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David Rozzell · Fritz Wagner

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Biocatalytic Production of Amino Acids and Derivatives

Edited by
J. David Rozzell and Fritz Wagner

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IMMOBILIZED ENZYMES: TECHNIQUES AND APPLICATIONS

J. David Rozzell

CONTENTS

13.1 INTRODUCTION

Immobilized enzymes have captured the interest of biotechnologists since the 1950s, but it was with the work by Katchalski-Katzir and Chibata and co-workers in the 1960s that research activities in this area began to accelerate, culminating in the First Enzyme Engineering Conference in 1971. Significant efforts towards improved immobilized-enzyme preparations continue today as new uses emerge. The first industrial application of enzymes in immobilized form was for amino acid production, as reported by Chibata and co-workers at Tanabe Seiyaku in Japan in 1969 [1]. This group immobilized L-aminoacylase for use in a packed-bed reactor in the resolution of various DL-amino acids into their corresponding optically pure enantomeric forms. Since that time, enzymes in immobilized form have become increasingly important as catalysts for the production of amino acids, as well as numerous other substances.

By way of definition, immobilized-enzyme biocatalysts consist of the enzyme, in varying degrees of purity, attached to or otherwise retained by a support matrix. At one extreme, intact dead cells (which are effectively bags of enzymes) may be bound to a support for use as a catalyst; at the other extreme, partially purified or purified forms of the enzyme(s) of interest may be immobilized. Driving the development of this technology is the fact that the immobilization of an enzyme can improve the economics of its application, improve the quality of the product produced, or both. By changing from batch to continuous operation, one can often significantly reduce the economics of an enzyme-catalyzed reaction. Other advantages, such as improved control of the reaction, leading to better uniformity of the product and greater ease of product recovery, are also often achieved through the immobilization of an enzyme.

In assessing the economics of a process using a biological catalyst (enzyme), the critical issue is not the cost of the biocatalyst itself but rather the contribution of the biocatalyst to the cost of the final product. Biocatalyst costs themselves depend on various components, including enzyme or cell production, support matrix, auxiliary reagents, and the loss of activity associated with immobilization. However, the important factors which determine the cost contribution of the biocatalyst are the yield of product, the volumetric productivity achieved in the process, the product concentration attained, and the useful lifetime of the biocatalyst under operational conditions. This chapter will survey immobilization methods, with special attention being paid to those which have been found useful in amino-acid production. The methods discussed here are not meant to be exhaustive but rather illustrative of what has been developed.

13.2 GENERAL IMMOBILIZATION METHODS

Five general techniques have been described for immobilizing enzymes:

1. adsorption;
2. covalent attachment;
3. cross-linking;
4. entrapment of an enzyme in a polymeric matrix; and
5. encapsulation or confinement of an enzyme in a membrane [2].

Each will be discussed along with its advantages and disadvantages. Although each immobilization technique is conceptually distinct, it is important to realize that there is often a certain amount of overlap or even combining of techniques in actual practice. Furthermore, the results of over thirty years of work by numerous researchers suggest that there is no one universally applicable immobilization technique; rather, a range of methodologies is available and must be evaluated on a case by case basis. There is still some art mixed with the science of enzyme immobilization. Certain enzymes may be immobilized successfully with one method but not with others. Each immobilization method has both advantages and disadvantages which must be considered within the context of the enzyme to be immobilized and the overall process in which the enzyme is to be used.

13.2.1 Adsorption

Adsorption is one of the most economical and operationally simple processes by which enzymes can be immobilized, and this simplicity is no doubt responsible for its attractiveness as an immobilization method. Adsorption was the technique used by Chibata and co-workers in their first commercial process involving an immobilized enzyme. However, even though this immobilization procedure may be straightforward to carry out, the interactions involved in adsorption are complex and not completely understood. A good definition of adsorption, given by Messing, is the adhesion of an enzyme to the surface of a support which has not been specifically modified for covalent attachment [3].

One of the principal advantages of adsorption is its ease to perform; simply contacting an aqueous solution of the enzyme with the support is all that is required. Occasionally, with judicious selection of the support, the desired enzyme will have an affinity for the support relative to other proteinaceous material such that a partial

[Refs. on p 318]

purification can be accomplished during the act of immobilization. Enzyme in varying states of purity (including whole cells and enzyme mixed with cell debris) may be used. Additionally, adsorbed immobilized enzyme preparations lend themselves readily to regeneration and reuse of the support material, if desired.

An enzyme may be immobilized by bonding to the external or internal surface of a support, or both. An important advantage of immobilizing the enzyme on a nonporous, external surface is that diffusional limitations are minimized. However, the disadvantages of adsorption on a nonporous material are:

1. a relatively low surface area for bonding may lead to low catalyst activity;
2. leaching, already a problem, may be worse because the enzyme is more susceptible to physical abrasion and sloughing off due to shear forces in the bulk solution; and
3. the enzyme is more exposed to microbial or proteolytic attack.

Although immobilization of an enzyme to the internal surface of a porous carrier has one marked disadvantage—that of diffusional limitations to the rate of the reaction catalyzed—certain advantages may be gained from this approach. An enzyme bound within a porous material is protected from sloughing off through abrasion and attack by adventitious microbial contamination. It has also been reported that, generally speaking, a more stable enzyme system may result from immobilization within a porous material [1].

Typically, support particles used in adsorption of enzyme will have diameters in the range of 0.5 to 100 micrometers. Smaller particles than this result in pressure drops that are unacceptably high; larger particles have such a reduced surface area for contact with substrate solutions that volumetric productivities are too low.

Supports used in the past for adsorption of enzymes include bentonite clay, chitosan, vermiculite, DEAE-cellulose and composites, ion exchange resins, titania, alumina, silica, glass, and granular activated carbon. With each support, the immobilization process depends on factors such as preconditioning of the surface with metal ions prior to contact with the enzyme, optimization of pH and ionic strength, and time. A typical procedure is given by Chibata *et al.* for the immobilization of porcine aminoacylase on DEAE-Sephadex [4], and illustrates the simplicity of the method.

At room temperature (20–25 °C), 100 g of DEAE-Sephadex A-25 (3.5 ± 0.5 meq/g, bead type) suspended in 1 l of distilled water is added to 5 l of 0.1 N NaOH, and the suspension is stirred for 3 h. After filtration, the precipitate is washed with water until the washing solution is neutralized, stirred with 5 l of 0.1 M phosphate buffer (pH 7.0) for 3 h, and kept standing for 16 h. The suspension is filtered, and the resulting precipitate is stirred with 1.67 l of aminoacylase solution (total enzyme activity: 167,000 μmol/h) for 3 h at room temperature. After filtration, 5 l of distilled water is added to the precipitate; the preparation is stirred for 1 h, then filtered. For further washing, 5 l of 0.2 M sodium acetate is added to the precipitate; the preparation is stirred for 1 h, then filtered. The precipitate is washed with distilled water until no

protein is detected in the washing solution. These washing procedures are carried out at room temperature. The resulting precipitate is ready for use in the enzyme-catalyzed reaction.

In order to store the immobilized preparation for a long period or to measure its enzyme activity, it is suspended in 2.5 l of distilled water and lyophilized. Using this procedure, 106 g of immobilized DEAE-Sephadex-aminoacylase is obtained. The activity of the preparation is reported to be about 700 μmol/h·g of preparation, under standard assay conditions.

The above method formed the basis for the first industrial process for the production of an amino acid by Tanabe Seiyaku Company in Japan. *N*-Acetyl-DL-methionine was passed through the column, where the L-isomer was deacetylated stereoselectively. The L-methionine was separated from the unreacted *N*-acetyl-D-methionine, which itself was recovered, racemized and recycled through the process. This process was successfully commercialized in 1969 and is considered to be the first commercial-scale immobilized-enzyme process.

In this example, the useful lifetime of the immobilized enzyme was approximately 30 days, and the support could be regenerated each month by adsorbing fresh enzyme on the column. It was reported that the same batch of support matrix could be used for a period of five years, with repetitive regeneration cycles.

Other enzymes useful for amino acid production which have been immobilized by the adsorption method include aspartase on DEAE-cellulose and silica gel [5], leucine aminopeptidase on hydroxylapatite [6], glutamic-aspartic transaminases on DEAE-Sepharose [7], and α-amino-ε-caprolactam racemase on DEAE-Sephadex [8]. There are numerous reports of the immobilization of other enzymes such as amylase, glucose isomerase, glucose oxidase, and proteases on materials such as activated carbon, bentonite, and alumina. Given the data base, it is probably fair to surmise that an appropriate material may be found that will allow the immobilization by adsorption of virtually any enzyme; however, the strength of the adsorptive forces binding the enzyme to the support will vary considerably from one enzyme to another. Adoption of such a catalyst in a process will likely depend on whether the mechanical properties and the operational lifetime of the catalyst are sufficient for commercial application.

13.2.2 Covalent Attachment

Covalent attachment of enzymes to surfaces is often employed when leaching of enzyme activity from the support is a concern. This method generally offers the advantage of an immobilized enzyme system that is more permanently anchored, and may also show greater stability and the ability to withstand a broader spectrum of pH conditions, ionic strengths, and temperatures. Enzymes immobilized by covalent attachment are also more resistant to attack by proteolysis. The main disadvantage

of this type of attachment is its somewhat greater complexity and higher cost to prepare. Cost notwithstanding, when covalent attachment results in a significantly more stable enzyme system or when the absence of enzyme in the product solution is of critical importance, it still may be the method of choice.

There are three different techniques by which covalent attachment can be effected. The first is through exposure of the enzyme to a support which has been preactivated to accommodate covalent binding. Operationally, once the activated support has been prepared, the immobilization proceeds like adsorption, but the result is an enzyme covalently bonded to the support matrix. The second technique involves exposure of the enzyme to the support in the presence of an activating or cross-linking reagent. Inevitably, some chemical modification or cross-linking of enzyme molecules occurs during an immobilization of this type which can lead to a loss in catalytic activity. A third possibility—much less commonly used—is to preactivate the enzyme and expose it to a support functionalized for covalent binding. The risk of inactivation of the enzyme by chemical processes during the preactivation procedure is significantly higher, and the procedure is less reproducible. Thus, virtually all practical systems for immobilization fall into one of the first two types.

Frequently, covalent coupling is preferred to other processes in cases where the enzyme is multimeric or contains prosthetic groups. There may be a reduced tendency to disrupt the complex nature of these enzymes since specific bonds can be formed with the functional group to bind the enzyme through multiple points of attachment to the support.

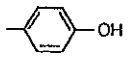
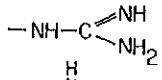
A list of the amino acid functional groups which are chemically reactive enough to participate in covalent binding reactions may be found in Table 13.1.

Of those functional groups listed, $-NH_2$, $-CO_2H$, and -SH are involved in most immobilization procedures due to their nucleophilicity. The phenolic ring of tyrosine is also extremely reactive in diazo-coupling reactions, and its hydroxyl group can be an excellent nucleophile at basic pH. The guanidino group of arginine can react with aldehydes. Histidine displays a lower nucleophilicity, but can sometimes react with supports activated with tosylates, tresylates, or other good leaving groups.

Functional groups of a wide range of types have been used for the covalent attachment of enzymes. The variety of chemistries available for covalent attachment allows the conditions of immobilization to be tailored to each enzyme system. The microenvironment of the enzyme may also be tailored by appropriate modification of the support surface; hydrophobic residues or ionically charged groups may be used to alter the support to affect in a desirable way the enzyme-catalyzed reaction of interest [9].

The range of support materials that has been used for covalent attachment, includes porous glass, porous ceramics, sand, charcoal, modified cellulose, polymeric resins, and metallic oxides. A few examples are described here to illustrate typical covalent attachment methodology.

TABLE 13.1 Useful Functional Groups for Covalent Attachment of Enzymes to a Support Matrix

Functional Group	Corresponding Amino Acid
$-NH_2$	Lysine, N-terminus
$-CO_2H$	Glutamic Acid, Aspartic Acid, C-terminus
$-SH$	Cysteine
	Tyrosine
	Arginine
	Histidine
$-CH_2OH$	Serine

13.2.2.1 Specific Examples of Covalent Coupling: Binding to Activated Carbohydrate Supports

One of the most commonly used procedures for the covalent coupling of enzymes to carbohydrate support matrices is based on a preactivation of a support with cyanogen bromide [10, 11]. The mechanism of this reaction has been studied extensively by Wilchek and Kohn [12, 13].

The activated carbohydrate will couple generally to an amino group of lysine on the protein or the free N-terminus of the protein to yield a covalently bound product. Supports which have been preactivated with cyanogen bromide can be prepared in advance and stored for periods of up to one year at freezer temperatures. Preactivated supports are also available commercially. Coupling of an enzyme to a CNBr-activated support requires no more than exposure of the enzyme to the support in aqueous solution for a few hours, followed by washing. This method, while extremely popular in lab-scale reactions, has not been widely used in large-scale systems due to the requirements to handle toxic reagents and the poorer mechanical stability of most carbohydrate gels and polymers compared to other support materials. The bond between the enzyme and the support is also potentially susceptible to hydrolytic cleavage.

13.2.2.2 Specific Examples of Covalent Coupling: Carbodiimide Coupling

Carbodiimide reagents activate carboxyl groups through the formation of an *O*-acylisourea intermediate, which reacts rapidly with nucleophilic functional groups of proteins to form amide, thioester, or ester linkages. Hydrolysis is a competing side reaction. This method has been described for the immobilization of transaminases useful for amino acid production [14]. The reaction typically forms a stable amide linkage between enzyme-bound carboxyl groups and support-bound primary amines, and the rate of the reaction is relatively rapid. One disadvantage of carbodiimide reagents is their relatively high cost. Chemical modification of the enzyme leading to a loss of catalytic activity is another potentially negative side reaction.

13.2.2.3 Specific Examples of Covalent Coupling: Amine-Bearing Supports

Amine-bearing supports are among the most useful and most utilized supports for the covalent attachment of enzymes, and a variety multifunctional reagents has been utilized for the covalent attachment of enzymes to supports bearing pendent amines. Difunctional reagents have most frequently been employed for binding. Glutaraldehyde, one of the least expensive reagents available in bulk, reacts in complex fashion to form Schiff bases with amine groups on the support and produces pendent aldehydes and α,β-unsaturated carbonyl functionalities through which proteins may attach. Other useful difunctional agents include diimidate esters and diisocyanates.

Inorganic supports bearing amine functionality have been widely used for the immobilization of proteins. Perhaps the most frequently used technique for the covalent attachment to inorganic surfaces is the preactivation and functionalization with aminosilane reagents [15, 16, 17]. This technique involves attaching an aminosilane to the inorganic surface, leaving the amine available for covalent attachment of the enzyme. The most frequently used silane, popularized through the developments at Corning Glass Works, is γ-aminopropyltriethoxysilane [16]. The terminal amino group may be functionalized by bifunctional coupling agents such as glutaraldehyde, resulting in a support which has been preactivated for enzyme attachment. Coupling of enzyme may also be achieved through the use of carbodiimide reagents, in which case it is through enzymic carboxyl groups that attachment is achieved.

Another process for immobilizing on amino-functionalized inorganic supports involves isocyanate bonding [18]. If the enzyme is attached under alkaline conditions, a substituted urea bond is formed between an amine on the protein surface and the isocyanate. If moderately acidic conditions are employed, then the isocyanate reacts

[Refs. on p 318]

Carbodiimide

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with a hydroxyl group on the enzyme and a urethane bond is formed. Isothiocyanates have also been used successfully [16].

Polyethyleneimine is a common polyamine, useful in providing amino group functionality for attachment. It has the advantage that it is inexpensive and that a wide range of supports including those other than inorganic particles may be used. Examples are alumina [19], carbon [20], diatomaceous earth [21], and poly(vinyl chloride)-silica composites [22, 23].

A typical procedure involving polyethylenimine activation, which has been used for the immobilization of recombinant transaminase, aspartase, and aspartate-β-decarboxylase in the industrial-scale production of L-amino acids, has been described by Rozzell [24]. The activity retained after immobilization approached 90%, with the operational half-lives of the immobilized biocatalysts ranging from 2 to 6 months.

Recently, Flaschel and co-workers have developed a method for the covalent attachment of enzymes involving a mineral or carbon particle coated with chitosan, providing a hydrophilic surface of attachment [25]. Chitosan, which is deacetylated chitin, contains available amino groups for chemical activation and is easily obtained at relatively low cost. Activation with a bifunctional reagent such as glutaraldehyde provides a stable immobilized-enzyme preparation. The use of rigid, incompressible particles on which the chitosan is deposited allows this catalyst to be used in both fixed-bed and fluidized-bed reactors. Yields of activity after immobilization of up to 90% have been reported.

13.2.2.4 Specific Examples of Covalent Coupling: Oxirane or Epoxy-Activated Polymers

Epoxy-activated polymers fall into the catagory of preactivated supports for covalent attachment, and they have gained attention as commercially useful support matrices for immobilized enzymes [26]. One advantage is the irreversible reaction by which enzymes may be attached to a support through epoxides; as the epoxide opens, in a displacement reaction involving a nucleophilic group on the enzyme, a nonhydrolyzable linkage is formed. Another advantage is the ability to activate a wide range of different surfaces with epoxides. Yet an additional advantage is that binding through epoxides does not appreciably change the charge state of the enzyme once it is bound. For example, the pK_a of the secondary amine formed after binding of an enzyme through a lysine side chain is not too different from that of the lysine side chain prior to coupling.

An epoxide-activated support has been developed by Rohm Pharma (Darmstadt, FRG), and is sold commercially under the trade name of *Eupergit*. The support is based on a methacrylic polymer bead bearing epoxide functionality. Eupergit has been successfully applied to the immobilization of many enzymes including penicillin acylase for use in the production of 6-aminopenicillanic acid [27].

13.2.3 Cross-Linking

Cross-linking of enzyme molecules with other proteins affords an insoluble preparation that can be readily handled or manipulated in a continuous reactor. These immobilized preparations are often produced in a particulate form, for use in packed bed reactors.

As enzymes are often stabilized by a concentrated protein environment, copolymerization of enzymes with other inexpensive proteins such as gelatin (using bifunctional reagents like glutaraldehyde) has been used effectively by companies such as Novo and Gist-Brocades to produce long-lived pellets of immobilized enzymes [28]. As an example of a commercially useful method, Novo takes cells which have been recovered from the fermentation broth in the form of a paste and extrudes them in the form of spaghetti. The strands are cut into uniform pellets and hardened in glutaraldehyde. This method is particularly useful because it gives particles of a controlled size for use in packed-bed reactor systems.

Although glutaraldehyde is the most popular cross-linking reagent, dimethyl adipimidate and diisocyanates have also been used. Diamines may also be used in conjunction with carbodiimides, which activate carboxyl groups on the proteins to react with amines.

The enzyme activity of immobilized preparations prepared by cross-linking with multifunctional reagents will depend upon the degree of cross-linking. If high concentrations of cross-linking agents are employed, more bonds will be formed, which will generally result in lower activities. The additional bonds formed at high concentrations may block the entrance of the substrate to the active sites and also restrict the conformational mobility of the enzyme severely. The effect of the quantity of the cross-linking agent, diazobenzidine, on the catalytic activity of papain was demonstrated by Silman and co-workers [29]. A similar effect of glutaraldehyde cross-linking on the activity of carboxypeptidase A was reported by Quiocho and Richards [30, 31]. Generally speaking, an increased level of cross-linking will lead to a more stable enzyme preparation, but often at the cost of part of its catalytic activity.

13.2.4 Entrapment in a Polymeric Matrix

Entrapment is perhaps best viewed as the physical confinement of an enzyme in a polymeric network or matrix. In this technique, an enzyme is typically added to a solution of monomer, and the resulting polymerization entraps the enzyme molecules in the matrix.

This immobilization technique has been extensively explored since the early work of Bernfeld and Wan using polyacrylamide [32]. The most frequently employed matrix for entrapment has in fact been polyacrylamide, which has had significant early success in Japan. However, various other materials have also been used. Bauman and

co-workers reported the immobilization of cholinesterase in polymerized starch [33]. Vieth and Venkatasubramanian investigated the entrapment of enzymes in collagen matrices [34]. Whitesides has published on the use of PAN (polyacrylonitrile) gels [35, 36].

One of the major concerns with respect to entrapped enzymes is that of leaching. The enzyme may migrate out of the pore if the pore is too large. In many cases, this leaching may be overcome by simply cross-linking the enzyme after entrapment with a bifunctional reagent such as glutaraldehyde.

