IMMOBILIZED CELLS *and* ORGANELLES

Volume II

Bo Mattiasson

CRC PRESS

Immobilized Cells and Organelles

Volume II

Editor
Bo Mattiasson, Ph.D.
Associate Professor
Department of Pure and Applied Biochemistry
Chemical Center
University of Lund
Lund, Sweden

CRC Press, Inc.
Boca Raton, Florida

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

Immobilized cells and organelles.

Bibliography: p.
Includes index.
Immobilized cells. 2. Immobilized cell organelles.
I. Mattiasson, Bo, 1945-
QH585.5.I45I47 1982 547.87 83-17870
ISBN 0-8493-6440-X (v. 1)
ISBN 0-8493-6441-8 (v. 2)

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

International Standard Book Number 0-8493-6440-X (Vol. 1)
International Standard Book Number 0-8493-6441-8 (Vol. 2)

Library of Congress Card Number 83-17870
Printed in the United States

PREFACE

Besides giving some insight into basic technology (immobilization procedures, etc.) these volumes also sum up the current know-how in the area and try to predict some future trends.

Cells and organelles are small units for biochemical synthetic purposes, often the smallest practically feasible unit since they contain coenzyme regenerating system, ordered enzyme sequences, etc.

The term "immobilized cells" covers everything from dead cells with a single active enzyme species to cells proliferating on or within a three dimensional polymer matrix. The practical handling of these structures make them useful in various applications, e.g., large-scale production of biomolecules, biodegration, analysis, etc.

In recent years immobilization techniques have become very mild so that, besides microorganisms, plant and animal cells can also be immobilized. When immobilized enzymes first appeared a bright future for the technology was predicted. During the last years some severe limitations have appeared, e.g., the problems to develop a practically feasible technology for coenzyme regeneration, the lack of methods to arrange enzyme molecules in ordered clusters to perform multistep enzyme catalyzed reactions. To all these problems immobilized cells and organelles offer exciting and promising alternatives.

Is it possible to manipulate the total metabolism by immobilization of the cell? This question is not fully answered yet, but strong evidence shows that changes occur.

Immobilized animal cells grown on microparticles of weak ion-exchangers have changed the field of cell-culture dramatically. This technique now makes it possible to exploit mammalian cell lines in biotechnology.

Bo Mattiasson

THE EDITOR

Bo Mattiasson, Ph.D., is an Associate Professor in the Department of Pure and Applied Biochemistry, Chemical Center University of Lund, Lund, Sweden. Dr. Mattiasson received his Ph.D. degree in biochemistry from the University of Lund in 1974. He has been teaching basic and applied biochemistry since 1971, and since 1976 has been an Associate Professor in biochemistry.

CONTRIBUTORS

Peter Brodelius, Ph.D.
Professor
Department of Pure and Applied Biochemistry
University of Lund
Chemical Center
Lund, Sweden

Julian Clark, Ph.D.
Technical Development Manager
Cell Biology Group
Pharmacia Fine Chemicals AB
Uppsala, Sweden

S. O. Enfors, Ph.D.
Department of Biochemistry and Biotechnology
Royal Institute of Technology
Stockholm, Sweden

Saburo Fukui, Ph.D.
Professor
Department of Industrial Chemistry
Faculty of Engineering
Kyoto University
Kyoto, Japan

Stina Gestrelius, Ph.D.
Section Head
Immobilized and Derivatized Enzymes R & D
Novo Industria A/S
Bagsvaerd, Denmark

Lena Häggström, Ph.D.
Department of Biochemistry and Biotechnology
Royal Institute of Technology
Stockholm, Sweden

Bärbel Hahn-Hägerdal, Ph.D.
Technical Microbiology
Chemical Center
University of Lund
Lund, Sweden

Michael D. Hirtenstein, Ph.D.
Product Group Manager
Cell Biology Group
Pharmacia Fine Chemicals AB
Uppsala, Sweden

