is of the utmost importance to select extraction media that do not interfere with the cells, or do so in a controlled and desirable way (see Chapters 6, 7, and 9). Here, one must differentiate between unspecific membrane permeabilization and toxicity effects on the cell. Organic solvents have been regarded as problematic in this respect, but more recent research clearly demonstrates the possibility of finding suitable organic extractants (22,23). More recent extraction systems, such as supercritical carbon dioxide (Chapter 6) and aqueous two-phase systems (Chapter 7), are regarded as more gentle to the cells.

VI. TECHNICAL CONSTRAINTS

That biotechnological processes by nature are slow and self-regulating and result in dilute aqueous product streams has caused a technical development toward more highly producing systems involving increased cell densities, better process control, and controlled substrate delivery. Controlled product removal, which is one of the aims of extractive bioconversion, adds yet another dimension to this process technology. These new conditions of course amplify many of the constraints mentioned earlier in this chapter and raise demands for efficient ways to handle them. This volume presents a wide variety of techniques applied in integrating bioconversion with downstream processing. We have deliberately excluded enzymes and cells immobilized in solid supports. This is done despite that product is continuously removed by the product stream. Many volumes have been published dealing with this matter, and we therefore refer to those (1-5). However, some experience from immobilized cells is discussed in relation to the other technologies treated in this volume.

When operating with high cell densities (in cell recycling, for example) substrate may be fed continuously and product removed continuously. Such an arrangement may cause problems; for example, some products may not be removed along with the product stream. Then there is an enrichment of by-products and eventually these affect the process, for example when glucose is fed to a *Saccharomyces cerevisiae* culture for production of ethanol. If the ethanol is removed by distillation, any glycerol produced with the ethanol remains in the medium and is recirculated back to the fermenter (24). Increasing concentrations of glycerol change the osmotic environment of the cells. A way around this problem is to operate with a bleed stream by which excess cells and some by-products may be removed. When operating with more complex media, such as molasses, nonbiodegradable compounds may be present and recirculated in the fermentation broth. A bleed stream is also the solution to problems of enrichment in this case.

Another situation in which it is advantageous to be able to remove the product stream continuously is when cells must be cultured at dilution rates that