

IMMOBILIZED ENZYMES
PREPARATION AND ENGINEERING
Recent Advances

J.C. Johnson

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NOYES DATA CORPORATION

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1979

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FOREWORD

The detailed, descriptive information in this book is based on U.S. patents, issued since July 1974, that deal with immobilized enzymes. This title contains new developments since our previous title *Immobilized Enzymes, Preparation and Engineering Techniques*, published in 1974.

This book serves a double purpose in that it supplies detailed technical information and can be used as a guide to the U.S. patent literature in this field. By indicating all the information that is significant, and eliminating legal jargon and juristic phraseology, this book presents an advanced, commercially oriented review of recent developments in immobilized enzymes.

The U.S. patent literature is the largest and most comprehensive collection of technical information in the world. There is more practical, commercial, timely process information assembled here than is available from any other source. The technical information obtained from a patent is extremely reliable and comprehensive; sufficient information must be included to avoid rejection for "insufficient disclosure." These patents include practically all of those issued on the subject in the United States during the period under review; there has been no bias in the selection of patents for inclusion.

The patent literature covers a substantial amount of information not available in the journal literature. The patent literature is a prime source of basic commercially useful information. This information is overlooked by those who rely primarily on the periodical journal literature. It is realized that there is a lag between a patent application on a new process development and the granting of a patent, but it is felt that this may roughly parallel or even anticipate the lag in putting that development into commercial practice.

Many of these patents are being utilized commercially. Whether used or not, they offer opportunities for technological transfer. Also, a major purpose of this book is to describe the number of technical possibilities available, which may open up profitable areas of research and development. The information contained in this book will allow you to establish a sound background before launching into research in this field.

Advanced composition and production methods developed by Noyes Data are employed to bring these durably bound books to you in a minimum of time. Special techniques are used to close the gap between "manuscript" and "completed book." Industrial technology is progressing so rapidly that time-honored, conventional typesetting, binding and shipping methods are no longer suitable. We have by-passed the delays in the conventional book publishing cycle and provide the user with an effective and convenient means of reviewing up-to-date information in depth.

The table of contents is organized in such a way as to serve as a subject index. Other indexes by company, inventor and patent number help in providing easy access to the information contained in this book.

15 Reasons Why the U.S. Patent Office Literature Is Important to You —

1. The U.S. patent literature is the largest and most comprehensive collection of technical information in the world. There is more practical commercial process information assembled here than is available from any other source.
2. The technical information obtained from the patent literature is extremely comprehensive; sufficient information must be included to avoid rejection for "insufficient disclosure."
3. The patent literature is a prime source of basic commercially utilizable information. This information is overlooked by those who rely primarily on the periodical journal literature.
4. An important feature of the patent literature is that it can serve to avoid duplication of research and development.
5. Patents, unlike periodical literature, are bound by definition to contain new information, data and ideas.
6. It can serve as a source of new ideas in a different but related field, and may be outside the patent protection offered the original invention.
7. Since claims are narrowly defined, much valuable information is included that may be outside the legal protection afforded by the claims.
8. Patents discuss the difficulties associated with previous research, development or production techniques, and offer a specific method of overcoming problems. This gives clues to current process information that has not been published in periodicals or books.
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10. Obtain licenses — many U.S. chemical patents have not been developed commercially.
11. Patents provide an excellent starting point for the next investigator.
12. Frequently, innovations derived from research are first disclosed in the patent literature, prior to coverage in the periodical literature.
13. Patents offer a most valuable method of keeping abreast of latest technologies, serving an individual's own "current awareness" program.
14. Copies of U.S. patents are easily obtained from the U.S. Patent Office at 50¢ a copy.
15. It is a creative source of ideas for those with imagination.

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INTRODUCTION

Because of the catalytic specificity of enzymes, considerable attention has been directed toward finding methods of using them in both laboratory and industrial applications. Enzymes are commonly water-soluble, and for that reason, many enzymes are uneconomical to use in large-scale batch-type operations since the enzymes can generally be used only one time in the absence of rather costly enzyme recovery and purification steps. In recent years, however, techniques have been devised to fix active enzymes on mostly water-insoluble materials that can be readily removed from a reaction, thus permitting reuse of the insolubilized or immobilized enzyme.