John Hopkinson, Ph.D.
Director
New Business Development
Amicon Corporation
Danvers, Massachusetts

S. B. Karkare
Department of Chemical and Biochemical Engineering
Rutgers University
New Brunswick, New Jersey

Bo Mattiasson, Ph.D.
Associate Professor
Department of Pure and Applied Biochemistry
Chemical Center
University of Lund
Lund, Sweden

Jose-Luis Ochoa, Ph.D.
Biotechnology Program
Centro de Investigaciones Biologicas
La Paz, Mexico

Atsuo Tanaka, Ph.D.
Senior Lecturer
Department of Industrial Chemistry
Faculty of Engineering
Kyoto University
Kyoto, Japan

K. Venkatasubramanian, Ph.D.
Director, Corporate Research
Technical Development
H. J. Heinz Company
Pittsburgh, Pennsylvania
Visiting Associate Professor
Rutgers University
New Brunswick, New Jersey

TABLE OF CONTENTS

Volume I

Volume II

Chapter 1

IMMOBILIZED NONVIABLE CELLS FOR USE OF A SINGLE OR A FEW ENZYME STEPS

Stina Gestrelius

TABLE OF CONTENTS

I. INTRODUCTION

For a process with only one or a few reaction steps the biocatalyst of choice may be a single enzyme, a couple of enzymes, or a cell containing the relevant enzyme(s). To date most industrial biocatalyzed processes are carried out either with soluble, cheap preparations of extracellular enzymes (as e.g., proteolytic, amylolytic or lipolytic enzymes) or by conventional fermentations (steroid conversions, production of gluconic acid, vinegar, etc.).

Very few intracellular enzymes are produced and used industrially, mainly due to severe problems with large-scale isolation procedures.[1] Especially membrane-bound enzymes are often solubilized and recovered in too low yields. For utilization of intracellular enzymes from microbial cells it has therefore been a natural approach to use the whole cell material, and a similar development can be expected for plant enzymes (see Chapter 3, Volume I).

In the early industrial applications the cells were used once only. However, by simple flocculation[2] or by choosing inherently stable spores[3] or fungal pellets[4] the cells could be used repeatedly.

By further physical and chemical treatment leading to a preparation of immobilized nonviable cells, the operational stability has often been improved, thus permitting reuse during increased periods of time (months) while the improved physical stability has allowed a wider choice of reactors and continuous processing. Yet the number of immobilized cell systems, viable or nonviable, that are presently used industrially is very modest. Table 1 lists such commercially operating immobilized nonviable cell catalysts, and it can be noted that the list would not be much longer even if immobilized cell-free enzymes or immobilized viable cells were included. However, except for processes like glucose isomerization where immobilized preparations of enzymes or cells are without competition, the introduction of particulate biocatalysts must be expected to occur only gradually when old plants are replaced, to fulfill new requirements of low energy consumption and increased in-house and environmental safety.

This paper will primarily deal with the interdisciplinary efforts required in developing new biocatalysts of the type immobilized nonviable cells for medium-size or large-scale applications where the keywords are high volumetric activity and good operational stability during continuous processing.

II. DEFINITION

An immobilized nonviable cell preparation contains, by the present definition, all or almost all of the original cell components, but may have lost more or less of the original cell organization. The cell structure may have become impaired before, during, or after immobilization, either by physical treatment such as homogenization, lysis, heat-treatment or freeze-thawing, or by treatment with solvents, detergents, or other chemicals.

III. RATIONALE FOR IMMOBILIZING NONVIABLE CELLS

In addition to the producer's advantage of immobilizing cells when the desirable enzyme(s) cannot be economically isolated or stabilized in a cell-free form, there must also be one or several technical advantages if the application of a nonviable cell biocatalyst is to become successful. Examples of such advantages include simplified catalyst re-use, smaller reactors, continuous processing, improved catalyst stability, withdrawal of catalyst from product stream, less unit operations in the product upgrading, and cheaper waste treatment.