The opposite effect is also a concern with entrapment: that of pore diffusion limitations. If the substrate is a rather large molecule, such as a protein, it may be restricted from entry into the pore and thus be inaccessible to the enzyme.

Dead cells containing enzymes have been very successfully immobilized by entrapment. The size of the cells prevents loss of catalyst due to diffusion out of the pores of the matrix. Fusee described the production of L-aspartic acid using polyurethane-immobilized cells containing aspartase [37]. Calton *et al.* reported the production of L-aspartic acid, L-phenylalanine, and L-alanine using cells entrapped in a polyazetidine matrix [38]. Chibata and co-workers have commercialized processes for L-aspartic acid and L-alanine using cells entrapped in carrageenan gels and hardened with hexamethylenediamine and glutaraldehyde [39]. Calcium alginate gel has similarly been used.

A novel method to overcome the problem of leaching out of activity was developed as a part of the polyazetidine method by Calton and co-workers. Cells containing enzyme are mixed with a polyazetidine polymer and cured by drying or mild heating. The polymer chains contain a reactive N-containing ring which opens under nucleophilic attack by cellular material or proteins. The result is believed to be a combination of entrapment and covalent attachment, rendering a more stable immobilized enzyme preparation in a hydrophilic environment, which is less prone to leaching [40]. Further enhancements to this technique have been developed by Novo [41].

13.2.5 Encapsulation or Confinement in a Membrane

Encapsulation is distinguished from entrapment methods by the fact that a solution of the enzyme is separated from the bulk solution by a membrane. In this approach, pioneered by Chang [42, 43], enzymes are encapsulated within membranes that are impermeable to the enzymes but permeable to the enzyme substrate. Such semi-permeable microcapsules have been used as artificial cells in which the enzymes, cofactors, organelles, and other bioactive materials are retained [30]. Encapsulation offers the opportunity to immobilize larger quantities of enzyme per unit volume of immobilized preparation than any other procedure [3]. Perhaps the biggest disadvantage of this immobilization technique is that only relatively small substrate molecules

can be utilized with the intact membranes. Depending on the type of membrane used, encapsulation can also be a relatively expensive way to immobilize enzymes due to the high cost of membranes.

Chang has described the encapsulation of enzymes in a variety of membranes [44]. Two of the membranes that he used most successfully were cellulose nitrate and nylon. In another example, the Snamprogetti Company in Italy has entrapped aminoacylase and hydantoinase, for the production of amino acids in hollow fibers of cellulose acetate [45]. The half-lives were greater than one month under operating conditions. Other enzymes have been similarly immobilized for producing a range of different products, showing wide applicability of this general method.

Another membrane system which has shown promising results in biocatalytic applications has been developed by Sepracor [46, 47]. This system has been particularly effective in facilitating contact between an aqueous phase and a non-water-miscible phase. The enzyme is entrapped against the membranes and held in place by a slight positive pressure. As long as the stability of the enzyme is high enough, this system can operate continuously with little down time. In addition, old, deactivated enzyme can be flushed out and new enzyme loaded while the membrane cartridge is in place.

13.3 CONCLUSIONS

Perhaps the most important conclusion one can draw from the past thirty years of work on enzyme immobilization is that, due to the range of methods that has been developed, it can be asserted with a high degree of confidence that a successful immobilization method can be developed for virtually any enzyme. That does not mean, however, that one method will be successful for almost all enzymes. In fact, the method of choice will likely vary from one case to another. All five methods surveyed and described here have been found useful in the past in certain instances. Even more importantly, a number of these methods have been proven successful at the commercial scale, thus giving the researcher several options to choose from which have withstood the "test of practicality" at the manufacturing scale.

Products manufactured today using immobilized enzymes include high-fructose corn syrup, 6-aminopenicillanic acid, certain fatty acids and derivatives, L-malic acid, and isomaltulose. Specifically, a number of amino acids are also made using immobilized enzyme catalysts, including L-aspartic acid from fumaric acid and ammonia, L-alanine from L-aspartic acid, D-4-hydroxyphenylglycine from the corresponding hydantoin, L-ornithine and L-citrulline from L-arginine, L-tryptophan from indole and L-serine. Depending on the economics, technology has been developed for other amino acids

as well, including L-lysine, L-phenylalanine, L-tyrosine, L-serine, L-4-phenyl-2-aminobutanoic acid, L-norvaline, and L-DOPA. Genetic engineering has played a key role in a number of cases in making enzymes available in quantity at costs that previously could not be achieved.

Future applications will likely have an increasing dependence on genetic engineering as a tool to enhance enzyme-catalyzed processes by allowing improvements in the properties of the enzymes: modified substrate specificity, improved stability, and more suitable temperature or pH optima. Figure 13.1 illustrates in flow chart form how this may be done.

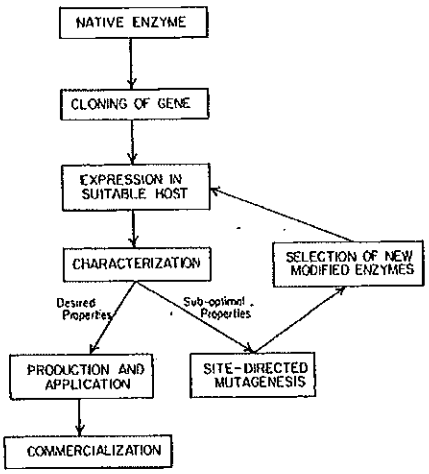

Figure 13.1. Using Genetic Engineering to Improve the Catalytic Properties of Enzymes.

The field of immobilized biocatalysts is rich with technology awaiting exploitation. Furthermore, biocatalysis is not standing still. The use of enzymes in non-aqueous solutions is rapidly expanding the scope of applications for biological catalysts. Continuing advances in genetic engineering, as mentioned above, and also in reactor design, should help to accelerate the realization of the potential benefits of this technology.

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ATTACHMENT D: "*Immobilized Aminotransferases for Amino Acid Production*": J. David Rozzell., "Methods in Enzymology" Volume 136, Pages 479-497, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.

lized pH- and glutaraldehyde-treated *P. dacunhae* cells. The substrate solution (adjusted to pH 8.5 with ammonia) consisting of 1.5 *M* ammonium fumarate and 1 m*M* Mg^{2+} is applied to the column containing immobilized *E. coli* cells at a flow rate of space velocity = 1.0/hr. After addition of PLP and pyruvic acid (these concentrations are 0.1 and 1 m*M*, respectively) into the effluent, pH of the solution is adjusted to 6.0 by addition of acetic acid. The solution is passed through the immobilized *P. dacunhae* closed column at a flow rate of space velocity = 0.06/hr and a pressure of about 8 kg/cm^2 achieved by plunger pump.

Crystallization of L-*Alanine from Column Effluent.* The effluent of appropriate volume is concentrated to about one-fourth of its original volume and cooled to 15°. L-Alanine crystallized is collected by centrifugation or by filtration and washed with 80% aqueous ethanol. The yield of L-alanine from ammonium fumarate is about 90% (theoretical). $[\alpha]_D^{20}$ = +14.8 (c = 10 in 6 *N* HCl).

Conclusion

In 1982, Tanabe Seiyaku Co. Ltd. successfully industrialized a continuous production system of L-alanine from ammonium fumarate, using a column reactor containing immobilized pH-treated *E. coli* cells and a closed column reactor containing immobilized pH- and glutaraldehyde-treated *P. dacunhae* cells.

By this system, L-alanine has been produced at low cost. This is considered to be the first industrial application of sequential enzyme reactions using two immobilized microbial cells.

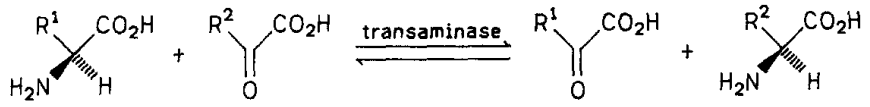
[44] Immobilized Aminotransferases for Amino Acid Production

By J. DAVID ROZZELL

Aminotransferases

Background

Aminotransferases (more commonly called transaminases, EC 2.6.1._) are a widely distributed class of enzymes. These enzymes catalyze the synthesis and breakdown of amino acids in microorganisms,



SCHEME 1.

plants, and animals by the transfer of an amino group from an α-amino acid to a 2-ketoacid as shown in Scheme 1.

The first evidence for aminotransferases was published by Needham[1] and Szent-Györgyi and co-workers[2] who noticed a relationship between the L-glutamic acid, L-aspartic acid, and oxaloacetic acid levels in pigeon breast muscle. Banga and Szent-Györgyi[3] demonstrated the reversibility of glutamic-pyruvic transaminase (EC 2.6.1.2, alanine aminotransferase) by chemically isolating the amino acid products L-glutamate and L-alanine. Since that time, a large number of aminotransferases have been discovered and characterized. One feature of aminotransferases is the requirement for the small molecule, pyridoxal 5′-phosphate, for catalytic activity, this cofactor being bound through a Schiff base linkage to the ε-amino group of an active-site lysine. Although the binding of pyridoxal 5′-phosphate to the enzyme is reversible, most aminotransferases show maximal catalytic activity at cofactor concentrations of 100 μM or less. Such low saturating concentrations of pyridoxal phosphate are an important property of aminotransferases; at concentrations of 100 μM or less, the cost of the cofactor in biocatalytic transamination processes is a relatively minor component of the total cost.

The mechanism of transamination is well known, and has been reviewed previously.[4] The reaction catalyzed by aminotransferases occurs as the result of two distinct half-reactions: the first involves transfer of the amino group of the L-amino acid donor to pyridoxal 5′-phosphate to yield a 2-ketoacid product which is released from the enzyme and an enzyme-bound pyridoxamine 5′-phosphate; the second is the binding of the 2-ketoacid to be transaminated to the enzyme and the transfer of the amino group from pyridoxamine 5′-phosphate to this 2-ketoacid to produce the desired L-amino acid and regenerate the pyridoxal 5′-phosphate. As a result, aminotransferases characteristically exhibit Ping-Pong kinetics.

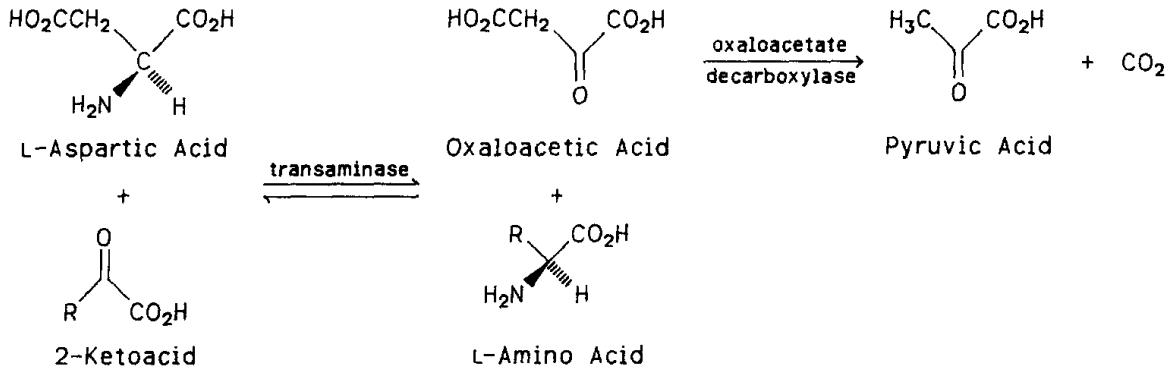
[1] D. M. Needham, *Biochem. J.* **24,** 208 (1930).
[2] E. Annau, I. Banga, A. Blazo, V. Bruckner, K. Laki, F. B. Staub, and A. Szent-Györgyi, *Z. Physiol. Chem.* **224,** 105 (1936).
[3] I. Banga and A. Szent-Györgyi, *Z. Physiol. Chem.* **245,** 118 (1937).
[4] A. E. Braunstein, "The Enzymes IX" (P. D. Boyer, ed.), Part B, pp. 379–481. Academic Press, New York, 1973.

Advantages and Disadvantages for Use in Biocatalysis

Although aminotransferases have been known for decades, these enzymes have seen little use as biocatalysts until now. However, since the normal function of aminotransferases is the biosynthesis and metabolism of amino acids, it is natural to look to these enzymes as potentially useful catalysts for the production of amino acids. In principle, almost any desired amino acid can be produced from the appropriate 2-ketoacid using an inexpensive amino acid as the amino donor. There are a number of advantages to the use of this kind of technology. (1) The aminotransferase enzymes catalyze the stereoselective synthesis of only L-amino acids from their corresponding 2-ketoacids. No D isomer is produced, and no resolution is required. (2) Aminotransferases have uniformly high catalytic rates, capable of converting up to 400 μmol of substrate/min per milligram of protein. (3) Many of the required 2-ketoacid precursors can be conveniently prepared by chemical synthesis at low cost. (4) The capital investment for an immobilized enzyme process using aminotransferases is much lower than for a fermentation process, and the productivity of the bioreactor is more than an order of magnitude higher. (5) The technology is generally applicable to a broad range of L-amino acids because aminotransferases exist with varying specificities. For example, there are enzymes specific for the transamination of amino acids with acidic side chains, aromatic side chains, branched alkyl side chains, etc. Such broad scope allows a number of different L-amino acids to be produced with the same equipment and often the same biocatalyst.

We have demonstrated laboratory-scale processes for the production of a variety of L-amino acids including L-alanine, L-phenylalanine, L-tyrosine, L-tryptophan, and several others. As an example, we have immobilized the commercially available glutamic-pyruvic aminotransferase from porcine heart on porous glass by covalent attachment, and obtained a stable biocatalyst with an activity of 400 International Units per gram. A column packed with 500 mg of this immobilized enzyme was operated continuously for 6 months and produced 160 mg L-alanine/hr from pyruvic acid as a starting material. This example illustrates the potential of immobilized aminotransferases applied to the production of L-amino acids.

There is one inherent disadvantage to the practice of this technology as described so far; as a catalyst, the aminotransferase can only accelerate the approach to equilibrium between the L-amino acid and 2-ketoacid precursors on one side of the equation and the 2-ketoacid and L-amino acid products on the other. Thus, the equilibrium constant for the generic transamination reaction as written in Scheme 1 is near unity, and the



SCHEME 2.

conversion of a 2-ketoacid feedstock to a desired L-amino acid will not proceed to completion in most cases. The key to the development of a general and commercially successful transamination process for the production of L-amino acids lies in overcoming this problem of incomplete conversion of a 2-ketoacid to the desired L-amino acid.

Driving the Reaction to Completion

Solving the problem of incomplete conversion of 2-ketoacid starting material to a desired L-amino acid required one important observation regarding the substrate specificity of aminotransferases. Although L-glutamic acid is generally considered to be the amino donor for aminotransferases that catalyze the transamination of a broad range of 2-ketoacids to L-amino acids, we have found that L-aspartic acid can also function competently as a general donor of an amino group with certain enzymes. We have worked extensively with an aminotransferase capable of using L-aspartic acid isolated from *Escherichia coli*.[5]

When L-aspartic acid is used as the amino group donor for the transamination of a given 2-ketoacid, oxaloacetic acid is coproduced along with the desired L-amino acid. Oxaloacetate, unlike 2-ketoglutarate, is a β-ketoacid, and as such can facilely be converted to pyruvic acid via an essentially irreversible decarboxylation step. This may be accomplished chemically by the use of certain metal ions or amines, thermally, or most preferably, enzymatically using the enzyme oxaloacetate decarboxylase. The coupled two-enzyme reaction is illustrated in Scheme 2.

The important feature of this process is the decarboxylation of oxaloacetate to pyruvate. It is this essentially irreversible decarboxylation that drives the entire process to completion to produce L-amino acids in quantitative yields from the appropriate 2-ketoacid precursors. The

[5] C. Mavrides and W. Orr, *Biochim. Biophys. Acta* **336**, 70 (1974).

pyruvic acid by-product is easily separated from the product mixture by crystallization of the L-amino acid or by ion-exchange methods.

We have investigated several methods for decarboxylating oxaloacetate, including catalysis by primary amines and divalent metal ions such as Mg^{2+}, Mn^{2+}, and Zn^{2+} and the enzymatic decarboxylation by oxaloacetate decarboxylase (OAD, EC 4.1.1.3). This chapter will focus on driving the overall reaction by the OAD-catalyzed decarboxylation of oxaloacetate to produce pyruvate and the desired L-amino acid.

Methodology

Sources and Production of Enzymes

Aminotransferases can be isolated from virtually any microbial, plant, or animal source. The most easily obtained enzymes are from porcine heart, yeast, and *E. coli*. However, the usefulness of the individual aminotransferases for amino acid production varies. The glutamic-oxaloacetic aminotransferase from porcine heart (EC 2.6.1.1, aspartate aminotransferase), although very stable and commercially available, is of limited utility for the production of amino acids because of its high specificity for L-glutamic acid, L-aspartic acid, and the corresponding 2-ketoacids as substrates.[6] Other substrates are not transaminated at reasonable rates.

Similarly, the commercially available porcine glutamic-pyruvic aminotransferase (EC 2.6.1.2, alanine aminotransferase) also exhibits the desirable properties of high stability, high specific activity, and lack of severe inhibition even at substrate concentrations up to 0.4 *M*, but the enzyme cannot use L-aspartic acid as the amino group donor. Thus, a highly productive immobilized biocatalyst can be prepared using this aminotransferase, and it can be used for the production of L-alanine from pyruvic acid and L-glutamate, but the reaction cannot be driven to completion by the coupling of oxaloacetate decarboxylase. These readily available enzymes have nonetheless been useful as model aminotransferases in the development and design of biocatalytic transamination processes, and data for the immobilization and use of these enzymes in bioreactors will be presented.

The microorganism *E coli.* is one of the most useful sources of aminotransferases. Of the four aminotransferases from this microorganism characterized to date,[7] we have found the so-called glutamic-oxaloacetic

[6] I. W. Sizer and W. T. Jenkins, this series, Vol. 5, p. 677.
[7] J. T. Powell and J. F. Morrison, *Eur. J. Biochem.* **87,** 391 (1978).

TABLE I
RELATIVE RATES OF PRODUCTION OF L-AMINO ACIDS USING ASPARTIC AMINOTRANSFERASE FROM *E. coli*[a]

L-Amino acid	Precursor	Relative rate
L-Glutamic acid	2-Ketoglutarate	225
L-Phenylalanine	Phenylpyruvate	100
L-Tyrosine	*p*-Hydroxyphenylpyruvate	130
L-Tryptophan	Indolyl-3-pyruvate	150
L-2-Aminoadipic acid	2-Ketoadipate	22
L-4-Phenyl-2-aminobutanoic acid	4-Phenyl-2-ketobutyrate	17
L-Histidine	Imidazole 3-pyruvate	3

[a] Amino group donor in all cases: L-aspartic acid.

aminotransferase (EC 2.6.1.1) to be an extremely useful catalyst for L-amino acid production. This enzyme is the most stable of the *E. coli* aminotransferases, and is produced constitutively.[8,9] The enzyme is capable of catalyzing the transamination of a large number of 2-ketoacids to L-amino acids using either L-glutamate or L-aspartate as cosubstrate. Table I lists the relative rate of transamination for the production of a variety of amino acids.

Interestingly, for use as a biocatalyst in the production of aromatic amino acids such as L-phenylalanine, the *E. coli* glutamic-oxaloacetic aminotransferase (GOA) is superior to the enzyme which carries out this function metabolically, the so-called aromatic-amino-acid aminotransferase (AA) (EC 2.6.1.5). The former enzyme is significantly more thermostable than the latter. In addition, the glutamic-oxaloacetic aminotransferase has catalytic rate constants for the transamination of phenylpyruvate to L-phenyalanine, *p*-hydroxypyruvate to L-tyrosine, and indole 3-pyruvate to L-tryptophan comparable to those for the aromatic aminotransferase. The K_m for these aromatic amino acids is approximately an order of magnitude higher for the GOA enzyme, but given that high concentrations of substrates are generally used in a biocatalytic process, the enzyme is functioning at its maximal catalytic rate.

The enzyme oxaloacetate decarboxylase (OAD) has been isolated from three different sources: *Pseudomonas putida* ATCC 950,[10] *Micro-*

[8] C. Mavrides and W. Orr, *J. Biol. Chem.* **250,** 4128 (1975).
[9] S. Chesne, N. Monnier, and J. Pelmont, *Biochimie* **60,** 403 (1978).
[10] A. A. Horton and H. L. Kornberg, *Biochim. Biophys. Acta* **89,** 381 (1964).

coccus luteus ATCC 4698,[11] and *Azotobacter vinelandii* ATCC 478.[12,13] We have purified all three of these enzymes, and have found the OADs isolated from *P. putida* and *M. luteus* as the most suitable for a biocatalytic process based on the criteria of specific activity, stability to operational conditions, activity after immobilization, and the ease of production and isolation. The enzymes may be obtained by modifications of published procedures.[10,11] Details of these purifications will be published elsewhere.[14]

Immobilization of Aminotransferases and Assays for Activity

A large number of immobilization methods have been examined for the immobilization of aminotransferases. Adsorption methods such as the binding of enzyme to ion-exchange resins falled to yield a stable immobilized enzyme preparation due to desorption of enzyme from the support. Entrapment in polymeric gels such as polyacrylamide produced active enzyme, but the activity was lower than that obtained using other methods. Also, this method suffered from the slow loss of activity due to diffusion of the enzyme out of the polymeric matrix. Covalent coupling of the enzyme to inert supports has proved to be the method of choice for the aminotransferases we have examined to this point, providing immobilized enzymes with high retention of activity, long-term operational stability, and good mechanical properties.

The successful immobilization of aminotransferases is highly dependent on the chemistry of the immobilization technique. Because of the requirement for the binding of the cofactor pyridoxal 5′-phosphate to the ε-amino group of a lysine residue, reagents such as glutaraldehyde, *p*-nitrophenyl esters, *N*-hydroxysuccinimidyl esters, and the like, which react with amines on the protein, can deactivate aminotransferases. For this reason, it is absolutely essential that the active site be protected during the immobilization by including pyridoxal 5′-phosphate and a 2-ketoacid (e.g., 2-ketoglutarate) in the reaction mixture. Alternatively, the coupling can be accomplished through carboxyl groups on the enzyme to amino groups on the support using a water-soluble carbodiimide.

Methods for the immobilization of aminotransferases involving the covalent binding of enzyme through its carboxyl groups to primary

[11] O. L. Krampitz and C. H. Werkman, *Biochem. J.* **35,** 595 (1941).
[12] S. S. Lee, R. H. Burris, and P. W. Wilson, *Proc. Soc. Exp. Biol. Med.* **50,** 96 (1942).
[13] G. W. E. Plaut and H. A. Lardy, *J. Biol. Chem.* **180,** 13 (1949).
[14] G. Edwards, J. Heier, and J. D. Rozzell, in preparation.

amines on the surface of an inert support gave immobilized aminotransferases of high activity. Typical supports include porous glasses, porous ceramics, and porous diatomaceous earths. Oxaloacetate decarboxylase can be immobilized using similar procedures, also with good retention of catalytic activity.

The following are sample procedures for the immobilization of various preparations of aminotransferase and oxaloacetate decarboxylase and procedures for assaying the immobilized enzymes.

Immobilization of Glutamic-Oxaloacetic Aminotransferase from E. coli for the Production of L-*Phenylalanine*. Controlled-pore glass with an average pore size of 500 Å is converted to the aminopropyl form by reaction with triethoxy-3-aminopropylsilane using the aqueous procedure of Weetall.[15] Aminopropyl glass (2.0 g) is added to 10 ml of a solution of sodium borate (5 m*M*) containing pyridoxal 5′-phosphate (0.5 m*M*). The pH is adjusted to 7.0, and 50 mg of a lyophilized glutamic-oxaloacetic aminotransferase from *E. coli,* which has been partially purified to a specific activity of approximately 2.5 units/mg (when assayed for the transamination of phenylpyruvate to L-phenylalanine) is added. After dissolution of the enzyme, ethyl dimethylaminopropylcarbodiimide hydrochloride (100 mg) is added, and the reaction mixture is shaken at room temperature for 45 min on a rotary shaker. At the end of this time the suspension is poured into a funnel with a glass frit (coarse porosity) and suction filtered. The support is washed successively with three portions of potassium phosphate buffer (50 m*M*, pH 7.0), three portions of 200 m*M* NaCl, and three more times with phosphate buffer. The immobilized biocatalyst has an activity of 120 units (60 units/g) when assayed for the transamination of phenylpyruvate to L-phenylalanine with L-aspartic acid as the amino donor. The activity retained on immobilization is approximately 95%.

Assays for the activity of free aspartic aminotransferase are carried out by a method similar to that described by Mavrides and Orr.[8] In a typical assay procedure, 0.700 ml potassium phosphate buffer (50 m*M*, pH 7.0), 0.100 ml L-aspartate (pH 7.0, 200 m*M*), 0.100 ml 2-ketoacid solution (pH 7.0, 100 m*M*), 0.030 ml NADH solution (5 mg/ml in H_2O), 0.050 ml malate dehydrogenase solution (1000 U/ml in 50 m*M* potassium phosphate buffer, pH 7.0), and 0.010 ml pyridoxal 5′-phosphate (pH 7.0, 10 m*M*) are pipetted into a cuvette. A background rate is determined by monitoring the change in the absorbance at 340 nm as a function of time, and the reaction is initiated by the addition of the aminotransferase sam-

[15] H. H. Weetall, this series, Vol. 34, p. 59.

ple (0.010 ml). Activity of the enzyme is calculated by the following equation:

$$\text{Activity (International Units/ml)} = (\Delta OD_{340}/\text{min})(100/6.2)$$

To assay the immobilized enzyme for activity, a weighed amount of immobilized enzyme (approximately 20–40 mg) is added to 1.0 ml of a solution containing potassium phosphate buffer (50 m*M*, pH 7.0), L-aspartic acid monosodium salt (100 m*M*), phenylpyruvic acid sodium salt (100 m*M*), and pyridoxal 5′-phosphate (0.1 m*M*). Aliquots of 10 μl are taken at 1-min time intervals and diluted into 990 μl of 2.5% NaOH (w/v). After mixing, 100 μl of this solution is added to a cuvette containing 900 μl of 2.5% NaOH, and the absorbance at 320 nm is read. The concentration of phenylpyruvate remaining at 1-min time intervals is calculated (ε_{320} = 17.5 mM^{-1} cm^{-1}) and from this data an aminotransferase activity can be calculated using the following equation:

$$\text{Activity (International Units)/g} = \frac{\Delta OD_{320}}{\text{min}} \frac{1000}{17.5 \times \text{grams of immobilized enzyme}}$$

An alternative assay is based on the quantitation of total oxaloacetate + pyruvate produced as a result of transamination (a small amount of pyruvate is produced by the spontaneous decarboxylation of oxaloacetate) by stoichiometric reduction with NADH using a combination of malic dehydrogenase and lactic dehydrogenase. The incubation of immobilized aminotransferase and substrates is carried out as above, and 20-μl aliquots are removed at 2-min time intervals and diluted into a cuvette containing 850 μl of 50 m*M* potassium phosphate buffer (pH 7.0), 50 μl of a 1000 unit/ml solution of lactate dehydrogenase, 50 μl of a 1000 unit/ml solution of malate dehydrogenase, and 30 μl of a 5 mg/ml solution of NADH. The net change in the absorbance at 340 nm is a measure of the consumption of NADH and therefore the total oxaloacetate + pyruvate produced (ε_{340} = 6.2 mM^{-1} cm^{-1}). Activity of the immobilized enzyme is calculated by the following equation:

$$\text{Activity (International Units)/g} = \frac{\Delta OD_{340}}{\text{min}} \frac{50}{6.2 \times \text{grams of immobilized enzyme}}$$

A small background correction must be made for the reduction of phenylpyruvate, which is a poor substrate for lactate dehydrogenase.

Immobilization of Glutamic-Oxaloacetic Aminotransferase from Porcine Heart. Aminopropyl controlled-pore glass prepared as described above (0.500 g) is suspended in 5 ml of sodium borate solution (5 m*M*) containing pyridoxal 5′-phosphate (0.5 m*M*) and the pH is adjusted to approximately 7. Glutamic-oxaloacetic aminotransferase (14 mg, specific activity 18.2 units/mg) is dissolved in the solution, followed by the addition of ethyl dimethylaminopropylcarbodiimide hydrochloride (50 mg). The reaction mixture is agitated at room temperature on a rotary shaker for 60 min, after which time assays have shown negligible aminotransferase activity in the solution. The controlled-pore glass particles are transferred to a coarse frit funnel and washed repetitively with 10-ml portions of water, potassium phosphate buffer (pH 7.0), three times with 0.2 *M* NaCl, and finally with phosphate buffer. The combined washings contain 8.8 mg of protein. The immobilized aminotransferase activity is 50 units when assayed for the transamination of L-aspartate and 2-ketoglutarate. The enzymatic activity retained after immobilization is 53%.

The assay for aminotransferase activity is carried out as described earlier for the quantitation of oxaloacetate + pyruvate produced using NADH, lactate dehydrogenase, and malate dehydrogenase.

Immobilization of Glutamic-Pyruvic Aminotransferase from Porcine Heart. Aminopropyl controlled-pore glass prepared as described above (0.500 g) is suspended in 5 ml of sodium borate solution (5 m*M*) containing pyridoxal 5′-phosphate (0.5 m*M*). The pH is adjusted to 7, and 30 mg of glutamic-pyruvic aminotransferase having a specific activity of 51 units/mg is dissolved in the solution. Ethyl dimethylaminopropylcarbodiimide hydrochloride (50 mg) is added, and the reaction mixture is shaken on a rotary shaker for 60 min at room temperature. At the end of this time, the reaction mixture is transferred to a glass frit funnel (coarse porosity) and the porous glass particles are washed with water, potassium phosphate buffer (50 m*M*, pH 7.0), three times with NaCl (200 m*M*), and again with potassium phosphate buffer. The combined washings contain 20 mg of protein (assayed by the method of Bradford[16]), indicating that 10 mg has been retained on the support. The activity of the immobilized enzyme was 200 units/0.5 g of support. The enzymatic activity retained after immobilization is 40% of the activity of the same quantity of native enzyme in solution.

Activity of the immobilized enzyme can be determined by quantitating the amount of pyruvate remaining in the reaction mixture after fixed times. A weighed amount of immobilized enzyme (approximately 20 mg) is suspended in a solution containing sodium pyruvate (100 m*M*), mono-

[16] M. Bradford, *Anal. Biochem.* **72,** 248 (1976).

sodium L-glutamate (100 m*M*), pyridoxal 5′-phosphate (0.1 m*M*), and potassium phosphate (50 m*M*). The pH of the solution is 7.

Aliquots of 0.020 ml are removed at 1.0-min intervals and diluted into 0.180 ml of potassium phosphate buffer (50 m*M*). Ten microliters of this solution is added to a cuvette containing 0.900 ml potassium phosphate (pH 7.0, 50 m*M*), 0.050 ml of NADH solution (5 mg/ml in H_2O), and 0.040 ml lactate dehydrogenase (1000 U/ml in potassium phosphate buffer, pH 7.0, 50 m*M*). The net change in the absorbance at 340 nm is measured, and the average ΔOD_{340} per minute is calculated. Activity of immobilized glutamic-pyruvic aminotransferase is calculated by the following equation:

$$\text{Activity (International Units)/g} = \frac{\Delta OD_{340}}{\text{min}} \frac{500}{6.2 \times \text{grams of immobilized enzyme}}$$

An alternative assay for the activity of immobilized glutamic-pyruvic aminotransferase is described below which measures the amount of 2-ketoglutarate produced as a function of time. Approximately 10 mg of immobilized enzyme is suspended in 1.0 ml of a solution containing monosodium L-glutamate (100 m*M*), sodium pyruvate (100 m*M*), potassium phosphate buffer (pH 7.0, 50 m*M*), and pyridoxal 5′-phosphate (0.1 m*M*). Aliquots of 10 μl are withdrawn at 1-min time intervals and diluted into a cuvette containing 800 μl of potassium phosphate buffer (pH 7.0), 50 μl of a 200 unit/ml solution of saccharopine dehydrogenase (Sigma Chemical Co., St. Louis, MO), 100 μl of a 100 m*M* solution of L-lysine, and 40 μl of a 5 mg/ml solution of NADH. The net change in the absorbance at 340 nm is determined ($\varepsilon_{340} = 6.2$ mM^{-1} cm^{-1}).

Immobilization of Oxaloacetate Decarboxylase from Pseudomonas putida. Cells of *Pseudomonas putida* are grown, harvested, and the enzyme purified as described elsewhere.[12] Aminopropyl glass (0.500 g) prepared as described earlier is suspended in 5 ml of 5 m*M* sodium borate buffer containing 10 m*M* $MgCl_2$, and 45 mg of a partially purified oxaloacetate decarboxylase having a specific activity of 30 units/mg is added. Ethyl dimethylaminopropylcarbodiimide hydrochloride (125 mg) is added and the reaction mixture is agitated on a rotary shaker for 60 min at room temperature. Assays have shown that by this time the decline of oxaloacetate decarboxylase activity in the supernatant (due to immobilization of the enzyme on the support) has slowed significantly. Independent controls show a negligible decrease in activity of the OAD due to chemical modification by the carbodiimide. The reaction mixture is transferred to a funnel with a glass frit (coarse porosity), and the immobilized

enzyme is washed with water, three times with Tris–HCl buffer (50 m*M*, pH 8.0), containing $MgCl_2$ (10 m*M*), three times with NaCl solution (200 m*M*), and finally with Tris buffer. The combined washings contain 14 mg protein. The immobilized oxaloacetate decarboxylase contain 275 units/500 mg support. The enzymatic activity retained after immobilization is 30%.

To determine the activity of immobilized oxaloacetate decarboxylase, a weighed amount of immobilized enzyme (approximately 10 mg) is added to 2.0 ml of a solution containing oxaloacetate (100 m*M*), Tris–HCl buffer (50 m*M*) with pH adjusted to 8.0, and $MgCl_2$ (10 m*M*). The mixture is shaken at 25°, 10-μl aliquots are withdrawn at 1- to 2-min intervals and diluted into a cuvette containing 990 μl of water, and the absorbance at 262 nm is measured. The net change in the absorbance at 262 nm corresponds to the consumption of oxaloacetate ($\varepsilon_{262} = 0.78$ mM^{-1} cm^{-1}). Activity of the immobilized enzyme is calculated by the following equation:

$$\text{Activity (International Units)/g} = \frac{\Delta OD_{262}}{\text{min}} \frac{100}{0.78 \times \text{grams of immobilized enzyme}}$$

Immobilization of Glutamic-Pyruvic Aminotransferase on Porous Diatomaceous Earth. Porous diatomaceous earth from Johns-Manville (R 640, 50/100 mesh) is boiled for 12 hr in 5% nitric acid and washed with deionized water; the fine particles are decanted, and dried for 12 hr in an oven at 110°. The support is then converted to the aminopropyl derivative by the aqueous activation procedure of Weetall.[15] Aminopropyl support (5.0 g) is suspended in sodium borate (5 m*M*) containing 2-ketoglutarate (0.5 m*M*), pyridoxal phosphate (0.5 m*M*), and 110 mg glutamic-pyruvic aminotransferase (specific activity 49 units/mg, Lee Scientific). Ethyl dimethylaminopropylcarbodiimide–HCl (250 mg) is added and the reaction mixture is placed on a rotary shaker for 2 hr at room temperature. At the end of this time, the support is transferred to a glass frit funnel (coarse porosity) and washed repeatedly with water and then 50 m*M* potassium phosphate buffer containing 0.1 m*M* pyridoxal phosphate. Assay for protein in the combined washings[16] has indicated that 80 mg of protein is bound to the support. The support contains 850 units of activity. The activity retained after immobilization is 22%.

Immobilization of Glutamic-Oxaloacetic Aminotransferase on Activated Porous Diatomaceous Earth. Aminopropyl porous diatomaceous earth (500 mg) prepared as described above is suspended in 2 ml of potas-

sium phosphate buffer (50 m*M*, pH 7.0) containing 2-ketoglutarate (2 m*M*), pyridoxal phosphate (0.1 m*M*), and 26 mg partially purified glutamic-oxaloacetic aminotransferase from porcine heart (specific activity of 14 units/mg). Ethyl dimethylaminopropylcarbodiimide hydrochloride (10 mg) is added and the reaction mixture is agitated on a rotary shaker for 1.5 hr. After washes as described above on a glass frit funnel with 50 m*M* phosphate buffer containing 0.5 *M* NaCl and finally phosphate buffer, 3.3 mg of protein is found to be bound to the support by quantitation of the recovered protein. Assay of the bound aminotransferase as described earlier gives 19 units of activity on the support. The specific activity of the immobilized enzyme is 41%.

Summary of Results. Using the carbodiimide method for immobilization, the three different aminotransferase (glutamic-oxaloacetic from porcine heart, glutamic-oxaloacetic from *E. coli,* and glutamic-pyruvic from porcine heart) could all be immobilized to porous supports bearing primary amine functional groups with good retention of activity. The results are summarized in Table II.

The stability to operational conditions is also quite high. For GPA immobilized on porous glass, the immobilized enzyme showed little loss of activity over 6 months of operation as described below.

Measurement of the Long-Term Stability of the Immobilized Enzymes. Measurements to determine the operational stability of immobilized enzyme were carried out by pumping substrate mixtures through a packed bed of biocatalyst at 25° and quantitating the amount of product in the effluent stream as outlined previously. In the case of glutamic-pyruvic aminotransferase, a solution of L-glutamate monosodium salt (200 m*M*), sodium pyruvate (400 m*M*), and pyridoxal phosphate (0.1 m*M*) with a pH between 7.0 and 7.5 was used as the substrate mixture. Activity of the

TABLE II
IMMOBILIZATION ON POROUS SUPPORTS

Enzyme	Support	Loading (mg/g)	Activity of immobilized enzyme (units/g)
GOA (*E. coli*)	Glass	25	60
GOA (porcine)	Glass	10	100
GPA	Glass	20	400
OAD	Glass	30	550
GPA	Diatomaceous earth	16	170
GOA	Diatomaceous earth	6	38

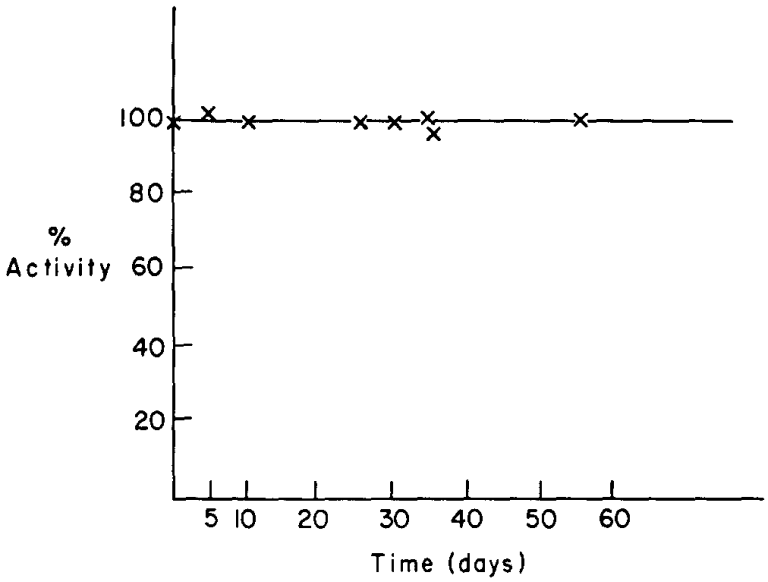


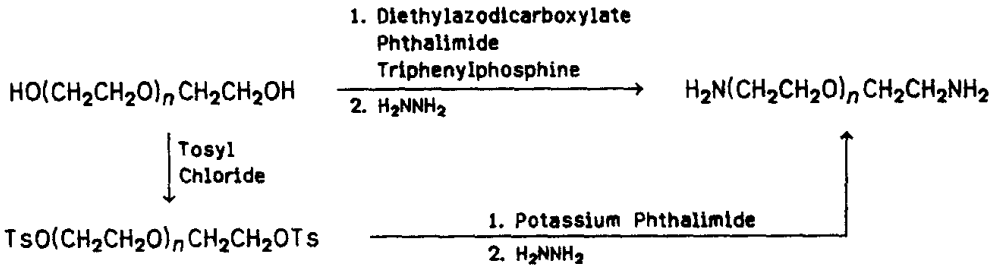
FIG. 1. Long-term operational stability of immobilized glutamic-pyruvic aminotransferase.

biocatalyst was determined by quantitating both the amount of 2-ketoglutarate produced and the amount of pyruvate consumed by the methods described earlier. The column was operated intermittently over 2 months for periods varying between 1 day and 1 week at a time. When not in use, the immobilized enzyme columns were stored at room temperature in substrate solution. The results of the experiments are shown in Fig. 1.

When immobilized glutamic-oxaloacetic aminotransferase from *E. coli* was mixed with immobilized oxaloacetate decarboxylase from *P. putida,* the resulting biocatalyst was shown to exhibit good stability and versatility for the production of a number of L-amino acids. As examples, L-glutamic acid, L-phenylalamine, L-tyrosine, L-tryptophan, L-2-aminoadipic acid, and L-4-phenyl-2-aminobutanoic acid could all be produced along with equimolar quantities of pyruvate using this two-enzyme system. Work to further expand the scope of this process is in progress.

Retention of the Pyridoxal 5′-Phosphate Cofactor in a Membrane Reactor

One of the ways in which the economics of the process just described could be improved would be to eliminate the requirement for continuous addition of the pyridoxal 5′-phosphate cofactor during the operation of the bioreactor. Because pyridoxal 5′-phosphate is a small molecule approximately the same size as an amino acid (MW = 247), retaining this molecule in a bioreactor using a membrane presents a problem. In the case



SCHEME 3. Synthesis of diaminopolyethylene glycol.

of nicotinamide cofactors, this problem of retention in a membrane reactor has been solved elegantly by Wandrey, Wichmann, Kula, Bückmann, and others[17–21] by covalently attaching the cofactor to a soluble polymer. The macromolecularized cofactor retains the majority of its catalytic activity after attachment to the polymer, and it can be retained along with enzymes in a bioreactor using a membrane which allows the passage of small molecule substrates and products (such as amino acids) but not higher molecular weight compounds.

We have successfully demonstrated a similar approach for retaining a catalytically active form of pyridoxal 5′-phosphate in a membrane reactor. Although a number of polymers can be used, we chose a modified polyethylene glycol of molecular weight 4000 or greater as the backbone polymer. The first step is the conversion of the polyethylene glycol to an α,ω-diamino derivative. This can be accomplished by one of two methods. The first method involves reacting the polyethylene glycol with *p*-toluenesulfonyl chloride to produce a ditosylate derivative, displacement of the tosylates with potassium phthalimide, and hydrazinolysis.[19,20] An alternative method uses the reaction of polyethylene glycol with diethylazodicarboxylate and triphenylphosphine in the presence of phthalimide[22] to product the α,ω-diphthalimido derivative directly, followed by hydrazinolysis or hydrolysis to produce the α,ω-diaminopolyethylene glycol. These two reaction schemes are illustrated in Scheme 3.

[17] R. Wichmann, D. Wandrey, A. F. Bückmann, and M.-R. Kula, *Biotechnol. Bioeng.* **23,** 2789 (1981).

[18] I. Urabe, N. Katayama, and H. Okeda, *Enzyme Eng.* **6,** 239 (1982).

[19] A. F. Bückmann, M.-R. Kula, R. Wichmann, and C. Wandrey, *J. Appl. Biochem.* **3,** 301 (1981).

[20] S. Furukawa, N. Katayama, T. Iizuka, I. Urabe, and H. Okada, *FEBS Lett.* **121,** 239 (1980).

[21] C. Wandrey, R. Wichmann, W. Leuchtenberger, M.-R. Kula, and A. Bückmann, *European Patent Application* 040,281.

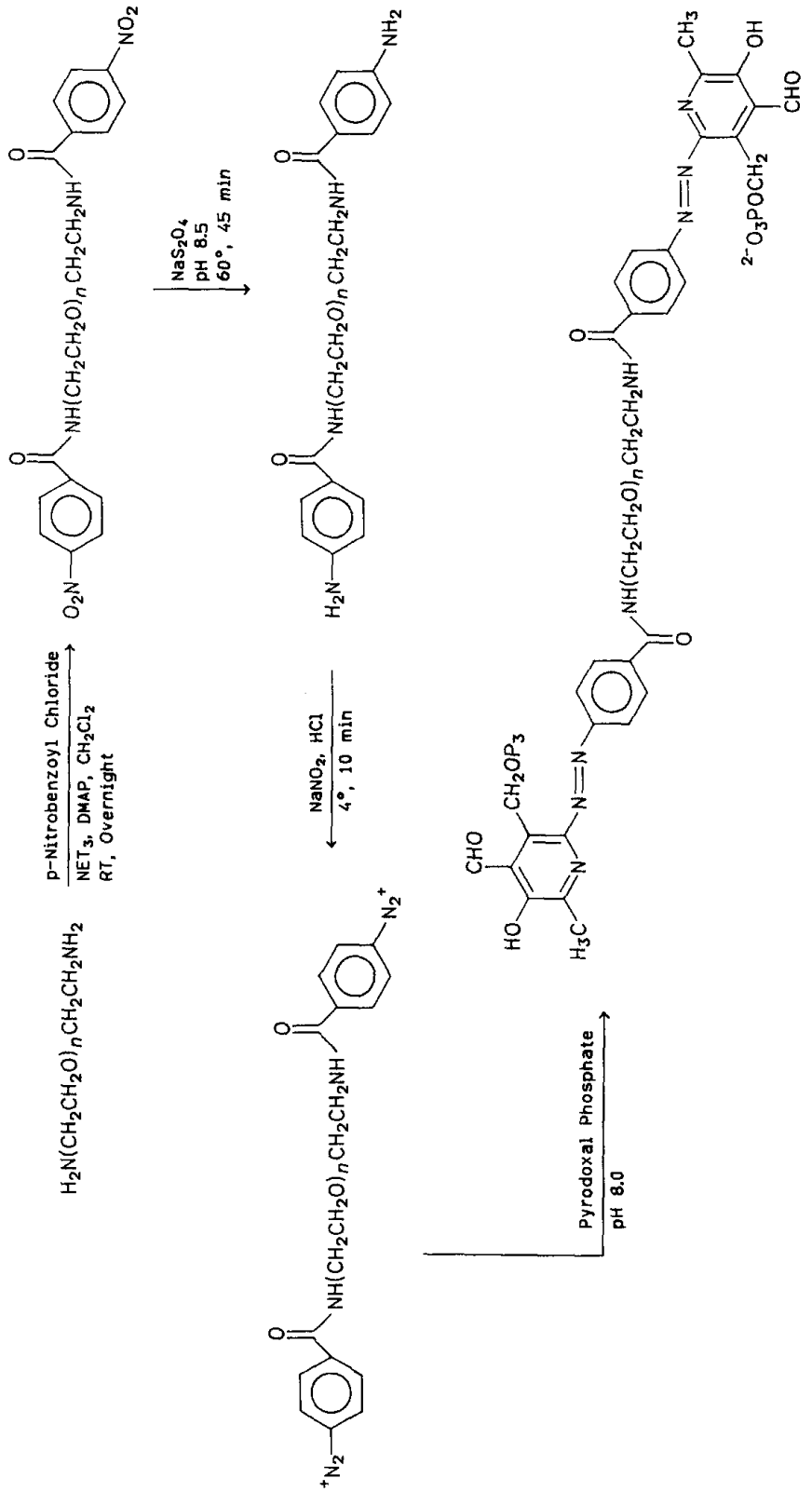
[22] O. Mitsunobu, M. Wada, and T. Sano, *J. Am. Chem. Soc.* **94,** 679 (1972).

Diaminopolyethylene glycol so produced is reacted with *p*-nitrobenzoyl chloride in the presence of the acylation catalyst 4-dimethylaminopyridine, followed by reduction of the nitro groups to amines with sodium dithionite. Diazotization of the bis-*p*-aminobenzoyl derivative is accomplished with HCl and $NaNO_2$,[23] generating the bisdiazonium derivative. Pyridoxal 5′-phosphate is very reactive in diazonium coupling reactions at pH values sufficiently basic to cause deprotonation of its phenolic hydroxyl group. Thus, incubation of the bisdiazonium derivative with pyridoxal phosphate at pH 8.0 produces a macromolecularized pyridoxal phosphate derivative in which the cofactor is attached to the modified polyethylene glycol polymer via a diazo linkage to the C-6 ring position. The synthesis of this derivatized, macromolecularized cofactor is outlined in Scheme 4.

The modified, macromolecularized pyridoxal phosphate has a maximum absorbance at 413 nm compared with 389.5 nm for the native cofactor, and when the stability of the modified cofactor to laboratory light was measured, it was found to be 20- to 30-fold more stable to photochemical decomposition at neutral pH values relative to unmodified cofactor.[24] The modified pyridoxal phosphate exhibited 65–80% of the activity of native pyridoxal phosphate when used under saturating conditions as a coenzyme with several enzymes including apoglutamic-oxaloacetic aminotransferase from porcine heart, apoaspartate aminotransferase from *E. coli*, and apotryptophanase from *E. coli*. The modified cofactor was used with an aminotransferase from *E. coli* in an ultrafiltration unit for 1 week with no loss in activity, producing L-phenylalanine from phenylpyruvic acid and L-aspartic acid. Procedures for the preparation and use of the macromolecularized pyridoxal 5′-phosphate cofactor are described below.

Synthesis of Diaminopolyethylene Glycol. Polyethylene glycol, MW_{av} 8000 (1.0 g), is dissolved in 10 ml dry CH_2Cl_2, and 50 mg phthalimide, which has been dried at 110° overnight, 90 mg triphenylphosphine, and 65 μl of diethylazodicarboxylate (Aldrich Chemical Co., Milwaukee, WI) are added. The reaction mixture is stirred at room temperature for 3 hr under a nitrogen atmosphere. At the end of this time, the contents of the reaction are poured into 150 ml anhydrous diethyl ether, and the flocculent white precipitate is collected and air dried. This product is then dissolved in 20 ml deionized water, and 1.1 ml of hydrazine hydrate is added. The resulting solution is refluxed for 18 hr. After cooling, the reaction mixture is extracted three times with CH_2Cl_2, and the extracts are combined and

[23] P. Cuatrecasas, *J. Biol. Chem.* **245,** 3059 (1970).
[24] C. Y. W. Ang, *J. Assoc. Off. Anal. Chem.* **62,** 1170 (1979).



SCHEME 4. Synthesis of macromolecularized pyridoxal phosphate.

dried over anhydrous Na_2SO_4. The diaminopolyethylene glycol is precipitated with anhydrous diethyl ether, and the precipitate is collected by suction filtration and air dried. Redissolution in CH_2Cl_2 and reprecipitation by the addition of diethyl ether yield a colorless product with a 65% conversion of free alcohols to amine end groups when assayed using trinitrobenzenesulfonic acid.[25]

Synthesis of Pyridoxal 5'-Phosphate Attached to a Soluble Polyethylene Glycol Derivative. Diaminopolyethylene glycol (0.5 g), prepared as described above, is dissolved in 15 ml anhydrous CH_2Cl_2, and *p*-nitrobenzoyl chloride (60 mg), *p*-dimethylaminopyridine (10 mg), and triethylamine (50 μl) are added. The reaction mixture is stirred at room temperature overnight under a nitrogen atmosphere. After the reaction is complete, saturated $NaHCO_3$ is added and the mixture is allowed to stand for 30 min. The CH_2Cl_2 layer is separated, washed three times with 15-ml portions of deionized water, and dried over anhydrous Na_2SO_4. The bis-*p*-nitrobenzoyldiaminopolyethylene glycol was precipitated by the addition of 200 ml of anhydrous diethyl ether. Assay for primary amines using trinitrobenzenesulfonic acid[25] indicates at 99% conversion of available amines. The recovery of product is 374 mg.

The bis-*p*-nitrobenzoyl derivative is dissolved in 20 ml of 0.5 *M* $NaHCO_3$, pH 8.5, and 450 mg of 85% $Na_2S_2O_4$ (Sigma) is added. The reaction mixture is stirred at 40° for 1 hr. The resulting solution is dialyzed for 36 hr against 4 liters of deionized water (three changes). Assay for primary amines as before has indicated a 61% conversion of nitro groups to aromatic amines. The bis-*p*-aminobenzoyldiaminopolyethylene glycol product is lyophilized and stored at 4° until needed.

Bis-*p*-aminobenzoyldiaminopolyethylene glycol (150 mg) is reacted with 2 ml of 0.5 *M* HCl at 4°, and 70 mg $NaNO_2$ in 0.5 ml H_2O prechilled to 4° is added. The reaction mixture is stirred for 7 min at 4°, and pH is immediately adjusted to 8.0 by the addition of chilled 0.5 *M* $NaHCO_3$ and NaOH, and 40 mg of pyridoxal 5'-phosphate is added. The resulting orange solution is stirred at 4° for 12 hr protected from light. The reaction mixture is dialyzed against 2 liters of deionized water (three changes), and the product is isolated by lyophilization. The derivatized pyridoxal 5'-phosphate has a reddish-orange color, and the absorbance maximum is 413.5 nm.

Use of the Macromolecularized Pyridoxal 5'-Phosphate Cofactor Derivative in a Continuous Flow Membrane Reactor. For membrane bioreactor studies, an Amicon 8MC ultrafiltration apparatus with a 100-ml reservoir and a 2-ml reaction changer was used. The reservoir was period-

[25] R. Fields, *Biochem. J.* **124,** 581 (1978).

ically refilled with substrate solution over the course of the experiment. An Amicon YM5 membrane having a molecular weight cutoff of approximately 5000 was used in the membrane reactor.

A substrate solution containing potassium phosphate buffer (50 m*M*, pH 7.0) sodium phenylpyruvate (50 m*M*), and L-aspartate (50 m*M*) was prepared, and 100 ml of this solution was used to fill the reservoir of the ultrafiltration unit. A solution of 0.5 ml of macromolecularized pyridoxal phosphate derivative prepared as above (concentration 8 mg/ml) containing 10 units of aspartic aminotransferase from *E. coli* and 1.5 ml of substrate solution were mixed in the reaction chamber. The flow through the membrane reactor was initiated by nitrogen gas pressure, and the solution in the reaction chamber was stirred continuously. The flow rate was maintained at 2 ml/hr. During continuous operation over 1 week the average conversion of phenylpyruvate to L-phenylalanine was 78–80%, as determined by quantitation of both phenylpyruvate consumed and pyruvate + oxaloacetate produced. The assays were carried out as described earlier.

[45] Phenylalanine Production via Polyazetidine-Immobilized *Escherichia coli*: Optimization of Cell Loading

By G. J. CALTON, L. L. WOOD, and M. L. CAMPBELL

The use of polyazetidine-immobilized microbial cells for the production of specialty chemicals has been commercialized as a result of the excellent economic and engineering characteristics this catalyst provides. These advantages include high retention of activity, excellent enzyme stability of the immobilized cell, high flow rate, and support rigidity which allows expanded column configurations.[1] Immobilization of a number of different microbes has been accomplished with increased lifetimes and/or retention of activity.[2]

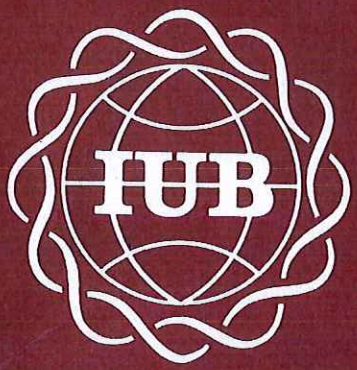
The potential use of phenylpyruvate as a starting material for the production of phenylalanine has been examined by a number of investigators. Ziehr *et al.* have used reductive amination as well as the trans-

[1] L. L. Wood and G. J. Calton, *Biotechnology* **1**, 1091 (1985).
[2] G. J. Calton, L. L. Wood, M. H. Updike, L. Lantz II, and J. P. Hamman, *Biotechnology*, in press (1986).

ATTACHMENT E: Enzyme Nomenclature 1978, published in 1979, Academic Press, New York, pp. 234-239.

ENZYME NOMENCLATURE

Recommendations (1978)
of the Nomenclature Committee
of the International Union of Biochemistry



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ENZYME NOMENCLATURE
1978

RECOMMENDATIONS OF THE NOMENCLATURE COMMITTEE OF THE INTERNATIONAL UNION OF BIOCHEMISTRY ON THE NOMENCLATURE AND CLASSIFICATION OF ENZYMES

This edition is a revision of the Recommendations (1972) of the IUPAC–IUB Commission on Biochemical Nomenclature, and has been approved for publication by the Executive Committee of the International Union of Biochemistry.

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80 81 82 9 8 7 6 5 4 3 2

Number	Recommended Name	Reaction

While the systematic name always includes 'hydrolase', the recommended name is, in most cases, formed by the name of the substrate with the suffix *-ase*. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

3.1 ACTING ON ESTER BONDS

The esterases are subdivided into those acting on carboxylic esters (3.1.1), thiolesterases (3.1.2), phosphoric monoester hydrolases, the phosphatases (3.1.3), phosphodiester hydrolases (3.1.4), triphosphoric monoester hydrolases (3.1.5), sulphatases (3.1.6), and diphosphoric monoesterases (3.1.7). The nucleases, previously included under 3.1.4, are now placed in a number of new sub-groups: the exonucleases (3.1.11-16) and the endonucleases (3.1.21-31).

3.1.1 CARBOXYLIC ESTER HYDROLASES

3.1.1.1	Carboxylesterase	A carboxylic ester + H_2O = an alcohol + a carboxylic acid anion
3.1.1.2	Arylesterase	A phenyl acetate + H_2O = a phenol + acetate
3.1.1.3	Triacylglycerol lipase	Triacylglycerol + H_2O = diacylglycerol + a fatty acid anion
3.1.1.4	Phospholipase A_2	A lecithin + H_2O = 1-acylglycerophosphocholine + an unsaturated fatty acid anion
3.1.1.5	Lysophospholipase	A lysolecithin + H_2O = glycerophosphocholine + a fatty acid anion
3.1.1.6	Acetylesterase	An acetic ester + H_2O = an alcohol + acetate
3.1.1.7	Acetylcholinesterase	Acetylcholine + H_2O = choline + acetate
3.1.1.8	Cholinesterase	An acylcholine + H_2O = choline + a carboxylic acid anion

...mber	Other Names	Basis for classification (Systematic Name)	Comments	Reference
3.1.1.1	Ali-esterase, B-esterase, Methylbutyrase, Monobutyrase, Cocain esterase, Procaine esterase	Carboxylic-ester hydrolase	Wide specificity. Also hydrolyses vitamin A esters	133, 202, 288, 425, 1371, 2874
3.1.1.2	A-esterase, Paraoxonase	Aryl-ester hydrolase	Acts on many phenolic esters; the enzyme from sheep serum also hydrolyses paraoxon	47, 136, 2056, 347
3.1.1.3	Steapsin, Tributyrase, Triglyceride lipase, Lipase	Triacylglycerol acylhydrolase	The pancreatic enzyme acts only on an ester-water interface; the outer ester links are preferentially hydrolysed	1764, 2023, 2911, 3081
3.1.1.4	Lecithinase A, Phosphatidase, Phosphatidolipase	Phosphatide 2-acylhydrolase	Also acts on phosphatidylethanolamine, choline plasmalogen and phosphatides, removing the fatty acid attached to the 2-position	672, 736, 919, 1175, 2272, 2895
3.1.1.5	Lecithinase B, Lysolecithinase, Phospholipase B	Lysolecithin acylhydrolase		574, 665, 855, 3527
3.1.1.6	C-esterase (in animal tissues)	Acetic-ester acetylhydrolase		47, 268, 1499
3.1.1.7	True cholinesterase, Choline esterase I, Cholinesterase	Acetylcholine acetylhydrolase	Acts on a variety of acetic esters; also catalyses transacetylations.	134, 269, 1928, 2324, 3852, 540
3.1.1.8	Pseudocholinesterase, Butyrylcholine esterase, Choline esterase II (unspecific), Benzoylcholinesterase	Acylcholine acylhydrolase	Acts on a variety of choline esters and a few other compounds	134, 136, 1732, 2324, 2931, 3259

Number	Recommended Name	Reaction
[3.1.1.9	*Deleted entry: Benzoylcholinesterase: a side reaction of EC 3.1.1.8]*	
3.1.1.10	Tropinesterase	Atropine + H_2O = tropine + tropate
3.1.1.11	Pectinesterase	Pectin + n H_2O = n methanol + pectate
[3.1.1.12	*Deleted entry: previously Vitamin A esterase, now believed to be identical with EC 3.1.1.1]*	
3.1.1.13	Cholesterol esterase	A cholesterol ester + H_2O = cholesterol + a fatty acid anion
3.1.1.14	Chlorophyllase	Chlorophyll + H_2O = phytol + chlorophyllide
3.1.1.15	Arabinonolactonase	L-Arabinono-γ-lactone + H_2O = L-arabinonate
[3.1.1.16	*Deleted entry: This reaction was due to a mixture of muconolactone isomerase (EC 5.3.3.4) and 3-oxoadipate-enol-lactone hydrolase (EC 3.1.1.24)]*	
3.1.1.17	Gluconolactonase	D-Glucono-δ-lactone + H_2O = D-gluconate
3.1.1.18	Aldonolactonase	L-Gulono-γ-lactone + H_2O = gulonate
3.1.1.19	Uronolactonase	D-Glucurono-δ-lactone + H_2O= D-glucuronate
3.1.1.20	Tannase	Digallate + H_2O = 2 gallate
3.1.1.21	Retinol-palmitate esterase	Retinol palmitate + H_2O = retinol + palmitate
3.1.1.22	Hydroxybutyrate-dimer hydrolase	3-D-(3-D-Hydroxybutyryloxy)-butyrate + H_2O = 2 3-D-hydroxybutyrate
3.1.1.23	Monoacylglycerol lipase	Hydrolyses glycerol monoesters of long-chain fatty acids
3.1.1.24	3-Oxoadipate enol-lactonase	4-Carboxymethylbut-3-enolide(1,4) + H_2O = 3-oxoadipate
3.1.1.25	γ-Lactonase	γ-Lactone + H_2O = 4-hydroxyacid

Number	Other Names	Basis for classification (Systematic Name)	Comments	Reference
3.1.1.10		Atropine acylhydrolase	Also acts on cocaine and other tropine esters	1062, 2269
3.1.1.11	Pectin demethoxylase, Pectin methoxylase, Pectin methylesterase	Pectin pectylhydrolase		708, 1979, 2234
3.1.1.13		Sterol-ester acylhydrolase	Also acts on esters of some other sterols	442, 1290, 1781, 3318
3.1.1.14		Chlorophyll chlorophyllidohydrolase	Also catalyses chlorophyllide transfer, *e.g.* converts chlorophyll to methylchlorophyllide	1343, 1712
3.1.1.15		L-Arabinono-γ-lactone lactonohydrolase		3641
3.1.1.17	Lactonase	D-Glucono-δ-lactone lactonohydrolase		391, 803
3.1.1.18		L-Gulono-γ-lactone lactonohydrolase		415, 507
3.1.1.19		D-Glucurono-δ-lactone lactonohydrolase		3706
3.1.1.20		Tannin acylhydrolase	Also hydrolyses ester links in other tannins	776
3.1.1.21		Retinol-palmitate palmitohydrolase		2045
3.1.1.22		3-D-(3-D-Hydroxybutyryloxy)-butyrate hydroxybutyrohydrolase		688
3.1.1.23		Glycerol-monoester acylhydrolase		2636
3.1.1.24		4-Carboxymethylbut-3-enolide (1,4) enol-lactonohydrolase		1819
3.1.1.25		γ-Lactone hydroxyacylhydrolase	The enzyme is specific for 4-8 carbon γ-lactones. It does not hydrolyse simple aliphatic esters,	887, 888

Number	Recommended Name	Reaction
3.1.1.26	Galactolipase	2,3-Di-*O*-acyl-1-*O*-(β-D-galactosyl)-D-glycerol + 2 H_2O = 1-*O*-(β-D-galactosyl)-D-glycerol + 2 fatty acid anions
3.1.1.27	4-Pyridoxolactonase	4-Pyridoxolactone + H_2O = 4-pyridoxate
3.1.1.28	Acylcarnitine hydrolase	*O*-Acylcarnitine + H_2O = a fatty acid + L-carnitine
3.1.1.29	Aminoacyl-tRNA hydrolase	*N*-Substituted aminoacyl-tRNA + H_2O = *N*-substituted amino acid + tRNA
3.1.1.30	D-Arabinonolactonase	D-Arabinono-γ-lactone + H_2O = D-arabinonate
3.1.1.31	6-Phosphogluconolactonase	6-Phospho-D-gluconate δ-lactone + H_2O = 6-phospho-D-gluconate
3.1.1.32	Phospholipase A_1	A lecithin + H_2O = 2-acylglycerophosphocholine + a fatty acid anion
3.1.1.33	6-*O*-Acetylglucose deacetylase	6-*O*-Acetyl-D-glucose + H_2O = glucose + acetate
3.1.1.34	Lipoprotein lipase	Triacylglycerol + H_2O = diacylglycerol + a fatty acid anion
3.1.1.35	Dihydrocoumarin hydrolase	Dihydrocoumarin + H_2O = melilotate
3.1.1.36	Limonin-D-ring-lactonase	Limonoate D-ring-lactone + H_2O = limonoate
3.1.1.37	Steroid-lactonase	Testololactone + H_2O = testolate
3.1.1.38	Triacetate-lactonase	Triacetate lactone + H_2O = triacetate

	Number	Other Names	Basis for classification (Systematic Name)	Comments	Reference
				acetylcholine, sugar lactones or substituted aliphatic lactones, *e.g.* 3-hydroxy-4-butyrolactone; requires Ca^{2+}	
osyl)-D-glycerol osyl)-D-glycerol	3.1.1.26		2,3-Di-*O*-acyl-1-*O*(β-D-galactosyl)-D-glycerol acylhydrolase	Also acts on 2,3-di-*O*-acyl-1-*O*-(6-*O*-α-D-galactosyl-β-D-galactosyl)-D-glycerol, and phosphatidylcholine and other phospholipids	1270, 1321
4-pyridoxate	3.1.1.27		4-Pyridoxolactone lactonohydrolase		427
fatty acid +	3.1.1.28		*O*-Acylcarnitine acylhydrolase	Acts on higher fatty acid (C-6 to C-18) esters of L-carnitine; highest activity is with *O*-decanoyl-L-carnitine	2046
+ H_2O = RNA	3.1.1.29		Aminoacyl-tRNA aminoacylhydrolase		1538
=	3.1.1.30		D-Arabinono-γ-lactone lactonohydrolase		2546
+ H_2O =	3.1.1.31		6-Phospho-D-gluconate-δ-lactone lactonohydrolase		1624
a fatty acid	3.1.1.32		Phosphatidate 1-acylhydrolase		997
glucose +	3.1.1.33		6-*O*-Acetyl-D-glucose acetylhydrolase		765
lglycerol + a	3.1.1.34	Clearing factor lipase, Diglyceride lipase, Diacylglycerol lipase	Triacylglycero-protein acylhydrolase	Hydrolyses triacylglycerols in chylomicrons and low-density lipoproteins. Also hydrolyses diacylglycerol	878, 1118, 796, 2293, 2411
elilotate	3.1.1.35		Dihydrocoumarin lactonohydrolase	Also hydrolyses some other benzenoid γ-lactones	1784
O =	3.1.1.36		Limonoate-D-ring-lactone lactonohydrolase		2055
te	3.1.1.37		Testololactone lactonohydrolase		1351
acetate	3.1.1.38		Triacetolactone lactonohydrolase		1608

ATTACHMENT F: U.S. Patent 6,265,191

US006265191B1

(12) **United States Patent**
Mizusawa et al.

(10) **Patent No.: US 6,265,191 B1**
(45) **Date of Patent: Jul. 24, 2001**

(54) **IMMOBILIZATION OF PSEUDOMONAS LIPASE ON SURFACES FOR OIL REMOVAL**

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(73) Assignee: **The Clorox Company**, Oakland, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/110,341**

(22) Filed: **Aug. 20, 1993**

Related U.S. Application Data

(63) Continuation of application No. 07/583,225, filed on Sep. 14, 1990, now abandoned.

(51) **Int. Cl.**[7] **C12N 11/02**; C12N 11/10; C12N 9/20; D06M 16/00

(52) **U.S. Cl.** **435/177**; 435/134; 435/178; 435/198; 435/263; 435/264; 435/877

(58) **Field of Search** 435/134, 177, 435/178, 198, 263, 264, 877

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,858,854	1/1975	Win et al.	252/89
4,006,059	2/1977	Butler	435/126
4,307,151	12/1991	Yamauchi et al.	428/373
4,472,503	9/1984	Matsuo et al.	435/179 X
4,703,872	11/1987	Cornette et al.	222/158
4,707,291	11/1987	Thom et al.	252/174.12
4,909,962	3/1990	Clark	252/547

FOREIGN PATENT DOCUMENTS

0206390	12/1986	(EP) .
0253487	1/1988	(EP) .
0268456	5/1988	(EP) .
0375102	6/1989	(EP) .
1442418	12/1973	(GB) .
8901032	7/1988	(WO) .
88-09367	12/1988	(WO) .

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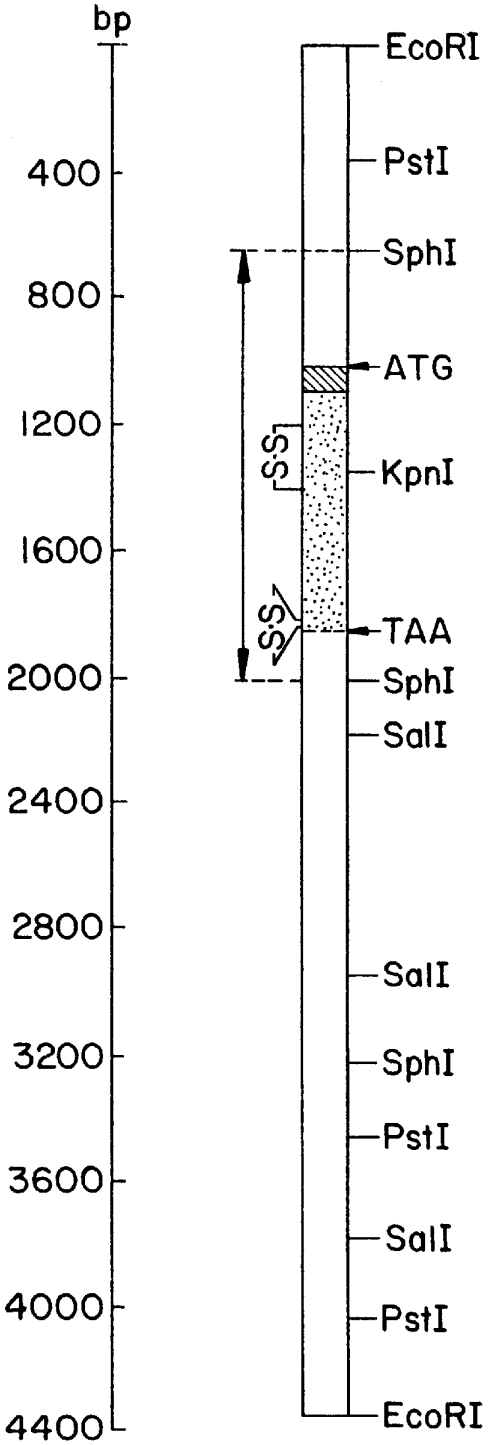
Ettinger, *Biochemistry*, (1987) 26:7883–7892.

Primary Examiner—David M. Naff
(74) *Attorney, Agent, or Firm*—Coudert Brothers

(57) **ABSTRACT**

Lipase is immobilized on surfaces to facilitate oil removal from the surfaces and to alter wettability of the surfaces. The lipase is isolatable from a Pseudomonas organism such as *Pseudomonas putida* ATCC 53552 or from an organism expressing a coding region found in or cloned from the Pseudomonas. A particularly preferred lipase has a molecular weight of about 30 to 35 kd and is resolvable as a single band by SDS gel electrophoresis. Lipase sorbed on fabric forms a fabric-lipase complex for oil stain removal. The lipase may be sorbed on fabric before or after an oil stain, and the lipase is active to hydrolyze an oil stain on dry fabric or fabric in laundering solutions. The sorbed lipase has enhanced stability to denaturation by surfactants and to heat deactivation, is resistant to removal from fabric during laundering, retains substantial activity after drying fabric at an elevated temperature, and retains activity during fabric storage or wear. Redeposition of oil and oil hydrolysis by-products during laundering of fabric is retarded by the lipase. Oil hydrolysis by-products are removable during laundering of fabric at a basic pH or in the presence of a surfactant.

22 Claims, 3 Drawing Sheets


bp
400
800
1200
1600
2000
2400
2800
3200
3600
4000
4400
EcoRI
PstI
SphI
ATG
S-S
KpnI
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SphI
PstI
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PstI
EcoRI

FIG. 1

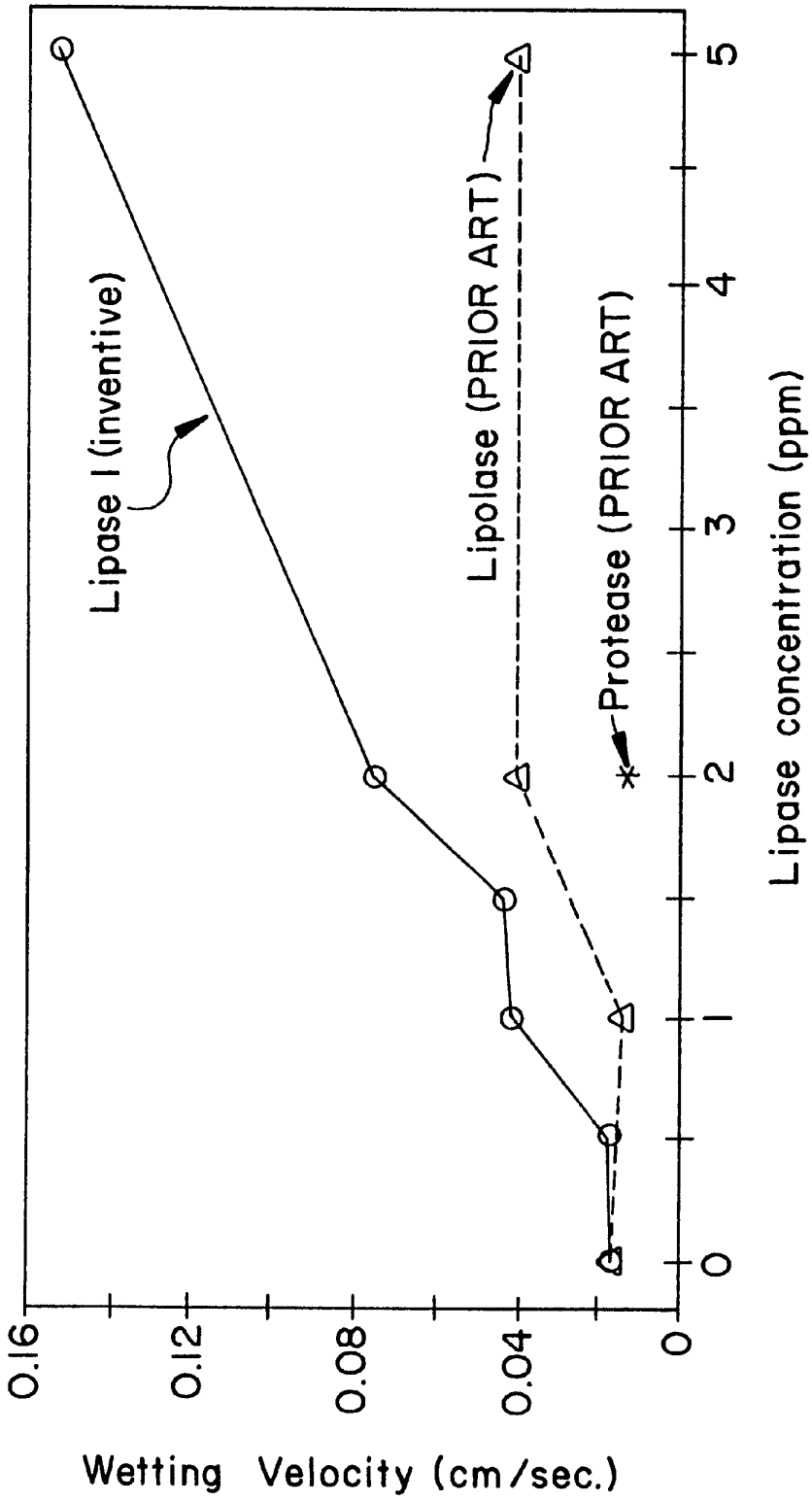
Lipase I (inventive)
Lipolase (PRIOR ART)
Protease (PRIOR ART)
Wetting Velocity (cm/sec.)
0.16
0.12
0.08
0.04
0
Lipase concentration (ppm)
0
1
2
3
4
5

FIG. 2

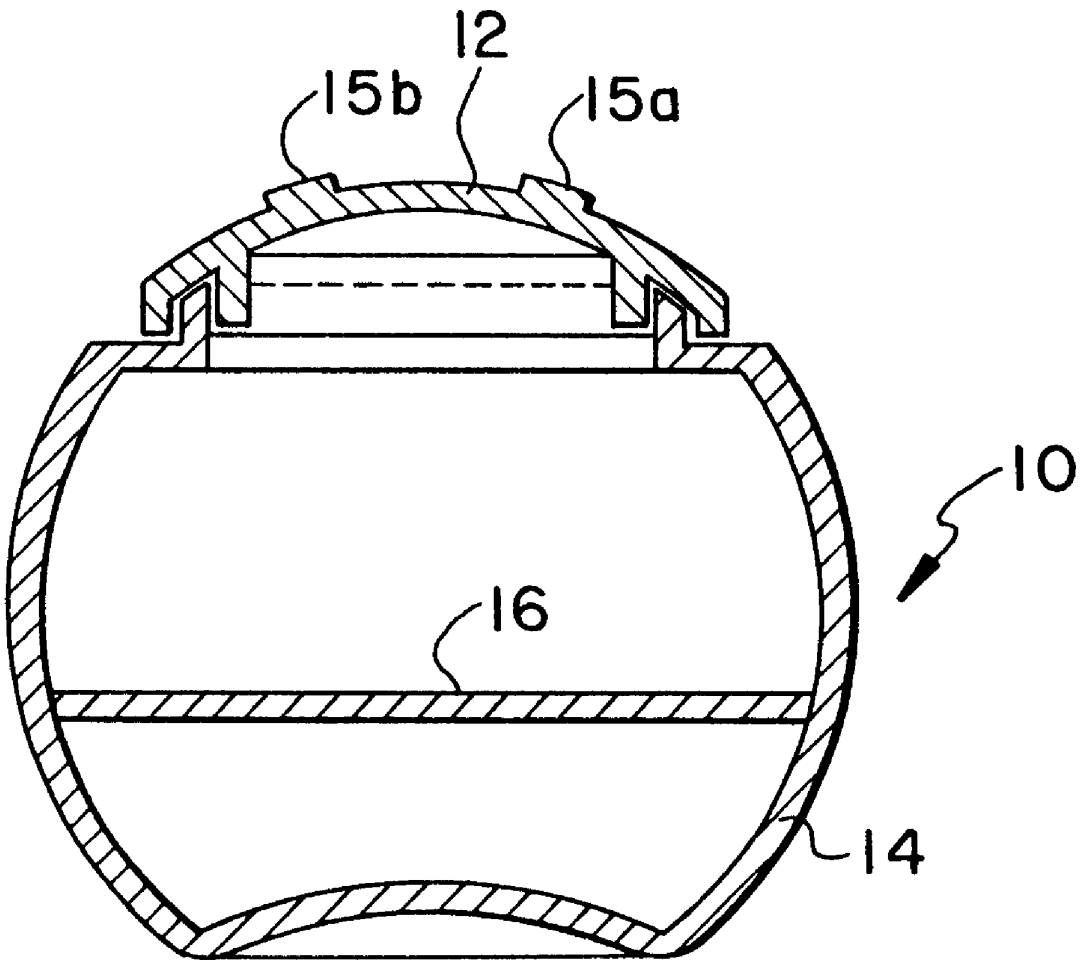

FIG. 3

IMMOBILIZATION OF PSEUDOMONAS LIPASE ON SURFACES FOR OIL REMOVAL

This is a continuation of application Ser. No. 07/583,225, filed Sep. 14, 1990.

FIELD OF THE INVENTION

The present invention relates to the field of use of lipases in laundry applications. More broadly, it relates to modification of surfaces such as for oil stain removal, improved wettability and anti-redeposition. More particularly, it relates to formation of hydrolase-fabric complexes which are stable and hydrolytically active during laundering, drying and use, and provide increased oil stain removal, wettability and anti-redeposition properties.

BACKGROUND OF THE INVENTION

Lipases are enzymes naturally produced by a wide variety of living organisms from microbes to higher eukaryotes. Fatty acids undergoing oxidation in tissues of higher animals must be in free form (that is, non-esterified) before they can undergo activation and oxidation. Thus, intracellular lipases function to hydrolyze the triacylglycerols to yield free fatty acids and glycerol. Enzymes useful in the present invention will be referred to as "lipases", but include enzymes described as being a "hydrolase" or "cutinase", as well as a "lipase", because the useful enzymes form hydrolysis by-products from oil substrates. All three terms and enzymes are contemplated and included by the use of the term "lipase" herein.

Bacterial lipases are classically defined as glycerolesterhydrolases (EC 3.1.1.3) since they are polypeptides capable of cleaving ester bonds. They have a high affinity for interfaces, a characteristic which separates them from other enzymes such as proteases and esterases.

Cutinases are esterases that catalyze the hydrolysis of cutin. For example, cutinase allows fungi to penetrate through the cutin barrier into the host plant during the initial stages of a fungal infection. The primary structures of several cutinases have been compared and shown to be strongly conserved. Ettinger, *Biochemistry.* 26, pp. 7883–7892 (1987). Sebastian et al., *Arch. Biochem. Biophys.,* 263 (1), pp. 77–85 (1988) have recently found production of cutinase to be induced by cutin in a fluorescent *P. putida* strain. This cutinase catalyzed hydrolysis of p-nitrophenyl esters of C_4–C_{16} fatty acids.

Because of this ability, lipases have long been considered as potential components in detergent compositions, and lipases obtained from certain Pseudomonas or Chromobacter microorganisms have been disclosed as useful in detergent compositions: Thom et al., U.S. Pat. No. 4,707, 291, issued Nov. 17, 1987 and Wiersema et al., European Patent Application 253,487, published Jan. 20, 1988. However, although lipases hydrolyze oil in solutions simulating laundry wash compositions, they have not proven to be very effective in removing oil stains from fabrics.

PCT application WO 88/09367 suggests the use of one of the lipases employed in the present invention in laundry applications. However, the method of use suggested merely comprises conventional use in laundry solutions or cleaning compositions. This lipase, so used by conventional methods, is no more effective than other lipases in removing oil stains from fabrics. Therefore, a need remains for effective utilization for the potential of lipases for removing oil stains in laundry applications.

Fabric treatments with non-enzyme compounds are known to alter the properties of fabric surfaces. For example, paralleling the development of durable-press and wash/wear fabrics, has been work on imparting oil and water repellency to fabrics. A widely used treatment utilizes a fluorochemical (sold by Minnesota Mining and Manufacturing Company under the mark Scotchgard) and another composition used for such fabric treatment is sold by E.I. du Pont de Nemours & Co. under the trademark Zepel. But oil and water repellant treated fabric have posed difficulties in removing stains by laundering, due to the fact that these repellant treatments make the fabric hydrophobic, and the oils forced onto such fabrics (particularly clothing at collar and cuffs) therefore are difficult to remove. One approach to this problem has been to treat the fabrics with soil release polymers. However, a need remains for imparting improved oil stain removal properties to surfaces, and particularly to fabrics exposed to significant oil staining, such as table cloths, aprons and clothing at body contact points such as collars and cuffs.

The use of lipases and/or cutinases in imparting oil hydrolysis activity during storage or wear has not been previously recognized.

When soil is released from fabric during laundering there is a further problem of redeposition of the oily soil on the previously cleaned fabric. This problem is well recognized. U.S. Pat. No. 4,909,962, issued Mar. 20, 1990, inventor Clark, attributes the redeposition of oily soil, in part, to phase separation (at least in the case of a pre-spotting composition when diluted with water in the wash bath). U.S. Pat. No. 4,919,854, issued Apr. 24, 1990, inventors Vogt et al., discloses detergent and cleaning preparations which include redeposition inhibitors described as water-soluble, generally organic, colloids (e.g. polymeric carboxylic acids and gelatin).

SUMMARY OF THE INVENTION

The present invention provides a novel use of the oil hydrolyzing potential of lipases for removing oil stains from fabrics more effectively than prior art attempts to utilize lipases for laundry cleaning applications.

In one aspect of the present invention, a method for modifying surfaces is provided to facilitate oil removal therefrom and comprises selecting a surface to be modified and then immobilizing (by chemical or physical means) an lipase onto the surface by forming a surface-lipase complex. The immobilized lipase is isolatable from Pseudomonas organisms. Suitable enzymes are lipases that are isolated from an organism expressing a coding region found in or cloned from *P. putida* ATCC 53552 or P. sp., more preferably from the *putida* species. A particularly preferred lipase is isolated with a molecular weight of about 30,000 daltons and is resolvable as a single band by SDS gel electrophoresis. The surfaces on which the enzyme is immobilized can be solid (e.g. glass) or can be fabrics (natural, synthetic, or metallic, woven or non-woven).

In another aspect of the present invention, a fabric is provided that is treated to have improved oil stain removal properties. The treated fabric has a lipase immobilized on the surface, forming a fabric-lipase complex. The fabric-lipase complex has substantial hydrolysis activity for oil stains during both subsequent use and laundering, and is resistant to removal during such use in laundering. Thus, although initial use of even the preferred lipases will not be effective for oil stain removal, the fabric-lipase-complex is effective for oil stain removal. The preferable lipase used to

form the fabric-hydrolase complex is isolated from *Pseudomonas putida* ATCC 53552, including modifications such as mutants or clones.

In yet another aspect of the present invention, a fabric treating composition, useful to improve oil stain removal of fabrics, comprises a solid or gelled carrier and the lipase described above. The lipase is dispersed in the carrier and can be applied to fabric, and once applied, the lipase sorbs and forms the fabric-lipase complexes.

Fabric having improved oil stain removal properties in accordance with the present invention can be repeatedly laundered without effective loss of such preparation because the lipase used is immobilized to the fabric, resists removal during laundering, and has substantial hydrolysis activity for oil stains on the fabric in both air and laundering solutions. The inventive treatments can be used to treat fabrics either before or after exposure to oily stains. The fabrics so treated need not be immediately laundered because the fabric-lipase complexes are hydrolytically active even on dry fabric in ambient air.

Other applications of the ability for the immobilized lipase to modify surfaces include uses to alter the wettability of the surface on which the lipase is sorbed. Thus, for example, solid plastic or glass surfaces having surface modifications in accordance with the invention may facilitate clog removal in plumbing, cleaning of windows, and other uses.

Other objects and advantages of the present invention will become apparent to persons skilled in the art upon reading the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** of this reference is a map of the 4.3kb *E. coli* fragment of a plasmid designated PSNE4, for a lipase useful in the present invention.

FIG. **2** graphically illustrates the increased wettability of polycotton fabrics when they are treated in accordance with the invention and contrasts this increased wettability with fabric washed in the presence of a prior art, commercially available lipase.

FIG. **3** is a sectional view of a vessel useful for generating a bleaching agent in accordance with the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Broadly viewed, the invention is a method for modifying surfaces by forming a lipase complex with the surface. One application of primary intent is to facilitate oil removal from or by a modified fabric surface. By "oil removal" is meant removal of oil which is deposited on the surface either before or after such surface modification, as well as the property of preventing or retarding redeposition of oil on the fabric such as during laundering. Surfaces that can be modified in accordance with the invention include glass, plastic, and metal solids as well as fabrics. Particularly preferred embodiments of the invention pertain to fabrics.

Thus, fabric treating compositions of the invention are useful to treat a wide variety of natural, synthetic or metallic fabrics whether viewed as textiles or woven or non-woven cloths. For example, among the different materials that have been treated in accordance with the invention so as to have sorbed enzyme on surfaces exposable to oils have been nylon, polycotton, polyester, woven polyester, double knit polyester, silk, vinyl, cotton flannel, rayon velvet, acrylic felt, wool blend (polyester/wool), synthetic blend (polyester/polyurethane), as well as pot cleaner materials such as cellulose sponge, nylon and stainless steel scrubbers and copper cloth.

The surfaces that have been treated in accordance with the invention can already be stained by (or carrying) oil before an enzyme-fabric complex is formed or the complex can be formed before such exposure. Examples of embodiments useful for the former applications include pre-wash liquid or gelled compositions that can be sprayed or directly applied to specific areas of oily stains. The garments or linens can then be stored in a laundry hamper, for example, and laundered in the normal course of a household's routine because degradation of the oily stain into hydrolysis by-products will be occurring during storage. Alternatively, fabric may be pretreated before use to convey improved oil stain removal properties.

Surfaces are modified in accordance with this invention by sorbing a lipase onto the surface. The sorbed lipase is isolatable from a Pseudomonas organism.

The suitable lipases can be viewed as glycerol ester hydrolases and are isolatable from certain Pseudomonas strains or from genetic modifications such as mutants or clones thereof. The particular Pseudomonas strains of interest are P. sp. and *P. putida* ATCC 53552, deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, on Oct. 15, 1986. It should be understood that the gene expressing the particular lipase of interest can be cloned into another organism, such as *E. coli* and *B. subtilis,* for higher levels of expression.

The previously noted European Patent Application 253, 487 of Wiersema et al. more fully describes the amino acid sequence of a specific suitable enzyme isolatable from the *P. putida* strain and further describes the cloning and expression of the gene coding for this enzyme. FIG. **1** of this reference is a map of the 4.3 kb *E. coli* fragment of a plasmid designated PSNE4 where the stippled region indicates the coding region (codons +1 to +258) for the mature polypeptide designated Lipase 1, which has a molecular weight of about 30,000 daltons and is resolvable as a single band by SDS gel electrophoresis. This EPA 253,487 is incorporated by reference, but for convenience the amino acid sequence of the specific enzyme ("Lipase 1") isolated from the *P. putida* strain is set out as follows:

Suitable enzymes can be modified with respect to the said amino acid primary structure.

Modifications preferably will be wherein the modified enzymes have an amino acid sequence substantially corresponding to the just-described lipase isolatable from *P. putida* ATCC 53552, but differing therefrom within certain parameters. Such preferred modifications are where there is at least one amino acid change occurring within (i) about 15 Å of serine 126, aspartic acid 176 or histidine 206 when the modified enzyme is in crystallized form or (ii) within about 6 amino acids of the primary structure on either side of serine 126, aspartic acid 176 or histidine 206. Such suitable modifications are as described in co-pending U.S. patent application Ser. No. 286,353, filed Dec. 19, 1988, now U.S. Pat. No. 5,108,457, entitled "Enzymatic Peroxyacid Bleaching System with Modified Enzyme", inventors Poulose and Anderson, which is incorporated herein by reference and is of common assignment herewith.

It is found that conventional initial washing with lipases, including the preferred lipases of the present invention, provides virtually no benefit over washing in the absence of lipase. The present invention nevertheless provides a method of employing lipases for effective removal of oil stains from fabric by utilizing a first wash cycle to form a

fabric-lipase complex, which remains active through subsequent drying and provides effective oil removal in subsequent wash cycles. An example of this is shown in Table 1 where no stain removal occurs in the first wash cycle, but does occur in subsequent cycles. Polycotton fabric swatches (65/35) were stained with triolein (5% by weight) and washed three times with two lipases of the invention. Table 1 summarizes the data of this study.

TABLE 1

	% Oil Stain Removal		
	1st Cycle	2nd Cycle	3rd Cycle
Lipase cloned from *P. putida*			
0 ppm	21	27	32
0.5 ppm	23	45	61
2.0 ppm	22	60	80
Lipase isolated from P. sp.			
0 ppm	21	27	32
0.5 ppm	21	37	46
2.0 ppm	20	44	56

As can be seen from the data summarized by Table 1, no oil stain removal is observed in the first cycle, while significant removal is observed in the second and third wash cycles.

Even increasing the enzyme concentration in the wash solution ten-fold to 20 ppm does not provide oil stain removal during initial use in the first cycle as might be expected. Surprisingly, however, the present invention provides significant oil stain removal in subsequent washings, even where no lipase is present in the subsequent wash cycles. This is demonstrated by Table 2.

Four replicate polycotton fabric swatches (2×2") were washed in 200 ml of 10 mM sodium carbonate containing 0.1 mM Neodol 25-9/0.2mM C_{12}LAS and various levels of lipase as indicated in Table 2. Wash solutions were at pH 10.5 and washed for 15 minutes at room temperature. Swatches were air dried before rewashing. Rewashing in cycles 2 and 3 were done without the addition of lipase.

TABLE 2

	Percent Soil Removal		
	Cycle 1	Cycle 2	Cycle 3
Control	15	23	27
Enzyme Treated:			
(2 ppm)	15	57	76
(5 ppm)	17	69	91
(10 ppm)	16	78	101
(20 ppm)	17	89	105

As is seen by the data of Table 2, polycotton fabric that had been treated with varying concentrations of lipase during the first laundering cycle demonstrated significant oil removal in the second laundering, and even better removal in the third laundering (where only surfactant was present in the second and third launderings). The data of Table 2 further shows that higher enzyme levels in the first cycle resulted in higher levels of oily stain removal in the second and third cycles. This demonstrates that oil removal observed in the second and third cycle is due to the presence of lipase in the first cycle. Furthermore, these data demonstrate that the lipase is adsorbed onto the fabric during cycle 1, and remains active and adsorbed through rinsing, drying, storage and use in cycles 2 and 3.

EXAMPLE 1

An experiment was performed that illustrates the use of lipase compositions to pretreat fabric before the fabric is exposed to oil. Three different enzyme concentrations for Lipase 1 were used to treat three separate sets of polyester/cotton (65/35) fabric swatches. The treatment consisted of washing four replicates in the wash solution described in Table 2 containing various lipases shown in Table 3. After air drying, each swatch was then stained with triolein (5 wt. % with respect to fabric weight). Control (untreated) swatches were similarly stained. The stained swatches were then washed once in a laundering simulation including detergent and the described levels of lipase. Table 3 summarizes the data.

TABLE 3

	% Stain Removal		
PreTreatment	(0.5 ppm)	(1.0 ppm)	(2.0 ppm)
Control(no lipase)	6	8	12
Lipase cloned from *P. putida*	20	26	33
Lipase P. sp	20	20	26
Novo Lipolase	9	7	11

As can be seen from the data summarized by Table 3, the fabrics pretreated (pretreated before oil exposure) with lipase cloned from *Pseudomonas putida* and the lipase isolated from Pseudomonas sp. resulted in about 2½ to almost 3 times better oil stain removal with respect to a control when both control and pretreated fabric were washed in a laundry simulation that included detergent and lipase.

The lipase-surface complexes have been shown to exhibit binding tenacity, and to retain activity binding on a broad spectrum of surfaces. This is illustrated in Table 4 where a wide variety of fabrics, several non-fabric woven surfaces, and several solid surfaces were soaked for 15 minutes in a buffered solution of lipase at pH 8. By calculation of the activity lost from solution, the amount of lipase sorbed onto the surfaces was determined. These fabrics and surfaces were then washed for 15 minutes in 5 mM phosphate at pH 8 and the amount of enzyme that had desorbed was similarly measured. Table 4 summarizes these sorption and desorption results.

TABLE 4

Fabric/ Surface Type	% sorbed from treating solution	% remaining sorbed after one washing
nylon	22	98
polycotton	32	92
grey polycotton	13	85
polyester	29	96
woven polyester	27	93
double knit polyester	53	97
silk (crepe de chenin)	8	87
vinyl	16	94
cotton flannel	19	93
rayon velvet	51	84
acrylic felt	38	100

TABLE 4-continued

Fabric/ Surface Type	% sorbed from treating solution	% remaining sorbed after one washing
polyester/wool	37	96
polyester /polyurethane	8	84
terry (85% cotton /15% polyester)	17	97
fleece (50% cotton /50% Polyester)	39	97
nylon pot cleaner	38	71
copper cloth pot cleaner	42	93
cellulose sponge	24	100
stainless pot cleaner	68	99
wax paper	17	99
unetched glass	39	99
etched glass	33	100
ABS pipe	22	1

As can be seen from this data, the lipase was sorbed from the treating solution, in varying amounts, depending on the surface, for a variety of different fabrics and surfaces. Furthermore, once sorbed the bound enzyme was substantially retained even after a 15 minute wash in phosphate buffer as described above. Four days after the laundering simulation, the enzyme activity of the surface-bound complexes was tested. All the examples summarized by the data of Table 4 were shown to be hydrolyticly active. This was demonstrated by contacting the lipase treated surfaces with p-nitrophenylbutryate, a substrate for the lipase, that is hydrolyzed to the yellow product p-nitrophenol.

Treating fabrics to improve oil stain removal in accordance with the invention normally begins by contacting the desired fabric with a lipase containing composition to sorb the lipase onto the fabric and to form fabric-lipase complexes. Factors which affect adsorbance of lipase onto surfaces include surface characteristics and solution components such as: surfactant composition, ionic strength, pH, and lipase concentration. The time of exposure of the surface to the lipase-containing solution also increases the amount of adsorbed lipase. We have found that adsorption is highest on polycotton fabric in the absence of surfactant, low ionic strength and alkaline pH. Under these preferred conditions, higher lipase concentrations in solution will provide higher adsorption of the lipase onto the fabric. In the presence of surfactants, mixtures of anionic/nonionic promote adsorption more efficiently than single surfactant systems.

Delivery of the lipase to the surface to form the surface-lipase complex can be effected in a number of ways. As previously discussed, one way is by contacting the surface with a lipase solution, either in by washing or spraying the surface with the solution. An example of a preferred aqueous solution suitable for application to fabric has a basic pH, most preferably pH 10.5, has the lipase preferably in an amount of about 20 ppm, and is buffered such as by 5 mM phosphate or 10 mM carbonate. Simply soaking or spraying such a composition on the fabric surfaces for which improved oil stain removal is desired will result in formation of fabric-lipase complexes with the desired laundering removal resistance and substantial hydrolysis activity already described.

Such delivery may be made prior to soiling, for instance as a finishing step in fabric manufacture, or in pretreatment of fabrics prior to use; or after soiling of the fabric. Localized treatment of oil stains prior to washing can be effected by spraying or by use of a solid or gelled carrier for the lipase in applications where the lipase is desired to be transferred to fabric by direct contact. For example, a consumer can use a gel stick applicator to directly apply the lipase to areas such as shirt collars. Various suitable solid, stick-like carrier compositions are illustrated in European Patent Application No. 86107435.9, published Dec. 30, 1986. For example, one preferred composition includes propylene glycol, nonylphenol ethoxylate, linear alcohol ethoxylate, dodecylbenzenesulfonic acid, and stearic acid. A particularly preferred embodiment for a solid or gelled carrier composition is as follows:

Component	Weight %
Propylene Glycol	42
Nonylphenol Ethoxylate	17
Linear Alcohol Ethoxylate	17
Polyethylene Glycol	2
Dodecylbenzenesulfonic Acid	6
Stearic Acid	10
Lipase	6

Although the reason the lipases of the present invention are not effective when merely added to a conventional laundry wash solution, but are effective when the surface-lipase complex of the present invention is formed, is not fully understood, it is believed, without being bound by this theory, that the structure of these lipases is altered to an active state when they are complexed to the surfaces. Therefore, a method of providing active lipase for use in a conventional laundry solution is also provided by the present invention. This comprises delivery of an article comprising a surface-lipase complex to the conventional wash solution. Such articles can include the lipase complexed with a fabric or non-fabric member. Preferably the non-fabric, particulate members are employed to provide adequate dispersion through the wash. Such particulate members should be hydrophobic surfaces onto which the lipases adsorb. Examples are stearate salts, methacrylate copolymers, hydroxybutylmethyl cellulose, and polyacrylamide resins.

The surface-lipase complex of the present invention preferably has the following characteristics: substantial hydrolysis activity during storage, enhanced stability compared to lipases in solution, and surface property modifications of the surface onto which it is immobilized. The following are examples illustrating these characteristics.

EXAMPLE 2

This example illustrates activity during storage. Polyester/cotton swatches were treated with a lipase containing solution to provide a fabric-lipase complex. The dry, treated swatches were soiled with triolein (5% by weight of fabric) and stored for two days at room temperature. The oil was then extracted from the swatches and the components of the extracted oil were determined by thin layer chromatography. This analysis showed that oleic acid, monoolein and diolein were present on the swatches. These products of lipolytic hydrolysis were not observed on "control swatches" (where there was no enzyme treatment prior to staining). The presence of oleic acid, monoolein, and diolein demonstrates that the fabric-lipase complex, in accordance with the invention, is active for hydrolysis of oily soil even on dry fabric.

EXAMPLE 3

The following experiments demonstrate that the inventive fabric-lipase complex displays enhanced stability towards:

A. High Temperatures

The bound lipase-fabric complexes retain activity despite drying of the laundered fabrics in hot (180° F.) dryers. This is illustrated by the data of Table 6.

TABLE 6

Drying conditions	% oil removed 3 Cycles
Inventive treated fabric - air dried	82
Inventive treated fabric - hot dryer	65
Control - air dried	20

As can be seen from the data of Table 6, although fabric dried three times in a hot dryer (following three launderings) did experience some enzyme activity loss with respect to an inventively treated fabric that was air dried, nonetheless oil removal for even the hot dried, inventively treated fabric was still over three times that of a control (untreated) fabric.

B. Surfactants

The lipase-surface complex has been shown to exhibit enhanced stability to denaturation by surfactants. This property can be useful in liquid formulations, for example, in conveying storage stability. Into a solution of surfactant and buffer an aliquot of hydrolase (Lipase 1) was incubated for 10 minutes at room temperature. The surfactant solution was 1 wt. % SDS, which was buffered by sodium carbonate to pH 10.5. The hydrolase was 2 ppm in solution. A second sample was similarly prepared except fabric was introduced into the surfactant/buffer solution before adding the aliquot of hydrolase. Both samples were then assayed for enzyme activity by removing aliquots at 2, 5, and 10 minutes and assaying for enzyme activity. In addition, the fabric from the second sample was removed and the fabric surface was assayed visually for yellow colored development after contacting with PNB.

We found that the first sample enzyme (which was simply in solution and incubated in the surfactant/buffer solution) was inactive at all time points tested. Similarly, the second sample had some enzyme remaining in solution (that had not sorbed to the fabric) and this solubilized hydrolase was also inactivated. But by contrast, assays of the fabric surface showed that the hydrolase having sorbed to the fabric surface remained active at all points of testing, including even after 10 minutes in the otherwise denaturing surfactant/buffer solution.

EXAMPLE 4

We have discovered that surfaces treated with lipase in accordance with the invention also causes a changed wetting characteristics of the surface. This is demonstrated for three surfaces:

A. Polycotton

Polycotton fabric treated with the lipases results in increased wetting velocity for that fabric when compared with untreated fabric. FIG. **2** shows the increased wettability of polycotton fabrics when treated in accordance with the invention. The FIG. **2** measurements were made using high speed videomicrography to observe and to measure the behavior of a water droplet as it contacts the fabric surface. The measurement of the contact angle as a function of time (msec) allows calculation of the velocity of wetting. Also shown in FIG. **2** is a comparison with polycotton that had been analogously treated with a commercially available Lipolase enzyme. Within the error of the experiments, the Lipolase enzyme treatment did not affect fabric wettability. A similar result (wettability not affected) was obtained in experiments involving a protease (commercially available as Savinase).

B. ABS Piping

These experiments used sessile drop shape analysis to evaluate the surface properties of ABS plastic pipe. The hydrolase solution used to contact the pipe surface was a solution containing 1 ppm hydrolase. After drying, the contact angle of a water drop as it spread over the pipe surface provided a measurement of the surface hydrophilicity. Table 7 summarizes the data.

TABLE 7

Treatment	Contact Angle
No hydrolase	66.7 ± 3°
Hydrolase	59.6°
	64.8°
	51.0°

Three different areas of the pipe were examined to test for homogeneity of sorption. The data suggests that hydrolase sorption was not homogeneous throughout the pipe surface, as can be inferred by the scatter in the contact angle measurements on the hydrolase treated pipe surface. No such scatter was observed on the surface of the untreated pipe. However, all three areas showed a lower contact angle with sorbed hydrolase. This lower contact angle indicates that the surface having sorbed hydrolase had become more hydrophilic and therefore was more easily wetted by water. This surface modification may provide preventative maintenance for drainage pipes.

C. Glass

Glass slides were also studied for sorption. Three compositions were prepared. The first composition was a control aqueous solution with 50 mM HPO_4^{--} buffer (pH 8.0). The second was a surface modifying composition of the invention to which 0.2 ppm lipase (isolated from a clone of *P. putida* organism) was added to the buffered control. The third composition was analogous to the second, but included 10 ppm of the lipase. The glass slides were soaked in one of the respective solutions for one hour, dried, and then the contact angle of a water drop as it spread over the glass slide surface was measured to indicate surface hydrophilicity. The slide soaked in the control solution had a contact angle of 53°, that soaked in the 0.2 ppm lipase composition had a contact angle of 44°, and that soaked in the 10 ppm lipase composition had a contact angle of 30°. These lower contact angles for glass surfaces treated in accordance with the invention indicate that the glass surfaces having sorbed hydrolase had become more hydrophilic and therefore the treated surfaces were more easily wetted by water. This characteristic may facilitate cleaning of surfaces such as floors, walls, tiles, mirrors, and window glass.

EXAMPLE 5

The fabric-lipase complex has also been shown to be effective in preventing redeposition of oily soils onto treated fabric surfaces. This is illustrated in this example.

Removal of oily soil from one fabric only to redeposit that oil (or its hydrolyzed derivatives) onto another, unsoiled

fabric during the wash is a particular problem in laundry containing mixed fabric types. Lipase 1 was shown to be useful as an anti-redeposition agent by the following example. 2" by 2" 100% cotton swatches were soiled with 95 mg of triolein. Two of these soiled swatches were then washed along with two clean polyester swatches (2" by 2") in a surfactant solution (0.3 mM C_{12}LAS/Neodol 25-9, 2:1 molar ratio) at pH 10.5 (buffered with 10 mM Na_2CO_3). The washes were at room temperature (25° C.) for a duration of 15 minutes. These swatches were then dried and oils on the swatches were measured gravimetrically by removing oil from the fabric with a solvent, evaporating the solvent, and weighing remaining oil. Following this procedure, the cotton swatches (originally soiled with 95 mg of triolein) retained 17 mg of triolein but the initially oil-free polyester swatches were found to have had 35 mg of triolein deposited onto them during the washing with soiled cotton swatches.

Two fabric treating methods using Lipase 1 were conducted. In the first fabric treating procedure the clean polyester swatches were pretreated with hydrolase by washing the clean polyester swatches in the above-described surfactant/carbonate solution but where the solution had added 1 ppm Lipase 1. After drying the clean polyester swatches were again washed in the presence of the oil stained cotton swatches as already described.

Another treatment procedure was where the 1 ppm Lipase 1 was simply added ("in situ") to the surfactant/carbonate wash while the oil stained cotton swatches were being washed along with the initially cleaned polyester swatches.

Table 8 demonstrates the control (no hydrolase treatment), the pretreatment, and the in situ treatment data following the procedures as have been just described.

TABLE 8

Hydrolase Treatment of Polyester Swatches	Cotton Swatch Oil Level Before Lauder-ing	Cotton Swatch Oil Level After Redeposition Laundering	Polyester Swatch Oil Level Before Laundering	Polyester Swatch Oil Level After Redeposition Laundering
None (control)	95 mg	17 mg	0 mg	34 mg
Pretreatment (1 ppm)	95 mg	17 mg	0 mg	2 mg
In situ (1 ppm)	95 mg	18 mg	0 mg	11 mg

As can be seen from the data of Table 8, treating the polyester swatches so as to sorb the hydrolase onto their surfaces before exposure to potentially redepositing oil (from the soiled cotton swatches) was effective to prevent most of the redeposition when the polyester swatches had already been treated, and substantially reduced the amount of oil redepositing when the treatment was in situ. This experiment demonstrates the efficacy of Lipase 1 as an anti-redeposition agent.

Effective surface modifying compositions of the invention preferably have enzyme within the range of 0.1 g/ml enzyme (0.1 ppm) and 20 g/ml enzyme. Of course, yet higher concentrations could be used. Efficacy of the lipase even when only 0.1 ppm lipase compositions are used for fabric treating is shown by the data in Table 9.

TABLE 9

	% of oil stain removed	
	2 Cycles	5 Cycles
fabric treated with lipase at 0.1 μg/ml (invention)	30	44
control	24	29

As can be seen from the data summarized in Table 9, even the very small amount of lipase (isolated from a clone of the *P. putida*) used in a treatment in accordance with the invention results in a statistically significant oil removal benefit for the fabric after two laundering cycles with respect to an untreated control. Indeed, the benefit increases upon multiple cycles and results in almost a 50% increase over the control (untreated fabric) after five laundering cycles.

In another aspect of the present invention a concentrated delivery system useful for generating a bleaching agent comprises a vessel, a surface structure disposed within the vessel, a lipase adjacent to or carried by said surface structure, and means for admitting a selected amount of oil and a selected amount of peroxygen to said vessel and into contact with said surface structure for generation of a peracid within the vessel via enzymatic catalysis. For example, for home laundering an embodiment of the inventive apparatus can serve both to generate a bleaching agent within the limited volume of the vessel as well as to dispense the bleaching agent generated into the laundering solution. A porous vessel can have lipase immobilized within the vessel interior. The lipase is preferably immobilized within the vessel interior, such as on a wall forming at least part of the vessel interior or a member defining a surface within the vessel, by both covalent and noncovalent coupling. Covalent coupling may be by various conventional means known to the art, such as through the N-terminal amine as is used for coupling antibody to membranes.

Referring to FIG. **3**, a generally spherical vessel **10** has a cover assembly **12** and a body **14**. Cover assembly **12** is fixed in a removable manner on body **14**, such as by a rotary-type mounting, or "twist-off" or any other quick and releasable mountings known to the art. Cover assembly **12** preferably includes a plurality of vents **15***a, b*. Body **14** has a surface structure **16** exposed to the interior on which lipase is immobilized (not illustrated). This structure **16** can take a wide variety of forms. In use, when the cover assembly **12** is removed, then the body **14** has the selected amounts of oil and of peroxygen added to a level sufficient to contact surface structure **16** with its immobilized enzyme for generation of peracid within the vessel **10**. As earlier noted, the immobilized enzyme is preferably bound to the structure **16** by both covalent and noncovalent coupling.

Noncovalent coupling is believed involved in forming enzyme-surface complexes through enzyme sorption as has earlier been described. When the consumer adds a selected amount of oil and a selected amount of peroxygen to the vessel interior and into contact with the immobilized lipase, then the lipase, its substrate, and the peroxygen will react to produce peracid in the limited volume of the vessel when in the presence of a substrate-solubilizing aqueous solution, such as a laundering composition. This is because a lipase, such as Lipase 1, will perhydrolyze substrates such as glycerides, ethylene glycol derivatives, or propylene glycol derivatives, which, in the presence of a source of hydrogen

peroxide, will form peracid. Such peracid bleaching systems utilizing these three essential components are more fully described in Ser. No. 932,717, filed Nov. 19, 1986, titled "Enzymatic Peracid Bleaching System," of common assignment herewith and incorporated hereby by reference. Example 6 illustrates this bleaching agent generation apparatus aspect of the invention.

EXAMPLE 6

A protocol was devised to determine whether a peracid (such as peroctanoic acid) in reasonably high concentrations could be generated using a lipase in a limited volume device that would be added to the wash when one desired laundry bleaching.

We prepared a surface structure with immobilized enzyme by pipetting 0.8 ml of 6.6 g/l lipase solution (isolated from a clone of the *P. putida* organism) into a weigh boat, added a fabric swatch and soaked the swatch in the solution overnight. The swatch was then treated by rinsing in sodium carbonate buffer at pH 11 for fifteen minutes with two water rinses to remove unbonded enzyme. This swatch, or surface structure with lipase carried on the surface, then was placed into contact with a selected amount of substrate for the lipase and a selected amount of peroxygen within a limited volume (a beaker). The substrate was 0.1 weight percent trioctanoin (in 200 ml, 0.2 g trioctanoin). The peroxygen was hydrogen peroxide (5000 ppm A.O. by calculation 6.5 ml/200 ml). Both the substrate (oil) and peroxygen were in an aqueous solution buffered with sodium carbonate (25 mM) to pH 10.8 with EDTA 0.2 ml/200 ml (50 μM). Liquid chromatography (Brinkman autoanalyzer) was used to determine the amount of peracid generated as a time function, as illustrated by Table 10.

TABLE 10

Elapsed Time (min)	ppm A.O. generated
6	4.8
12	7.8
18	8.8
25	9.3

A control (with no enzyme present) resulted in the generation of 0.05 ppm A.O. in 12 minutes. Thus, while only an insignificant amount of chemical perhydrolysis (between substrate and peroxygen) occurred, the immobilized enzyme placed into contact with substrate and peroxygen generated peracid within the vessel via enzymatic catalysis.

Another composition was prepared in which the substrate oil was increased to 52 g/200 ml. EDTA was present as 0.6 ml in 600 ml, there was 2% PVA, and the solution was prepared with 350 ml water. The hydrogen peroxide was also increased (10 ml into 150 ml emulsion sample) and the initial pH of the emulsion was raised (using 50% NaOH) to 10.8. The enzyme amount was 6.8 mg/swatch which is equivalent to about .1 ppm in a 70 liter wash. The amount of available oxygen generated for this system was again calculated and the results are shown as is shown in Table 11.

TABLE 11

Elapsed Time (min)	ppm A.O. generated
1	175
4	390
7	360
10	340
14	404

A control with no immobilized enzyme resulted in no peracid being detected after 14 minutes. These experiments indicate that peroctanoic acid at high concentrations (30 mM) can be generated by immobilizing a lipase in accordance with the invention and employing the immobilized enzyme as a catalyst for a reaction system with hydrogen peroxide (2%) and oil substrate trioctanoin (8.7% g/100 ml).

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

1									10		
ala	pro	leu	pro	asp	thr	pro	gly	ala	pro	phe	pro
							20				
ala	val	ala	asn	phe	asp	arg	ser	gly	pro	tyr	thr
					30						
thr	ser	ser	gln	ser	glu	gly	pro	ser	cys	arg	ile
			40								
tyr	arg	pro	arg	asp	leu	gly	gln	gly	gly	val	arg
	50										60
his	pro	val	ile	leu	trp	gly	asn	gly	thr	gly	ala
									70		
gly	pro	ser	thr	tyr	ala	gly	leu	leu	ser	his	trp
							80				
ala	ser	his	gly	phe	val	val	ala	ala	ala	glu	thr
					90						
ser	asn	ala	gly	thr	gly	arg	glu	met	leu	ala	cys
			100								
leu	asp	tyr	leu	val	arg	glu	asn	asp	thr	pro	tyr
	110										120
gly	thr	tyr	ser	gly	lys	leu	asn	thr	gly	arg	val
									130		
gly	thr	ser	gly	his	ser	gln	gly	gly	gly	gly	
							140				
ile	met	ala	gly	gln	asp	thr	arg	val	arg	thr	thr

-continued

					150						
ala	pro	ile	gln	pro	tyr	thr	leu	gly	leu	gly	his
			160								
asp	ser	ala	ser	gln	arg	arg	gln	gln	gly	pro	met
	170										180
phe	leu	met	ser	gly	gly	gly	asp	thr	ile	ala	phe
									190		
pro	tyr	leu	asn	ala	gln	pro	val	tyr	arg	arg	ala
							200				
asn	val	pro	val	phe	trp	gly	glu	arg	arg	tyr	val
					210						
ser	his	phe	glu	pro	val	gly	ser	gly	gly	ala	tyr
			220								
arg	gly	pro	ser	thr	ala	trp	phe	arg	phe	gln	leu
	230										240
met	asp	asp	gln	asp	ala	arg	ala	thr	phe	tyr	gly
									250		
ala	gln	cys	ser	leu	cys	thr	ser	leu	leu	trp	
ser	val	glu	arg	arg	gly	leu					

What is claimed is:

1. A treated fabric having improved oil stain removal, consisting essentially of:

a fabric; and

a lipase sorbed on the fabric surface, the lipase being isolatable from a Pseudomonas organism.

2. The treated fabric as in claim **1** wherein the sorbed lipase forms fabric-lipase complexes having substantial hydrolysis activity for oil stains.

3. The treated fabric as in claim **1** or **2** wherein the sorbed lipase alters the wettability of the fabric surface.

4. The treated fabric as in claim **1** or **2** wherein the lipase is isolated from an organism expressing a coding region found in or cloned from *Pseudomonas putida* ATCC 53552, the lipase having a molecular weight of about 30 to 35 kd and being resolvable as a single band by SDS gel electrophoresis.

5. The treated fabric as in claim **4** wherein the. sorbed lipase retards redeposition of oil and hydrolysis by-products during oil removal from the surface in the presence of aqueous solutions.

6. The treated fabric as in claim **2** wherein the sorbed lipase retains at least some hydrolysis activity when the fabric is exposed to drying at elevated temperatures.

7. The treated fabric as in claim **4** wherein the sorbed lipase is resistant to removal during laundering of the fabric.

8. The treated fabric as in claim **4** wherein the sorbed lipase alters the wettability of the fabric.

9. A method for modifying surfaces to facilitate oil removal, consisting essentially of:

selecting a surface to be modified;

immobilizing a lipase onto the surface, the lipase being isolatable from a Pseudomonas organism.

10. The method as in claim **9** wherein the lipase is isolated from an organism expressing a coding region found in or cloned from *Pseudomonas putida* ATCC 53552 or genetic mutants thereof, the lipase having a molecular weight of about 30 to 35 kd and being resolvable as a single band by SDS gel electrophoresis.

11. The method as in claim **9**, wherein the immobilized lipase forms surface-lipase complexes on the surface having substantial hydrolysis activity for oil stains.

12. The method as in claim **11** wherein the immobilized lipase forms surface-lipase complexes on the surface having enhanced stability to denaturation by surfactants and to heat deactivation.

13. A method of treating fabric to improve oil stain removal, consisting essentially of:

selecting a fabric to be modified;

sorbing a lipase onto the fabric, the lipase being isolatable from a Pseudomonas organism.

14. The method as in claim **13** wherein the sorbed lipase forms fabric-lipase complexes having substantial hydrolysis activity for oil stains on the fabric while in the presence of air.

15. The method as in claim **13** or **14** wherein the lipase is isolated from an organism expressing a coding region found in or cloned from *Pseudomonas putida* ATCC 53552, the lipase having a molecular weight of about 30 to 35 kd and being resolvable as a single band by SDS gel electrophoresis.

16. The method as in claim **15** wherein the sorbed lipase retards redeposition of oil and hydrolysis by-products during laundering of the fabric.

17. The method as in claim **15** wherein the sorbed lipase retains at least some hydrolysis activity when the fabric is exposed to drying at elevated temperatures.

18. The method as in claim **15** wherein the sorbed lipase is resistant to removal during laundering of the fabric.

19. The method as in claim **15** wherein the sorbed lipase alters the wettability of the fabric.

20. The method as in claim **14** wherein at least some of the hydrolysis by-products are removable during laundering of the fabric at basic pH or in the presence of surfactant.

21. The method as in claim **14** wherein at least most of oil stains when present on the fabric are removed via hydrolysis by-products after three launderings.

22. The method as in claim **15** wherein the lipase is sorbed by contacting the fabric with an lipase containing composition having the lipase in an amount between about 0.1 ppm to about 2,000 ppm.

* * * * *

ATTACHMENT G:

1. "*Optical resolution of dl-menthol by entrapped biocatalysts*": Saburo Fukui and Atsuo Tanaka, "Methods in Enzymology" Volume 136, Pages 293-302, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.;

2. "*Industrial operation of immobilized enzymes*": M.J. Daniels, "Methods in Enzymology" Volume 136, Pages 371-379, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.;

3. "*Regiospecific interesterification of triglyceride with celite-adsorbed lipase*": Shigeru Yamanaka and Takashi Tanaka, "Methods in Enzymology" Volume 136, Pages 405-411, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.; and

4. "*Production of aspartame by immobilized thermoase*": Kiyotaka Oyama, Shigeaki Irino and Norio Hagi, "Methods in Enzymology" Volume 136, Pages 503-516, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.

[27] Optical Resolution of *dl*-Menthol by Entrapped Biocatalysts

By SABURO FUKUI and ATSUO TANAKA

l-Menthol is widely used in the cosmetic, food, and pharmaceutical industries. *l*-Menthol can be isolated from peppermint oil or other mint oils; it can also be produced by optical resolution of *dl*-menthol mixtures obtained chemically by hydrogenation of thymol. Chemically synthesized *dl*-menthol, however, contains four isomers: *dl*-menthol, *dl*-isomenthol, *dl*-neomenthol, and *dl*-isoneomenthol. Thus, the separation of *l*-menthol from its isomers and racemates is industrially important. Resolution of menthyl ester racemates by microbial esterases in aqueous systems has been reported.[1,2] However, neither menthyl ester nor menthol is soluble in water. Hence, it would be advantageous to employ an appropriate organic solvent for the stereoselective enzymatic hydrolysis of menthyl esters. In addition, racemates of menthol and other terpenoids can be resolved optically by stereoselective esterification of these racemates with lipase. In this case, it is essential to choose appropriate organic solvents both to dissolve the reactants and to shift the equilibrium toward esterification.

The reaction will only succeed, however, if the used biocatalysts are not inactivated by organic solvents. As immobilization could render the biocatalysts more stable in organic solvents, we have entrapped both yeast cells and lipase in polymer gels.

This article deals with optically selective hydrolysis of *dl*-menthyl succinate by gel-entrapped yeast cells (Fig. 1) and stereoselective esterification of *dl*-menthol by gel-entrapped lipase (Fig. 2).

Stereoselective Hydrolysis of *dl*-Menthyl Succinate[3]

Materials and Methods

Prepolymers. Two kinds of water-miscible urethane prepolymers (PU-3 and PU-6) are prepared by Toyo Tire & Rubber Co., Japan.[4-7] PU-3

[1] T. Oritani and K. Yamashita, *Agric. Biol. Chem.* **37,** 1695 (1973).
[2] Y. Yamaguchi, A. Komatsu, and T. Moroe, *J. Agric. Chem. Soc. Jpn.* **51,** 411 (1977).
[3] T. Omata, N. Iwamoto, T. Kimura, A. Tanaka, and S. Fukui, *Eur. J. Appl. Microbiol. Biotechnol.* **11,** 199 (1981).
[4] S. Fukushima, T. Nagai, K. Fujita, A. Tanaka, and S. Fukui, *Biotechnol. Bioeng.* **20,** 1465 (1978).

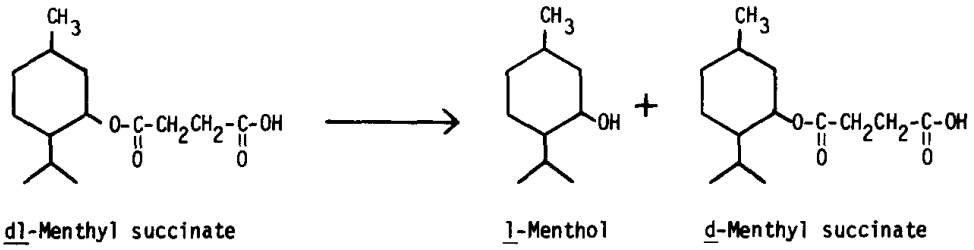

FIG. 1. Stereoselective hydrolysis of *dl*-menthyl succinate catalyzed by *R. minuta* var. *texensis* cells.

gives a hydrophobic gel and PU-6 a hydrophilic gel. A photo-cross-linkable resin prepolymer, ENT-4000, is synthesized by Kansai Paint Co., Japan.[7,8]

Cultivation of Yeast. Various microorganisms, especially those belonging to the genera *Rhodotorula* and *Bacillus*, show high hydrolytic activity using menthyl succinate as a substrate. Among the strains examined *Rhodotorula minuta* var. *texensis* IFO 1102 was selected because it showed both high hydrolytic activity and satisfactory stereoselectivity in the catalyzed process. *Rhodotorula minuta* var. *texensis* is cultivated with shaking (220 rpm) at 30° in 500-ml shaking flasks each containing 100 ml of a medium of the following composition (per liter): treacle, 50 g; corn steep liquor, 50 g; ammonium sulfate, 5 g; mineral mixture, 10 ml. The mineral mixture was (g per liter): $MgSO_4 \cdot 7H_2O$, 20; $FeSO_4 \cdot 7H_2O$, 5; $CaCl_2$, 2; $MnCl_2 \cdot 4H_2O$, 0.2; $NaMoO_4 \cdot 2H_2O$, 0.1 and NaCl, 0.1. The pH of the medium is adjusted to 7.0 with 1 *N* NaOH before sterilization. Cells are harvested by centrifugation after 70 hr of cultivation, washed twice with 20 m*M* potassium phosphate buffer (pH 7.0), mixed thoroughly, and stored in sealed sample bottles at −20°. The frozen cells maintain their original hydrolytic activity for at least one month.

Immobilization of Cells. Thawed cells (1 g wet cells) suspended in 3 ml of water are entrapped wih 1 g of ENT-4000[6,7]; 1 g wet cells suspended in 2 ml of water is immobilized with 1 g of PU-3, PU-6, or mixtures of these prepolymers.[6,7] The gels so prepared are cut into small pieces (~1 × 1 mm for ENT-4000, thickness, ~0.5 mm; ~1 × 1 × 1 mm for PU-3 and PU-6) and used for the reaction.

[5] K. Sonomoto, I.-N. Jin, A. Tanaka, and S. Fukui, *Agric. Biol. Chem.* **44,** 1119 (1980).
[6] T. Omata, T. Iida, A. Tanaka, and S. Fukui, *Eur. J. Appl. Microbiol. Biotechnol.* **8,** 143 (1979).
[7] S. Fukui, K. Sonomoto, and A. Tanaka, this series, Vol. 135 [20].
[8] A. Tanaka, S. Yasuhara, M. Osumi, and S. Fukui, *Eur. J. Biochem.* **80,** 193 (1977).

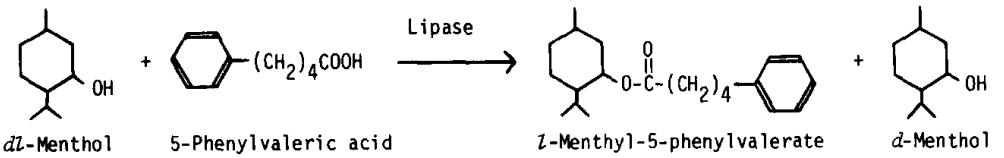

FIG. 2. Stereoselective esterification of *dl*-menthol catalyzed by *C. cylindracea* lipase.

Reaction Conditions. Free yeast cells (1 g wet cells) are suspended in 10 ml of 20 m*M* potassium phosphate buffer (pH 7.0) containing 39 m*M* *dl*-menthyl succinate ammonium salt. The reaction is carried out at 30° with shaking (180 strokes/min) for the indicated period. Most of *l*-menthol formed does not dissolve in the aqueous buffer and accumulates on the surface of the cells, thereby decreasing the apparent activity of the cells. As shown in Table I, the hydrolytic activity of the cells is reduced significantly in the presence of organic cosolvents, except for 10% methanol, probably due to denaturation of the enzyme (esterase) in the yeast cells.

TABLE I
EFFECT OF SOLVENTS ON HYDROLYSIS OF *dl*-MENTHYL SUCCINATE BY FREE CELLS OF *Rhodotorula minuta* VAR. *texensis*[a]

Substrate	Solvent	Conversion ratio (%)
dl-Menthyl succinate ammonium salt	KPB[b]	71
	KPB–Methanol (75 : 25)	48
	KPB–Methanol (90 : 10)	66
	KPB–Dimethylformamide (50 : 50)	14
	KPB–CH_3CN (50 : 50)	5
	KPB–CH_3CN (65 : 35)	14
	KPB–CH_3CN (80 : 20)	30
dl-Menthyl succinate	Water-saturated benzene	1
	Water-saturated chloroform	0
	Two-phase system (KPB–solvent, 1 : 5)	
	t-Butyl acetate	0
	Benzene	22
	Benzene–*n*-heptane (50 : 50)	25
	Benzene–*n*-heptane (40 : 60)	33
	Benzene–*n*-heptane (30 : 70)	38
	Benzene–*n*-heptane (20 : 80)	45
	Benzene–*n*-heptane (10 : 90)	53
	n-Heptane	73

[a] From Omata *et al.*[3]

[b] 20 m*M* Potassium phosphate buffer (pH 7.0). The cells (1 g wet cells) were incubated for 48 hr in 10 ml of KPB, KPB–water-miscible solvents, or water-saturated organic solvents (39 m*M* substrate), or in two-phase systems composed of 10 ml of water-immiscible organic solvent (39 m*M* substrate) and 2 ml of KPB.

Hydrolysis of menthyl succinate in water-immiscible organic solvents catalyzed by the free cells was not successful, because the cells could not be suspended well in such solvents. Therefore, two-phase systems composed of the phosphate buffer and organic solvents containing 39 m*M* substrate (1 : 5, v/v) were tested for the optically selective hydrolysis of *dl*-menthyl succinate by the free cells (Table I). After 48 hr of incubation in a two-phase system composed of 20 m*M* potassium phosphate buffer (pH 7.0) and *n*-heptane (1 : 5, v/v), the conversion ratio was almost the same as in the phosphate buffer containing *dl*-menthyl succinate ammonium salt. Hence, *n*-heptane was selected as the reaction solvent using the phosphate buffer–*n*-heptane two-phase system for the free cells and water-saturated *n*-heptane for the gel-entrapped cells. In this solvent system both substrate and products show satisfactory solubility, and under these conditions there is minimal inactivation of the hydrolytic enzyme in the cells. Concentration of *dl*-menthyl succinate in the organic solvent is 39 m*M*. The reaction is carried out at 30° with shaking (180 strokes/min).

Analytical Methods. *l*-Menthol produced from *dl*-menthyl succinate is assayed by gas chromatography with a JEOL-20 KFL gas chromatograph equipped with a hydrogen flame ionization detector. The steel column (2 m × 2 mm I.D.) is packed with LAC-2R-446 (20%, w/w) on Celite 545, 60-80 mesh (Gasukuro Kogyo, Japan). The temperatures at the injector, in the column, and at the detector are 175, 140, and 240°, respectively. The flow rate of the carrier gas, helium, is 44 ml/min. γ-Valerolactone is used as the internal standard for the determination of *l*-menthol. *l*-Menthol is isolated from the reaction mixture as follows. The reaction mixture is washed with an equal volume of 0.1 *N* NaOH and dried on calcium chloride, and the solvent is removed *in vacuo* to yield white crystals which are recrystallized from ethanol by adding water. The melting point of *l*-menthol so obtained is 40–41° (mp in literature, 41–43°). Optical rotation of the product is measured in ethanol with a DIP-SL automatic polarimeter (Japan Spectroscopic Co.). The specific rotation $[\alpha]_D^{25}$ is estimated using the following equation:

$$[\alpha]_D^{25} = (a/lc)100$$

where a is the observed rotation; l, light path (dm); and c, concentration of *l*-menthol (g/100 ml).

Synthesis of Menthyl Succinate. Succinyl anhydride is used as the acyl donor for the ester synthesis because succinate produced by the enzymatic reaction can be easily recovered as succinic anhydride in high yield and because menthyl succinate can be solubilized in water as the ammonium salt. Finely powdered succinic anhydride (15.7 g) is added to a

solution of *dl*-menthol (24.5 g) in *o*-xylene (30 ml). The reaction is carried out at 80° for 3 hr, after which the solvent is removed. The white crystals thus obtained are recrystallized from benzene (yield, 86%). The product is identified by spectrophotometric analyses (NMR, IR, and mass-spec.). The results show that the compound is indeed *dl*-menthyl succinate without contamination by esters of *dl*-isomenthol, *dl*-neomenthol, and *dl*-isoneomenthol. The water-soluble ammonium salt of *dl*-menthyl succinate is prepared as follows. *dl*-Menthyl succinate (8.3 g) is neutralized with 60 ml of 2.2 *M* ammonium hydroxide, and the pH is adjusted to 7.0 with 5.1 *N* HCl. The final concentration of *dl*-menthyl succinate ammonium salt is estimated to be 390 m*M*. This solution is diluted to 39 m*M* with 20 m*M* potassium phosphate buffer (pH 7.0).

Properties of Gel-Entrapped Cells

Activities of Cells. The rate of *l*-menthol formation by the hydrophobic gel (PU-3)-entrapped cells was 2.5 μmol/hr-g wet cells, that of the hydrophilic gel (PU-6)-entrapped cells was 2.0 μmol/hr-g wet cells, while that of the free cells was 6.1 μmol/hr-g wet cells.

Effect of Gel Hydrophobicity. When the hydrophobicity of the gels was changed by mixing PU-3 and PU-6 in different ratios, the activity increased as the content of the hydrophobic PU-3 increased.

Effect of Reaction Temperature. The optimal temperature for the free and entrapped cells was 40°. In most cases, however, the cells were used at 30° to minimize the thermal inactivation of the enzyme during long periods of operation; the activity of the cells at 30° was about 75% of that at 40°.

Stability of Hydrolytic Activity. When menthyl succinate ammonium salt was hydrolyzed by the free cells, about 80% of *l*-menthol produced by the cells was accumulated on the cell surface. Extraction of *l*-menthol by an organic solvent, such as benzene, significantly damaged the hydrolytic activity of the cells, thus rendering repeated use of the cells difficult. Addition of 10% methanol to the aqueous reaction mixture improved the productivity of *l*-menthol by the free cells, and their activity was stable for at least 10 reaction batches (total operational period, 240 hr). Figure 3 illustrates the repeated use of the free cells in the buffer–*n*-heptane system and of PU-3-, PU-6-, and ENT-4000-entrapped cells in water-saturated *n*-heptane for the hydrolysis of menthyl succinate. The free cells were recovered from the reaction mixture by centrifugation, and the immobilized cells by filtration. The half-life of the activity of the free cells was 50 hr, while that of the ENT-4000-, PU-6-, and PU-3-entrapped cells was estimated to be 560, 1315, and 1520 hr, respectively. Thus, immobili-

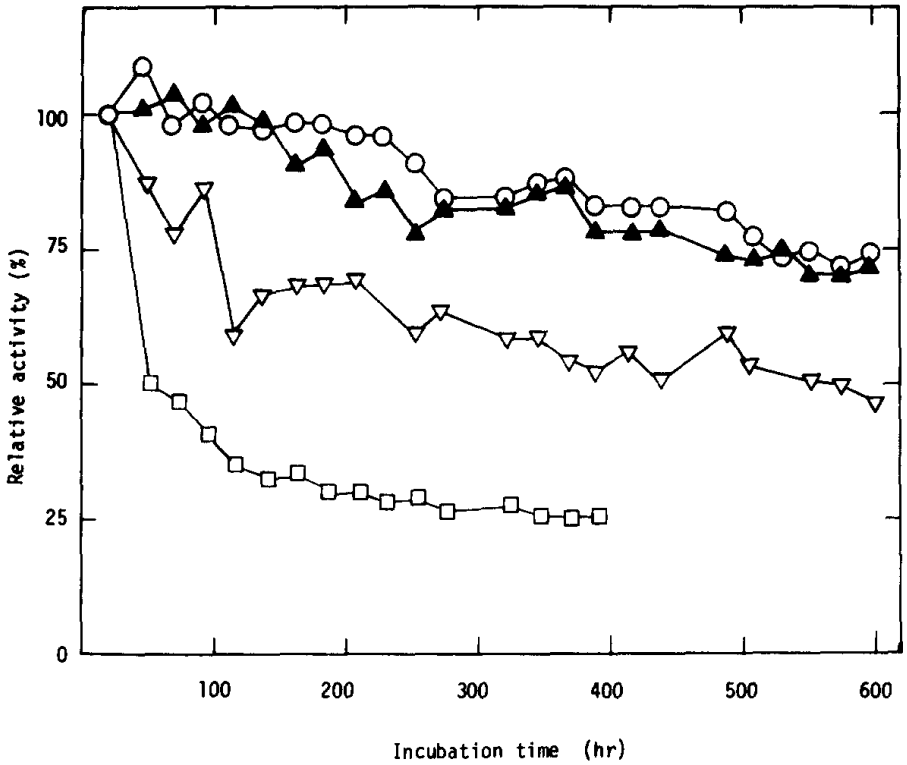

FIG. 3. Repeated use of free and gel-entrapped *R. minuta* var. *texensis* cells in hydrolysis of *dl*-menthyl succinate. Each reaction was carried out at 30° by shaking the reaction mixtures (180 strokes/min) for 24 hr as described in the text. (□) Free cells; (○) PU-3-entrapped cells; (▲) PU-6-entrapped cells; (▽) ENT-4000-entrapped cells. From Omata *et al.*[3]

zation greatly improved the operational stability of the hydrolytic enzyme in the yeast cells.

The specific rotation of *l*-menthol ($[\alpha]_D^{25} = -51°$) produced from *dl*-menthyl succinate by the free and entrapped cells showed that the product was pure *l*-menthol. The initial optical yield were maintained even after 10 reaction batches in every case.

Production of l-Menthol

The acids used to form the *dl*-menthyl esters have to be carefully selected because they influence the optical purity of the hydrolysis product.[9] Recovery of the corresponding acids from the reaction mixture should also be high. We employed the succinate ester as the substrate because of the 100% optical purity of *l*-menthol produced by the enzyme of *R. minuta* var. *texensis* and because of the high recovery (75%) of

[9] Y. Yamaguchi, T. Oritani, N. Tajima, A. Komatsu, and T. Moroe, *J. Agric. Chem. Soc. Jpn.* **50,** 475 (1976).

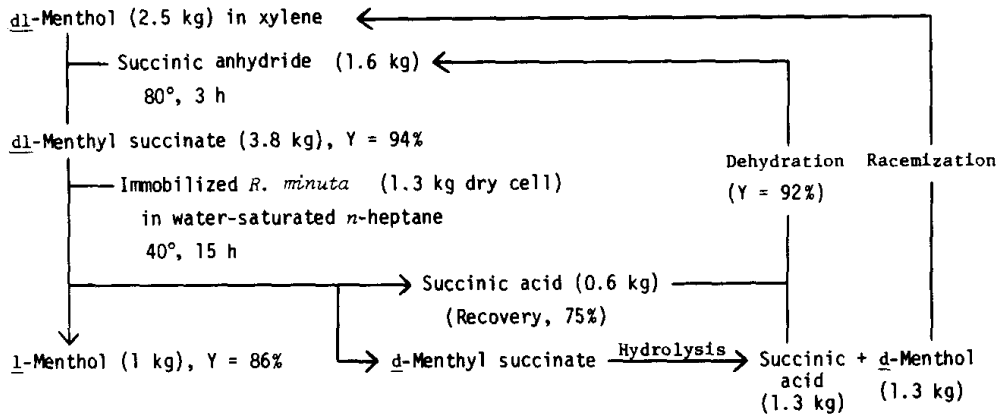

FIG. 4. Flow diagram of *l*-menthol production by gel-entrapped *R. minuta* var. *texensis* cells. From Omata *et al.*[3]

succinic acid after transformation to succinic anhydride. The remaining *d*-menthyl succinate was hydrolyzed with KOH. The *d*-menthol thus obtained was racemized by heating at 260–280° for 4.5 hr under hydrogen pressure of 65–72 kg/cm^2 in the presence of a catalyst such as copper chromite.[10] The yield of *dl*-menthol was ~46%. Based on the results mentioned above we have tried to produce *l*-menthol on a large scale. The flow diagram for the large scale production of *l*-menthol is shown in Fig. 4.

Stereoselective Esterification of *dl*-Menthol[11]

Materials and Methods

Prepolymer. Water-miscible urethane prepolymer (PU-3)[7] prepared by Toyo Tire & Rubber Co., Japan, is used. PU-3 gives a hydrophobic gel.

Enzyme. Lipase from *Candida cylindracea* (MY and OF 360; Meito Sangyo Co., Japan) and porcine pancreas (Sigma, USA) shows a high synthetic activity in the production of *l*-menthyl 5-phenylvalerate with a high stereoselectivity. On the other hand, enzymes from *Aspergillus niger* (Nagase Seikagaku Co, Japan) and *Rhizopus delemar* (Seikagaku Kogyo Co., Japan) are almost inactive in catalyzing the menthyl ester formation. When lipase preparations from hog pancreas (Sigma) and porcine pancreas (Wako Pure Chemicals Co., Japan) are entrapped with PU-3, rigid gels could not be obtained using these enzyme preparations. Pancreas lipase (Sigma; 19 units/mg powder) and lipase MY (30 units/mg powder) have relatively low activity, and it is rather difficult to prepare immobi-

[10] T. Yoshida, A. Komatsu, and M. Indo, *Agric. Biol. Chem.* **29,** 824 (1965).
[11] S. Koshiro, K. Sonomoto, A. Tanaka, and S. Fukui, *J. Biotechnol.* **2,** 47 (1985).

lized enzyme preparations showing high enzyme activity. Therefore, lipase OF 360 was selected as the enzyme for the esterification.

Immobilization of Enzyme. Lipase OF 360 (100 mg; 36,000 units) dissolved in 0.2 ml of deionized water is entrapped with 0.5 g of PU-3.[6,7] The prepared gel is cut into small pieces (~3 × 3 × 3 mm) and is ready to be used in the esterification experiments. In some cases, 100 mg of lipase in 0.2 ml of water is adsorbed on 0.25 g of Celite 535. