A Rosevear
John F Kennedy
Joaquim M S Cabral

IMMOBILISED ENZYMES AND CELLS

Adam Hilger

Immobilised Enzymes and Cells

A Rosevear

Biotechnology Group, The Harwell Laboratory, Didcot, UK

John F Kennedy

Department of Chemistry, University of Birmingham, UK

Joaquim M S Cabral

Department of Chemical Engineering, Technical University of Lisbon, Portugal

Adam Hilger, Bristol and Philadelphia

British Library Cataloguing in Publication Data

Rosevear, A.
Immobilised enzymes and cells.
1. Immobilised enzymes Laboratory manuals
I. Title II. Kennedy, John F. (John Frederick) III. Cabral, Joaquim M. S.
547.7'58'028 QP601

ISBN 0-85274-515-X

Published under the Adam Hilger imprint by IOP Publishing Ltd
Techno House, Redcliffe Way, Bristol BS1 6NX, England
242 Cherry Street, Philadelphia, PA 19106, USA

Typeset by Mathematical Composition Setters Ltd, Salisbury
Printed in Great Britain by J W Arrowsmith Ltd, Bristol

Immobilised Enzymes and Cells

Preface

WHO IS THIS BOOK AIMED AT?

A great deal of research literature and many specialist reviews and conference proceedings exist on the topic of immobilised enzymes and cells. However, no single book brings together the information on how best to go about immobilising and using these biocatalysts. This handbook aims to provide an overview of the subject, as well as practical guidance on the choice and relative merits of the many techniques. The general principles are exemplified with selected examples taken from the literature with additional advice based on the authors' experience.

The book is intended not only as an introduction to this topic for the new research student but also as a source of practical details on efficient immobilisation methods for the worker whose only interest is in exploiting the techniques in his or her particular field. Wherever possible, the references given are the most definitive papers on any particular subject and have been selected so that those wishing to go more deeply into a topic can gain rapid access to the key texts.

This is primarily a laboratory book and as such does not go deeply into the fascinating theoretical and mechanistic problems which are part of this technology. It should be found, dog-eared and stained, on a laboratory bench rather than gathering dust on a library shelf.

Alan Rosevear
John F Kennedy
Joaquim M S Cabral

Contents

1

Introduction

1.1 WHAT IS IMMOBILISATION?

In a biochemical context, immobilisation is a generic term used to describe the retention of a biologically active catalyst within a reactor or analytical system. The biocatalyst, be it a single enzyme, mixture of enzymes, or enzymes contained inside a living cell, is confined within or on a support material. The immobilised complex takes on the physical characteristics of the support while retaining the basic biochemical activity of the free catalyst, thus improving the handling properties of the catalyst and improving the efficiency with which it is used in bioconventions.

Immobilisation provides an insoluble complex on a specialised module through which fluids can pass easily, transforming substrate to product in a controlled enzymatic reaction and facilitating the removal of catalyst from the product as it leaves the reactor (figure 1.1). Its main object is to apply the benefits of heterogeneous catalysis to the soluble enzymes and homogeneous cell suspensions used routinely in academic bioscience and industrial biotechnology.

During the past 20 years the immobilisation of enzymes, and more recently that of cells, has developed into a major topic of theoretical and practical importance. This technology unites the disciplines of chemistry, biochemistry and cell biology on the one hand with biochemical and process engineering on the other. As well as having an importance in its own right, its use has already made an impact on downstream processing of biochemicals and its specialised requirements are making new demands on the supply of biocatalysts which can only be used to advantage in this form.

Immobilisation has generated its own literature (Appendix 1) and conferences, and it is thanks to the Enzyme Engineering Conferences organised by the Engineering Foundation that a considerable degree of cohesiveness exists in this area. In particular the agreement on general terms such as '*immobilisation*' has facilitated the systematic searching and key wording

of this technology. Ambiguous terms such as *insoluble*, *bound*, *complexed* and *conjugated* which appeared in the early literature are now rarely used as general terms. For the sake of clarity and to speed the dissemination of information the use of '*immobilisation*' as the principal key word should be encouraged and will be used throughout this book.

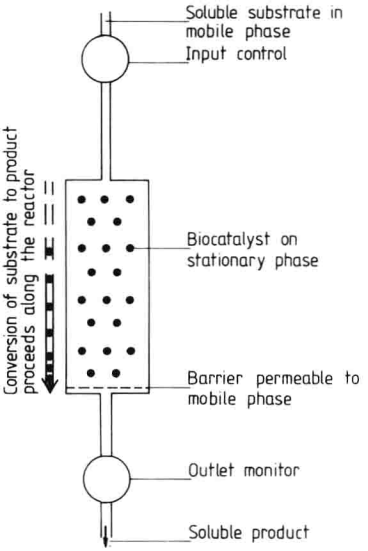

Figure 1.1 Principle of biocatalyst immobilisation.

1.2 INFORMATION ON IMMOBILISATION

Appendix 1 lists those journals which regularly carry papers on immobilised enzymes and cells. A scan of this literature reveals a bewildering number of immobilisation techniques, the vast majority of which are rarely used outside the inventor's laboratory. The patent literature, particularly in Japan, is replete with methods of supposed economic and technical value, yet in practice few of these are used even by those who have invested heavily in their development. There are also the few immobilisation systems which can be bought off the shelf either as activated supports or on ready immobilised systems. These are listed in Appendix 2 and often involve a series of straightforward laboratory procedures which guarantee success in the preparation of some sort of immobilised biocatalyst. However, by their very nature they are general techniques, not optimised to fulfil any particular role, and they are often costly.

In choosing methods to include in this book, we have tried to select representative techniques from each of the major types. Because of their simplicity and practicality, the proprietary immobilisation methods are

represented but the majority of the techniques which will be covered are derived from the research literature and have stood the test of repeated use. For those who wish to search further for more esoteric methods or theoretical treatise, table 1.1 lists the most prominent general reviews on immobilisation. Appropriate reviews of specific areas are mentioned at the beginning of the relevant chapters.

The quick reference summary drawn up regularly by Sturgeon and Kennedy provides a convenient update of the literature for those who do not wish to face the deluge of relevant references which the key word '*immobilisation*' generates fortnightly from the UKCIS file of Chemical Abstracts (The Royal Society of Chemistry, UKCIS, The University of Nottingham).

Table 1.1 Reviews of specific aspects of immobilised biocatalysts.

General
Trevan (1980)
Royer (1980)
Mosbach (1980)
Suckling (1977)
Sturgeon and Kennedy (1979–86)
Atkinson (1974)
Klibanov (1983)
Immobilised cells
Cheetham (1980)
Venkatsubramanian (1979)
Rosevear and Lambe (1983)
Kolot (1981)
Klein and Wagner (1978)
Supports
White and Kennedy (1980)
Manecke and Vogt (1980)
Cofactors
Lowe (1978)
Reactors
Flaschel and Wandrey (1979)

1.3 OVERVIEW OF IMMOBILISATION

Immobilisation is the application of heterogeneous catalysis to biological systems. Most existing biochemical processes and techniques use a soluble enzyme or finely divided cell monoculture to convert a substrate (low value material or precursor) into product (high value material or measurable

parameter). Immobilisation involves confining these soluble protein catalysts or individual, neutral bouyancy cells, in a reactor system which can repeatedly treat fresh charges of solutions. The immobilised material might be retained in a column reactor through which a fluid is passed continuously, the solutes being converted as they pass through the bed and emerging continuously as a catalyst-free product. Alternatively (figure 1.2) the immobilisation can aim to provide a catalytic material which can be moved repeatedly from one container of process fluid to another, converting substrate to product on a batch principle.

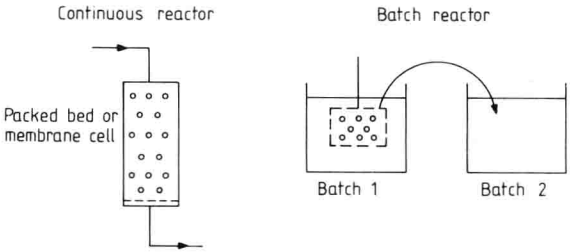

Figure 1.2 Basic modes of operation. In a continuous reactor conversion varies with position in the reactor but the character of the output does not change with time. In a batch reactor conversion throughout each batch varies with time.