Table 1
COMMERCIAL IMMOBILIZED NONVIABLE CELL PREPARATIONS

Utilized enzyme	Application	Microorganism	Immobilization method	Producer (trade name)	Ref.
Aspartase (E.C. 4.3.1.1)	Production of L-Asp. from ammonium fumarate	*E. coli*	Entrapment in K-carrageenan, treatment with hexamethylenediamine + glutaraldehyde	Tanabe Seiyaku Co. Ltd., Japan	5
Fumarase (E.C. 4.2.1.2)	Production of L-malic acid from fumaric acid	*Brevibacterium flavum*	Entrapment in K-carrageenan	Tanabe Seiyaku Co. Ltd., Japan	6
Alpha-Galactosidase (E.C. 3.2.1.22)	Hydrolysis of raffinose (in molasses) to galactose and sucrose	*Absidia regneiri*	Cross-linking after freeze-thawing	Nippon Beet Sugar Mfg. Co. Ltd. Japan	7
		Mortierella vinacea	Cross-linking with glutaraldehyde	The Great Western Sugar Co., U.S.A.,	8
Beta-Galactosidase (E.C. 3.2.1.23)	Hydrolysis of lactose (in whey) to glucose + galactose	*Bacillus sp.*	Cross-linking with glutaraldehyde	NOVO Industri, Denmark (Novozym 231)	9
Glucose isomerase (E.C. 5.3.1.5)	Production of high fructose syrup from glucose	*Actinoplanes missouriensis*	Occlusion in gelatine, cross-linking with glutaraldehyde	Gist-Brocades, Netherlands (Maxazym)	10
		Bacillus coagulans	Cross-linking with glutaraldehyde	NOVO Industri, Denmark (Sweetzyme)	11
		Streptomyces olivacens	Cross-linking with glutaraldehyde	Miles Laboratories Inc., U.S.A. (Taka Sweet)	12
		Streptomyces albus	Heat treatment Filter reactor	Clinton Corn Proc. Co., U.S.A.	13
Invertase (E.C. 3.2.1.26)	Hydrolysis of sucrose to fructose and glucose	Selected yeast strain	Occlusion in gelatine, cross-linking with glutaraldehyde	Gist-Brocades, the Netherlands (Maxinvert Immob.)	14
Penicillin acylase (E.C. 3.5.1.11)	Production of 6-APA from penicillin G or V	*E. coli*	Entrapment in polyacrylamide	Tanabe Seiyaku Co. Ltd., Japan	15
		Proteus rettgeri	Binding with glutaraldehyde to glycidylmethacrylate polymer	Pfizer Inc., U.S.A.	16
		Selected microorganism	Cross-linking with glutaraldehyde	NOVO Industri, Denmark (Novozym 217)	17
		E. coli	Entrapment in gelatine, cross-linking with glutaraldehyde	Shanghai, China	18

A number of limitations have also been pointed out, such as difficulties with high molecular weight reactants where soluble enzymes are generally preferred, and difficulties with cofactor retention and regeneration where viable cells may be preferred. Finally such applications, e.g., in medical treatment, where a well-defined catalyst composition is required and a pure (immobilized) enzyme is regarded safer.

For single enzymes without need for cofactors, the immobilization of *viable* cells is chosen mainly to secure a long lifetime of the catalyst through intermittent reactivation in suitable medium (see Chapter 2, Volume II). However, the application of living cells necessitates the use of very mild immobilization procedures and virtually aseptic application conditions to preclude microbial contamination during reactivation, making this type of catalyst attractive for only few industrial branches. *Nonviable* cells, on the other hand, can be treated with heat and chemicals to the extent that the enzyme of interest can withstand, permitting the use of a larger number of immobilization methods and sterilization treatments. Other advantages include facilitated mass transport to and from the enzymes after rupturing or permeabilizing the cells, and the potential of inactivating disturbing enzymes to obtain cleaner reactions and improved operational stability (the latter after removing proteolytic enzymes).