Prior methods for immobilizing enzymes have usually been classified as: (1) physical adsorption; (2) ionic bonding as on an ion exchange resin; (3) physical entrapment such as inclusion in a microporous gel or fiber or by microencapsulation; (4) crosslinking of the enzymes; and (5) covalent bonding of the enzyme to a support. With increasing sophistication, the technology now combines one or more of these techniques to produce the immobilized enzymes, and the ligands or coupling groups used to covalently bind the enzyme to a support are designed to place the enzyme at optimum distance from the support. Thus, the immobilization methods used as chapter headings in this review must not be regarded as rigid classifications.

This review covers approximately 200 processes disclosed in 211 patents issued since July 1974. Covalent bonding of the enzyme to a support is the means of immobilization in the majority of the processes. Emphasis is also placed on the production of enzyme products that can be used in continuous processes; that is, they permit continuous flow of substrate over the immobilized enzyme in a column or reactor without disintegration of the support with plugging of the column.

Industrial applications of the immobilized enzymes are found in the production of sugars from starch and in the production and analysis of various pharmaceutical and drug-related products. Increasing attention is being shown to the use of coupled enzymes in affinity chromatography and immunoassay procedures.

Definitions of abbreviations:

- DE Dextrose equivalent refers to the total reducing sugar content of the dissolved solids in starch hydrolysates expressed as percent dextrose.
- GU Glucose activity unit is the amount of enzyme which catalyzed the production of one gram of dextrose per hour at 60°C at pH 4.5.
- IU The amount of pullulanase which catalyzed the liberation of 1 μ mol of maltotriose per minute from a 0.5% of pullulan at pH 5.0 at 45°C.
- IGIU International glucose isomerase unit is that amount of glucose isomerase needed to convert 1 μ mol of glucose to fructose per minute at 60°C at pH 6.85.

The abbreviations used to designate enzyme depositories and their catalog numbers are:

- ATCC American Type Culture Collection, Rockville, Maryland.
- NRRL Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois.
- IFO Institute of Fermentation, Osaka, Japan.

IMMOBILIZATION BY ADSORPTION

Adsorption of enzymes to water-insoluble supports, whether organic or inorganic, has been the simplest insolubilization technique. It has been attractive because it requires merely exposing the enzyme in solution to the support material. The ease of adsorption, however, is offset by the corresponding ease of desorption.

USE OF POROUS ALUMINA-MAGNESIUM OXIDE SUPPORT MATERIALS

Porous Alumina with Specific Amounts of Magnesium Oxide for Supporting Isomerase

D.L. Eaton and R.A. Messing; U.S. Patents 3,982,997; September 28, 1976 and 3,992,329; November 16, 1976; both assigned to Corning Glass Works have found that a very high enzyme loading per gram of carrier for an immobilized glucose isomerase composite can be achieved by incorporating a critical amount of MgO in a porous Al_2O_3 enzyme support material. Specifically, it has been found that a very efficient composite can be prepared by adsorbing glucose isomerase enzymes to the internal surfaces of a high surface area (at least $5 \text{ m}^2/\text{g}$), porous, inorganic carrier having an average pore diameter of 100 to 1000 Å and comprising by weight, between 0.84 and 3.80% MgO and Al_2O_3 . Preferably, the porous MgO- Al_2O_3 enzyme carrier is in particulate form having an average particle size (U.S. Standard Sieve), preferably between 30 and 45 mesh with the average pore diameter being between 150 and 250 Å.

The general method for preparing the MgO- Al_2O_3 porous carriers involves starting with alumina particles having an average particle size of 300 ± 200 Å. These particles are then mixed with a solution consisting of varying amounts of magnesium ions to form a slurry which is mixed well. The magnesium ions can be added from a variety of available sources such as $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or $\text{Mg}(\text{OH})_2$. The slurry is then gently dried to remove water. This drying step tends to shrink the individual particles together such that the ultimate dried product is porous and has an average pore size approximating the average particle size of the starting materials. The gentle drying can be accomplished via a number of methods

such as simple air drying, drying with gentle heat ($\sim 100^\circ\text{C}$), spray drying the slurry, and like methods. The main requirement in the drying step is that it be gentle enough to preserve the skeletal pore structure formed as the particles shrink together.

After drying, the porous body is strengthened by firing it to a temperature below the sintering point; e.g., fired to 400° to 600°C for 1 to 16 hours. The resulting product can then be comminuted, if necessary, and the individual porous particles sorted according to desired mesh size range which is preferably between 30 and 45 mesh, U.S. Standard Sieve. Alternatively, the slurry can be spray dried to the desired particle size range prior to firing.

After the porous particles are prepared, they can be used to immobilize the glucose isomerase molecules by adsorption to internal surfaces of the porous bodies. By using porous particles having an average pore diameter of less than 1000 Å, in particle sizes of 30 to 45 mesh, a very high surface area per gram (e.g., greater than about $5\text{ m}^2/\text{g}$) is assured for maximum enzyme loading. It was found that enzyme loading is increased significantly if, prior to adsorption of the enzymes, the carriers are reacted with an aqueous citrate solution (e.g., 0.1 M citric acid or sodium citrate solution, pH 7.0).

Examples 1 through 10: Ten sample carriers were made consisting of alumina and from 0 to 28.6% MgO (by weight). By using the amounts of each ingredient as shown in Table 1, the sample carriers were made by first adding to the distilled or deionized water sufficient glacial acetic acid to bring the solution to 0.1 M. Then a slurry was formed by adding the alumina with vigorous stirring. The stirring was continued until a smooth, creamy mixture was obtained, approximately 15 to 30 minutes. The pH was then adjusted to 2.0 to 3.0 and the magnesium compound added to the slurry either as a liquid, or as a solid. This mixture was then blended at a high speed for an additional 15 to 30 minutes.

The resulting blend was then formed into particles or spheres by either slip casting or spray drying. The slip cast material was then broken and sorted according to particle size by conventional means. Both the slip cast and spray-dried material were fired at 600°C for 16 hours. Prior to adsorption of the enzymes, the effect of MgO additions on the pH of the carriers was determined by mixing 1 gram of each carrier with 9 grams of distilled water for 15 minutes to achieve an equilibrium, and then measuring the pH of the mixture with a conventional pH meter.

Table 1

Example	Ingredients				Final Product		
	H ₂ O	Al ₂ O ₃	MgCl ₂ ·6H ₂ O	Mg(OH) ₂	Carrier Shape (30-45 mesh)	pH	MgO (% by wt)
1*	489	400	0	0	spheres	4.4	0
2**	100	100	0	0	particles	4.4	0
3**	100	99.16	4.3	0	particles	7.0	0.84
4*	244	200	14.2	0	spheres	7.5	1.4
5*	244	200	22.3	0	spheres	8.2	2.2
6*	244	200	29.4	0	spheres	8.1	2.9
7*	244	200	38.6	0	spheres	8.4	3.8
8**	100	100	0	10.3	particles	8.8	6.65

(continued)

Table 1: (continued)

Example	Ingredients				Final Product		
	H ₂ O	Al ₂ O ₃	MgCl ₂ ·6H ₂ O	Mg(OH) ₂	Carrier Shape (30-45 mesh)	pH	MgO (% by wt)
9**	100	100	69.2	0	particles	8.9	12.0
10**	100	100	0	58.1	particles	9.3	28.6

*Pounds **Grams

Note: All porous bodies had an average pore diameter within range of 150-250 Å.

Each of the above carrier samples was used to immobilize glucose isomerase by reacting the enzyme preparation with each carrier to adsorb the enzyme onto the internal surfaces of the pores. About 15 ml of the enzyme preparation was used for each 15 grams of carrier. Prior to the actual adsorption step, each carrier sample was initially washed with distilled water by fluidizing the carrier sample in a column. The washed carriers were then reacted with a 0.1 M citrate solution in a shaking bath for 1 hour. Then, the enzyme preparation was added and the adsorption was allowed to proceed for 24 hours with shaking to facilitate the adsorption process.

The final product was then rinsed with distilled water and the individual samples were assayed with the following results where E_0 represents the enzymatic activity per gram and E_0 (equiv) represents a normalized value associated with an increased loading observed using the irregularly shaped particles (cf spheres).

Table 2

Ex. No.	MgO (%)	pH*	Shape	E_0	E_0 (equiv)
1	0	4.4	Spheres	203	203
2	0	4.4	Particles	387	(200)
3	0.84	7.0	Particles	805	(600)
4	1.4	7.5	Spheres	650	(650)
5	2.2	8.2	Spheres	898	898
6	2.9	8.1	Spheres	899	899
7	3.8	8.4	Spheres	909	909
8	6.65	8.8	Particles	916	(720)
9	12.0	8.9	Particles	768	(600)
10	28.6	9.3	Particles	200	(50)

*Of carrier.

To be commercially feasible, the immobilized glucose isomerase should have an enzymatic loading of at least 500 units of activity per gram of carrier under a continuous isomerization (flow-through) process. As can be seen from Table 2, this loading level is obtained when the percent by weight MgO in the MgO-Al₂O₃ porous carrier is 0.84 to 12.0% MgO with best results obtained when the carrier consists of 0.84 to 3.8% MgO. Although the exact mechanism(s) whereby the MgO content results in improved loading is not fully understood, it can be appreciated from the data in Table 2 that the carrier pH may play a role in determining loading amount since the desired minimum loading of at least 500 activity per gram occurs on carriers having a pH of 7.0 to 8.9. Hence, it is thought that the addition of MgO may not only serve to satisfy a portion of the enzymes Mg⁺⁺ needs but also set carrier pH parameters which limit both higher and lower loadings.

Regenerating Isomerase Supports by Pyrolysis plus Citrate Washing

Highly porous $\text{MgO-Al}_2\text{O}_3$ support materials useful for the immobilization of glucose isomerase can be regenerated for reuse in the process disclosed by L.R. Bialousz, E.R. Herritt, D.J. Lartigue and W.H. Pitcher, Jr.; U.S. Patent 3,965,035; June 22, 1976; assigned to Corning Glass Works. The regeneration comprises pyrolysis under conditions sufficient to remove substantially all carbonaceous matter, followed by treatment with a neutralized citrate solution.

Preferred carrier materials consist of highly porous particles consisting of $\text{MgO-Al}_2\text{O}_3$ having incorporated between 0.84 and 12.0% by weight MgO , the particles having an average pore diameter, preferably between 150 and 250 Å and an average particle size, preferably of 30 to 45 mesh, U.S. Standard Sieve. The preferred regeneration steps involve subjecting such particles to a temperature of 500° to 900°C in the presence of oxygen for a period of time sufficient to remove substantially all carbonaceous matter from the carrier.

Thereafter, the particles are allowed to cool and are then exposed to a neutralized aqueous solution of citrate ions long enough to remove contaminants such as metal ions which would minimize economical reuse of the carrier. Preferably the citrate solution consists of 0.1 molar citrate solution at a pH between 6.0 and 10, very preferably a sodium citrate solution at a pH of 7.0, and the pyrolyzed carrier is incubated with the citrate solution for at least 15 minutes, preferably at room temperature. The glucose isomerase solution used was an aqueous solution with a glucose isomerase activity of 2,700 IGIU/ml. The enzyme was derived from a *Streptomyces* sp. organism.

The composites were prepared by reacting 10 ml of the enzyme solution with 10 grams of porous carrier as follows: The carriers are initially washed with distilled water in a fluidizing column. The carrier is then placed in a flask to which 10 ml/g carrier of 0.05 M magnesium acetate is added and the flask is placed in a shaker bath for 1 hour.

The solution is then decanted and the enzyme is added and this mixture is allowed to react in the shaker bath for 24 hours to facilitate enzyme adsorption. The product is then rinsed with distilled water and the immobilized enzyme composite can be stored in water or as a wet cake until used.

In preparing the composites of the examples, approximately 10-gram (wet weight) quantities of each composite were prepared by the above methods. Nine separate composites were prepared with new carrier (unregenerated). Each new carrier was then spent by placing it in a plug flow-through column through which the glucose solution was flowed continuously under assay conditions for at least 30 days. Then, the indicated number of samples was regenerated by pyrolysis, and pyrolysis followed by citrate treatment, as indicated. The pyrolysis step involved heating the carriers to a temperature of 500° to 600°C for 1 hour in the presence of an oxygen source. The citrate solution treatment involved pumping 0.1 M citric acid solution neutralized to pH 7.0 (with NaOH) through a packed bed (plugged flow-through column) of the pyrolyzed carrier for about 1 hour, the amount of citrate solution being about 3 ml/g of pyrolyzed carrier treated.