It is intriguing to note that the accepted, traditional systems for using dilute solutions of free enzyme are themselves a rather artificial way of utilising biocatalysts. In the cellular environment many enzymes are attached to membrane structures or generated at high concentration close to the cell. However, it is those enzymes which are soluble and easily isolated which are available to the biochemist and it is around these that industrial biochemistry in particular has grown up. Consequently in studying immobilised biocatalysts it should be remembered that one is often recreating the conditions under which many of these materials first evolved, either in cellular films or whole tissue, and that one is merely forming a well defined, artificial matrix in which properties lost during generation of the homogeneous catalyst are regenerated.

1.4 BENEFITS AND LIMITATIONS

Before entering into the details of how this immobilised state can be achieved we will consider some of the benefits and limitations which stem from this approach. These characteristics differ only in magnitude between the various application areas, and the selection of a particular immobilis-

ation method for a specific situation will reflect the relative importance of these general points.

The benefits will be considered under five headings—those resulting from

(*a*) retention of the catalyst in the reactor,
(*b*) high concentration of catalyst in the reactor,
(*c*) control of the catalyst microenvironment,
(*d*) quantitative and rapid removal of the catalyst,
(*e*) separation of catalyst production from its use.

The limitations result from

(*a*) loss of catalytic activity during immobilisation
(*b*) mass transfer problems,
(*c*) physical discrimination between catalyst and fluid,
(*d*) prolonged operation,
(*e*) empirical nature of immobilisation technology.

These general points will be expanded below while later chapters will go into detailed discussion of the practical implications.

1.4.1 Benefits

Retention of catalyst

In any attempt to use free biocatalysts in a continuous process, the flow of substrate solution through the reactor inevitably sweeps the active components along with it. In the case of a homogeneous, soluble enzyme this loss of catalyst is exponential and results in contamination of the product with active proteins which often require removal or deactivation. The situation is more tolerable with free cells since rapid cell division can maintain a critical concentration of active biomass in a continuous fermenter despite loss of cells in the product stream. However, at dilution rates (the reciprocal of retention times in the reactor) higher than a critical value, wash out of even this biomass occurs and a situation akin to the soluble enzyme exists (figure 1.3).

Thus the use of free enzymes and cells is almost exclusively limited to batch operation, the biocatalyst being lost or destroyed in an arbitrary fashion when all substrate in the tank is converted. In contrast immobilised enzymes and cells can be exploited for their full lifetime under ideal conditions without loss or unnecessary deactivation. This is achieved by flowing substrate through a reactor containing the bound enzyme or by repeatedly transferring the active catalyst to fresh batches of substrate. The product is unlikely to suffer post-reaction changes or deterioration and no potentially toxic or antigenic material reaches the product. Changes in flow rate only affect the contact time of substrate and catalyst and do not change the con-

centration of catalyst within the system. This is particularly important in flow reactors where high fluid velocities aid mass transfer.

Immobilisation ensures that maximum use is made of the catalyst, a particularly important factor for expensive enzymes and cells. Furthermore, quantitative retention of biocatalytic debris in the reactor is an important advantage where contamination of the product is undesirable for stability or antigenic reasons.

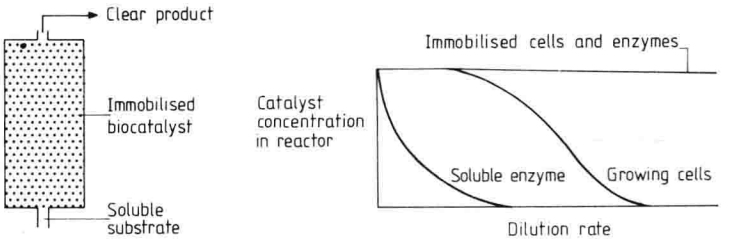

Figure 1.3 Benefits of immobilisation—retention of catalyst.

High concentration of biocatalyst

Confining the enzyme or cell inevitably increases the local concentration of catalyst. The overall activity per unit volume of reactor is consequently higher than is normal for homogeneous systems, while in the immediate environment of the catalyst the density of enzymes greatly exceeds that at any point in the free catalyst reaction. In practice, immobilised systems are used to treat small elements of fluid sequentially with a concentrated mass of all the catalyst, in contrast to homogeneous systems where the catalyst is used at low levels in a single operation to treat huge volumes of substrate. As figure 1.4 illustrates, the contact time between enzyme and a given element of substrate solution is markedly reduced by this increase in process intensity.

Since many of the non-enzymatic reactions which occur alongside the catalysed reaction are time related, minimising the time the solution spends in the reactor significantly reduces side reactions. This has obvious benefits for labile molecules but is also relevant for a cost-sensitive bulk product where expense is saved at present by using a small quantity of enzyme to convert a large volume of substrate by employing very long reaction times. Such procedures generate substantial amounts of unwanted by-products which must be removed downstream. In an immobilised system, the full charge of enzyme can be used to completely convert small volumes of fluid very rapidly, dramatically reducing by-product formation and cutting purification costs (Daniels 1983).

Furthermore, the high catalytic density results in a more compact reactor system, reducing capital costs but also reducing the volume of reactor which

has to be specially treated or kept sterile. The high volumetric flow through such systems places great demands on the immobilisation matrix but inevitably improves mass transfer and increases the efficiency with which inhibitors, including product, are swept from the system. In the case of immobilised cells the process intensification is particularly important since growing cells often reach a limiting density well short of that which makes efficient use of the reactor volume.

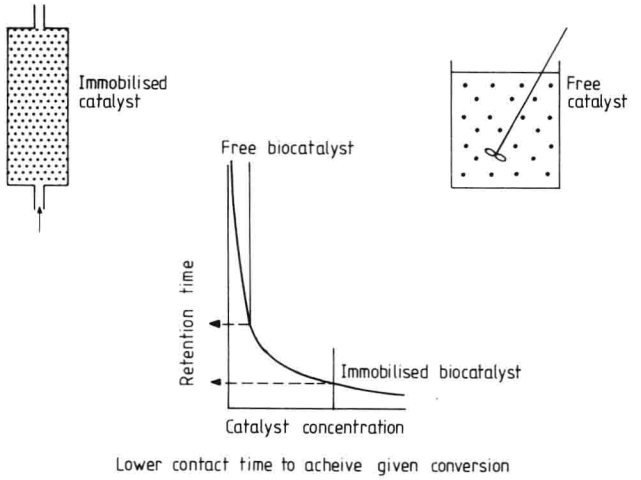

Figure 1.4 Benefits of immobilisation—high biocatalyst density.

Microenvironmental control

The environment around an immobilised catalyst differs significantly from the homogeneous equivalent. Surface charge or hydrophobic regions on the matrix will interact specifically with the catalyst, and even where this does not occur effects on ionic balance, pH, water activity and osmolarity will have an impact on the aqueous environment. These effects can substantially increase the thermostability of the enzymes by reducing the degrees of freedom for the protein chain. The movement of proteases, which might cause autolysis in soluble preparations, is restricted by immobilisation, thus dramatically reducing this mode of activity loss. In addition, toxins and inhibitors can be excluded from the matrix (figure 1.5) while the biocatalyst is mechanically protected. This is particularly valuable in the case of large eukaryotic cells which are easily damaged by shear.

The apparent pH optimum of a biocatalyst can be altered by immobilisation on a charged matrix. For instance an acidic support will maintain near its surface a lower pH than that of the bulk fluid. Thus it is possible to operate enzymes with potentially incompatible pH optima in the same

system, or to prevent decomposition of labile solutes. It should, however, be noted that such phenomena only become apparent when the buffering capacity of the solution is low.

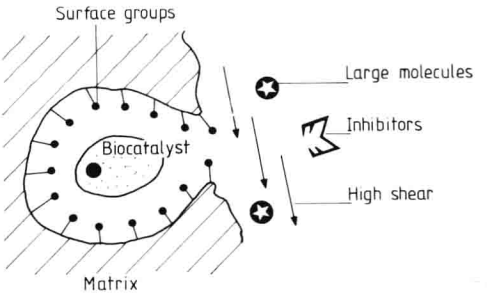

Figure 1.5 Benefits of immobilisation—microenvironmental control.

Catalyst removal

Since biocatalyst is held back in the reactor, the enzyme-induced changes cease as soon as the fluid leaves the system (figure 1.6). This is a very useful property for preparing intermediates or partially degraded products (e.g. polypeptides). The relationship between substrate flow rate and conversion can be computed and then controlled by established process engineering techniques. Since contact time in the system is low the process is very responsive to control parameters (e.g. temperature, pH, flow, concentration) making automation easy to apply. The system can also operate continuously, integrating conveniently into other unit operations or being seen as a convenient source of product which can be switched on and off according to need.

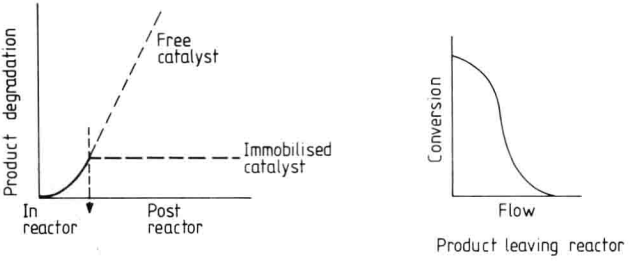

Figure 1.6 Benefits of immobilisation—fine control.

Separation of biocatalyst and product generation

The separation of any biological material from an aqueous solution is a difficult and costly process. Thus free biocatalyst systems seek to avoid such expense by using the crudest acceptable grade of enzyme and not attempting

to recover it at the end of the process. In the case of free cells this policy leads to the growth and use of the cells in the same liquor. Immobilisation makes such compromises unnecessary since the more efficient use of the biocatalyst makes greater expenditure on catalyst preparation justifiable. Before immobilisation the biocatalyst must be recovered from growth medium and is subject to a complete change in its fluid environment, a step greatly facilitated by the immobilisation process.

Catalyst binding conditions are usually optimised on the desired biochemical activity and contaminating activities are either washed away, immobilised at different sites or selected against by choice of binding conditions. Thus with enzymes some purification through selection can be expected. In the case of cells the separation of the growth phase from the production phase makes it possible to select conditions for biomass formation in the former while minimising biomass and encouraging product formation in the latter (figure 1.7).

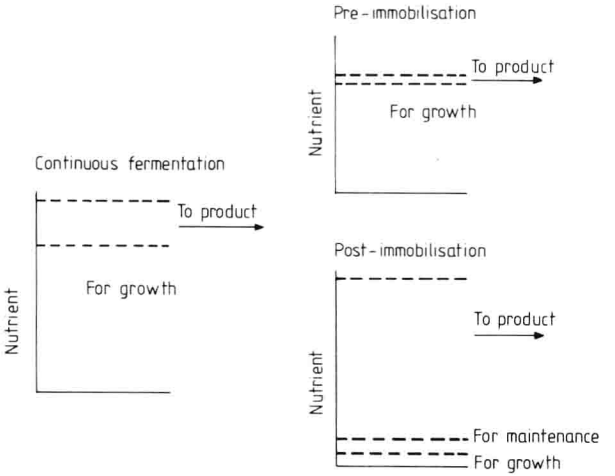

Figure 1.7 Benefits of immobilisation—uncouples growth and production. Comparison of nutrient utilisation with free and immobilised biocatalyst.

1.4.2 Limitations

The success of immobilisation technology illustrates that, in general, the benefits of the technique far outweigh any disadvantages. Although the points covered in this section are often significant factors they are to be seen as things to avoid rather than being absolute restrictions on operation. Methods by which these limitations might be overcome are referred to briefly here.