Thus, for many single enzyme applications there is a potential of finding a combination of microorganism strain and immobilization procedure that will permit production of a cheap immobilized nonviable cell catalyst with as high volumetric activity and clean reaction pattern as most immobilized enzyme preparations. Also for some multienzyme applications, e.g., of consecutively working enzymes without cofactor requirements, the concept of immobilizing one type of nonviable cells containing all the desired enzymes may prove very favorable.

IV. PRODUCTION OF IMMOBILIZED NONVIABLE CELLS

A. Selection of Cells

Like in all enzyme production a screening program is normally undertaken in order to find the best enzyme source for the application of interest.[19]

Special emphasis must, however, be put on selecting a cell that is suitable not only for producing the best enzyme, but also for becoming an integrated part of the immobilized preparation. A necessary condition is, of course, that the enzyme of interest is intracellular. While a high enzyme activity per cell is a generally desirable criterium that can often be met by mutational improvement of a strain, the stability during immobilization and the subsequent performance under application conditions are properties that are difficult both to predict and correct.

Takata et al.[20] have described a screening program for K-carrageenan immobilized fumarase where 4 strains out of 241 were chosen for thorough investigation of immobilization yields and operational stabilities of the immobilized cells. Each of the four strains *(Brevibacterium ammoniagenes, Brevibacterium flavum, Proteus vulgaris,* and *Pseudomonas fluorescens)* was fermented on a separately optimized medium, and the strain that turned out the best choice for catalyst activity and stability was not the one with the highest activity on screening medium. Thus, even with a prechosen immobilization method the selection of the most favorable strain is very time-consuming. When both the cell strain and the immobilization method are to be selected for developing a new nonviable cell catalyst, an iterative approach may be useful[21] since test runs with a suboptimal catalyst may help outlining the evaluation criteria. Safety regulations for production and application of microbial cell material (especially food industry regulations) require that microbial strains and fermentation media are selected with great care.[22] Pathogenicity and toxicity testing is thus becoming a routine procedure in the industrial strain selection programs. The choice of asporogenic organisms or mutants may reduce viable counts of enzyme/nonviable cell preparations and increase safety in production.[19]

The absence of interfering enzyme activities is a very important property of cells to be immobilized. Yamamoto et al.[23] thus selected a *Pseudomonas putida* (L-arginine deiminase) for citrulline production since this strain lacked the undesirable ornithine

transcarbamylase, and Jack and Zajic[24] chose a *Micrococcus luteus* strain that was free of urocanase for urocanic acid production with His-ammonia lyase.

Permeabilizing treatment causing loss of cofactors has proved effective in removing side activities by cofactor-dependent enzymes. A classical example is bile extract treatment of *Brevibacterium ammoniagenes* (fumarase catalyst) as described by Yamamoto et al.,[25] leading to suppression of unwanted succinic acid formation. Cell rupture treatments in connection with immobilization will be discussed in detail in Section IV.C. below (for references see Table 4).

Heat and pH shocking can sometimes be a selective method for removing hydrolytic enzymes,[26-28] but a selected strain or mutant lacking the interfering enzyme(s) offers the most convenient and permanent solution to the side activity problem.

B. Production and Recovery of Cells

After the microorganism and strain have been selected, and maybe improved, much attention must be paid to the properties of the fermentation broth since it will strongly influence the quality of the final immobilized cell preparation. For example the choice of raw materials, sterilization method, and foam control method may affect not only the yield and activity of the catalyst, but also the operational and physical stability.

Cheap media often have high contents of solids such as ground whole grains or flakes which may be difficult to separate from the cell sludge prior to immobilization. It should be remembered that the dry cell yield in a typical large-scale enzyme fermentation is often 0.5 to 2% while residual nutrients and metabolites constitute 5 to 10% of the broth at the end of a fermentation.[19] The cells are generally recovered from the broth by centrifugation or filtration prior to immobilization, but some immobilization methods comprising flocculation,[29] chelation,[30] or cross-linking[31] as initial steps can also be performed without previous separation. Such procedures can be favorable if the cells are difficult to recover.

For the manufacture of carrageenan-immobilized *Brevibacterium flavum*/fumarase (catalyst for malate production), Takata et al.[20] have established the optimum culture conditions including carbon source, nitrogen source, vitamins, effect of corn steep liquor, aeration rate, temperature, and time. Maximum operational stability of the immobilized cells was found for the same culture condition as maximum activity of free (and immobilized) cells with a few exceptions. A change in the concentration of corn steep liquor had only effect on activity while changes in the aeration rate had the most pronounced effect on the operational stability.

Fermentation time can sometimes be a very important parameter for activity yield and stability of immobilized cell preparations, e.g., for cells that easily lyse and leak intracellular enzymes if they are not harvested at the proper stage. The choice of certain cells and fermentation techniques may simplify immobilization. It has, for example, been proposed to use autoflocculating cells or marine bacteria that have an inherent tendency to attach to surfaces.[32] Kennedy et al.[33] found it easy to chelate a cellulosic slime-producing *Acetobacter* strain with hydrous titanium oxide while nonflocculating strains could not be chelated by the same method. Also the induction of filamentous growth of bacteria has been used to facilitate cell immobilization.[34]

The good inherent stabilities of spores and conidia have been utilized for preparing immobilized cell catalysts by adsorption,[35] entrapment, or mixing with filter aid.[36] (Entrapment of spores followed by germinating treatment has recently become a valuable method for aseptical production of viable cell catalysts[37,38]). Finally, mycelium pellets have been transformed into stable biocatalyst particles by cross-linking with glutaraldehyde.[39]

C. Selection of Immobilization Method

While hundreds of cell-immobilization methods have been described, including en-

Table 2
DESIGN CRITERIA FOR INDUSTRIAL IMMOBILIZED NONVIABLE CELL PREPARATIONS FOR USE OF A SINGLE OR A FEW ENZYMES

Item	Requirement of biocatalyst or immobilization method
Application (e.g., food; pharmaceuticals; organic synthesis; waste treatment)	Toxicity testing. No leakage. Choice of harmless reagents. Sufficient productivity.
Reactor (e.g., type: fixed-bed, fluid-bed, stirred tank; size)	Physical stability. Suitable density, size, shape. Sufficient volumetric activity.
Reaction conditions (e.g., pH; temperature; solvents)	Chemical resistance, thermostability. Hydrophilicity/hydrophobicity.
Reactant properties (e.g., molecular weight, purity, solubility or volatility of substrates and products)	Suitable pore size and particle size. Elasticity.
Cell type (e.g., unicellar organism; cell size; robustness)	Suitable porosity of carrier. Rupturing treatment of cells.
Enzyme properties (e.g., location in cell; sensitivity to oxygen or chemicals; cofactor requirement)	Rupturing treatment or mild immobilization. Utilization of several enzymes (for cofactor regeneration)
Catalyst manufacture (e.g., scale; continuous/discontinuous, safety requirements, acceptable cost)	Simple upscaling. Cheap and nontoxic reagents. High yield. Good storage stability. Suitable form for transportation.

trapment, aggregation, adsorption, cross-linking, and chemical coupling to carriers (see Chapter 2, Volume I), the guidelines for selecting a suitable method are seldomly discussed. Table 2 lists a number of criteria that are important to consider in designing an industrial biocatalyst of immobilized nonviable cell type.

1. Catalyst Application

Although it may be tempting to judge a preparation from its activity and immobilization yield, it is generally safer to start from the other end and make sure that the method and preparation will be compatible with the potential application.

Even if immobilized biocatalysts will qualify as processing aids and not as additives in food or drugs, new product approval laws demanding extended toxicity testings are presently being passed in many countries.[40] Support materials, such as edible proteins and polysaccharides, and chemical reagents that are already being used in food processing (e.g., as cleaning agents) may then be more easily accepted than synthetic polymers and new chemicals.

In this context any leakage from a preparation is essential to control. Any leakage of cells will, of course, lead to decreased catalyst activity, but loss of cell or carrier material can also be serious in polluting reaction mixtures or cause problems in downstream processing. Entrapment and covalent coupling techniques are therefore often preferred to physical adsorption or aggregation.

For a certain industrial application the immobilized cell catalyst will be judged from its productivity in a particular reactor. The productivity concept denotes the accumulated amount of product formed per unit catalyst (kg or liter) during the operational lifetime. This lifetime is, of course, a compromise between production rate and catalyst cost, but two half-lives, i.e., until the catalyst has lost 75% of its original activity, is often the minimum time. For most industrial applications using fixed-bed reactors the useful lifetime should be in the order of months. This can, however, seldomly be attained unless microbial contamination can be avoided (contaminations will plug porous catalysts, destroy flow patterns, change pH, or decompose reactants or the catalyst itself), and it is therefore very important that the catalyst can be used under extreme conditions or can tolerate the addition of preservatives or intermittent washing procedures.

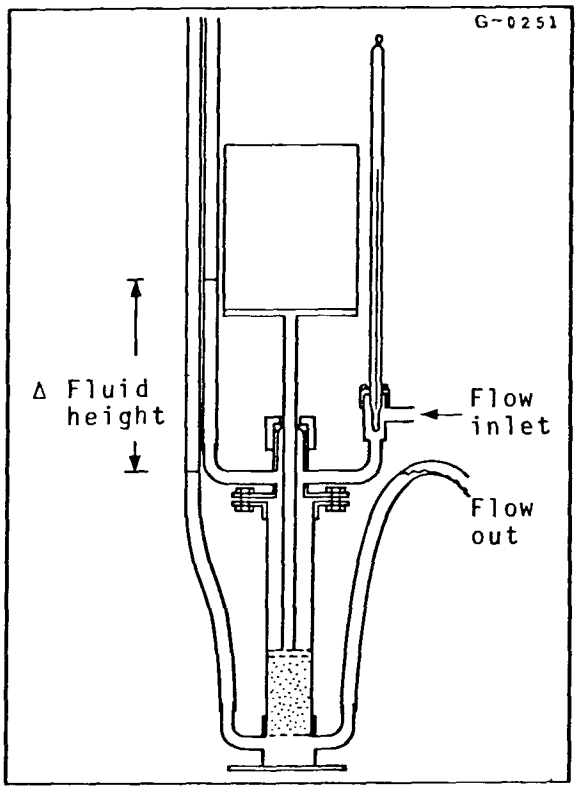

FIGURE 1. Pressure test apparatus. (From Norsker, O., Gibson, K., and Zitton, L., *Starch/Starke*, 31, 13, 1979. With permission.)

2. Reactor Type

Even if the reactor in principle can be chosen to suit a certain type of catalyst, as e.g., a spiral-wound biocatalytic reactor module for membranes[41] or a radial reactor for fibers,[42] it seems to be more common that the reactor design depends on industrial practice and investment costs. Packed beds are used in most large-scale applications due to a high volumetric efficiency and ease of operation. Where pH or temperature control are critical, an arrangement of rapid recycling[43] or a series of smaller packed beds with intermittent adjustment[17] can approach the stirred tank performance without the abrasion problem often seen in stirred tank systems. Batch-run or continuous stirred tanks are, however, also used, especially for penicillin acylase applications.[44]

Dependent on bed height as well as feed stream velocity and viscosity, the application of a biocatalyst in a fixed-bed reactor will require a certain physical stability. While glucose isomerase preparations are loaded into 5 to 7 m beds, a full-size application of a steroid-converting biocatalyst may require less than 0.5 m beds. Figure 1 shows a laboratory scale pressure test apparatus that has been developed by Norsker et al.[45] to simulate the behavior of particles in a large fixed-bed reactor. The apparatus measures the permeability of the true substrate through a sample of catalyst as a function of time at different compression forces, and it is used by NOVO Industri, Denmark, both for testing experimental products in the development stage and for routine quality control of commercial preparations. Similar set-ups have been described by Cheetham[46] and Klein and Kluge.[47] Compression tests on polymer blocks or single particles[48] give rapid estimations of pressure stabilities, but cannot be regarded as useful methods for testing the behavior of particles in a reactor.[49]

Stirred tank reactors and fluidized beds can be expected to subject an immobilized cell preparation to even harsher treatment than fixed-bed reactors. Klein and Eng[50] have described a method for measuring the abrasion of biocatalyst particles in a well-stirred vessel by following particle scattered Tyndall light with a nephelometer.

The many different test methods and reaction conditions applied makes it very difficult to use published data to compare biocatalyst particles. However, there seems to be a common finding for gel-entrapped preparations that both compression and abrasion resistance of particles decreases with increasing cell loading. High cell loading is not necessarily an advantage for fixed-bed reactors where the increased flow rate (to obtain unchanged substrate conversion) will require *further* improvement of the pressure stability of the particles. On the other hand, a maximum loading is desirable for stirred tanks since it leads to an increased volumetric activity and lowered volume fraction of the catalyst ($V_{particle}/V_{total}$), resulting in less contact between particles and stirrer and consequently less abrasion.[50] A similar difference between requirements for fixed bed and stirred tank reactors is also found for particle size since large particles are generally more pressure stable, but more easily abraised than smaller particles.

Several recent papers and patents have dealt with the improvement of particle strength. Thus, addition of small pre-formed particles[51] or polyamine polymers[52] during cross-linking of cells, hardening treatment of polysaccharide gel preparations with polyamines[53-54] or polyamine plus glutaraldehyde,[55] and drying of polysaccharide gels[56,57] are examples on procedures that have resulted in better physical stability of the preparations.

3. Enzyme Reaction Conditions and Reactant Properties

For a number of applications the processing parameters such as substrate concentration and purity, pH, temperature, presence and concentration of buffers or solvents, etc., are completely or partially fixed regardless of the properties of the biocatalyst.

Many examples are found in food processing (e.g., milk, beer, juice, wine), but also pharmaceutical production may be restricted by a lack of stability or solubility of the reactants. Thus, the application of penicillin acylase for 6-APA production is limited to temperatures below 40°C, a narrow pH range, and a minimum buffer concentration (the latter in order to facilitate product isolation).

Steroid conversions are often performed in the presence of solvents due to their low water solubility. Alcohols[58-60] or water-immiscible solvents[60-61] have been added to many gel-entrapped preparations (polyacrylamide, alginate, agar, photocross-linked resins, etc.). Omata et al.[62] immobilized thawed cells of *Nocardia rhodocrous* containing 3-beta-hydroxy steroid dehydrogenase in a number of photocross-linkable resin prepolymers or urethane prepolymers of varying hydrophobicity and tested the preparations on four different substrates in a benzene/*n*-heptane solvent mixture. The hydrophobic preparations showed significantly higher activities than the hydrophilic ones, and the effect was most pronounced for the steroids with an aliphatic side chain at position C-17 (most hydrophobic) since these were oxidized by hydrophobic gel preparations only. Recently, hydrophobic gels were also reported to be favorable for hydrolysis of dl-menthyl succinate in water-saturated *n*-heptane by entrapped *Rhodotorula minuta* cells[63] and for transglycosylation (production of adenine arabinoside, a slightly soluble antiviral agent) by entrapped *Enterobacter aerogenes*.[64] Furthermore, solvents have been added in order to solubilize indole in tryptophan production.[65] However, even when the processing parameters can be varied freely to optimum for biocatalyst utilization, the negative effects of microbial contamination will often urge the use of extreme pH values, extreme temperatures or the presence of solvents or preservatives to preclude growth. Naturally, the immobilization matrices must be resistant to such extreme conditions and preferably also to microbial attacks. Carbohydrate processing with glucose isomerase, inulinase or lactase (the latter when used in

deproteinized whey) is preferentially carried out above 57°C, and the primary concern is to stabilize the cell preparations at these temperatures.

4. Cell Type and Enzyme Properties

When all the above-mentioned "nonenzyme" criteria have been allowed to limit the potential number of immobilization methods, the further selection can be attempted from optimized cell loading, activity yield, and operational stability plus, of course, economy.

The cell loading will naturally depend on the microorganism as well as the immobilization method. Messing et al.[66,67] have established the optimum pore size ranges of inorganic supports for maximum surface loading of biomass. They point out that the size distribution of a microorganism within the same culture may be wide (major dimension of *E. coli* was found to vary between 1 and 6 microns) and that additional space must be available if reproduction of the microorganisms within the carrier is desired. Such growth inside a carrier or gel matrix can be utilized for production of nonviable cell catalysts. Wada et al.[68] have demonstrated that growth inside K-carrageenan gel particles will produce a dense layer of cells close to the surface and result in a more active catalyst. Thus, the aspartase activity of an immobilized *E. coli* cell layer after cell rupture was reported to be 50% higher than that of the same number of homogeneously distributed immobilized cells.[68]

Table 3 compares cell loading by different immobilization methods. Adsorption or covalent binding to carriers will generally result in much lower loading (0 to 10 g wet cells/ℓ) than gel entrapment or cross-linking (100 to 1,000 g wet cells/ℓ), as measured from cell mass per catalyst volume (void volume excluded). Cross-linking and entrapment are therefore the methods of choice, in spite of a sometimes quite high diffusion restriction in gels and tightly cross-linked particles. Roels et al.[77] have even described how particle diffusion can be varied in gelatine-entrapped (glutaraldehyde cross-linked) cell preparations in order to tailor-make biocatalysts with predetermined half-life.

The location of the enzyme of interest within the cell and its intrinsic stability to various chemicals can have a profound influence on the activity yield. Cell surface-bound enzymes are more exposed to immobilization reagents (prior to cell rupture) and may require a mild immobilization procedure. Many of the published immobilization methods have been modified to allow such mild treatment. Thus, polymerization of acrylic monomers can be performed rapidly and be initiated by various chemicals[78,79] or by radiation,[80] ionotropic gels can be formed with different ions,[81] and glutaraldehyde cross-linking can be performed in the presence of inert proteins[41,82] or polyamines.[31] Addition of 0.4% polyethyleneimine to the whole broth of *Bacillus pasteurii* prior to cross-linking with glutaraldehyde raised the urease activity yield from 0.3% to 34% without impairing the physical stability of the preparation, and similar positive effects were found for other glutaraldehyde sensitive enzymes.[31]

Some of the mild immobilization methods may leave a high degree of residual cell integrity (or viability) in the preparations, which requires additional treatment to rupture the cells. Table 4 shows that cell rupture treatments can be performed before, during, or after immobilization, and that many positive effects have been achieved. Loss of viability *per se* is a desirable effect for many large-scale applications where growing cells would complicate the processing (the problems with microbial contaminations). Cell rupture before immobilization may lead to preparations with improved physical stabilities. Undesirable side activities, especially from cofactor-dependent enzymes or sensitive enzymes, may be removed by rupture, but can also be prevented by selection of microorganism strains or mutants lacking these activities (see Section IV.A.).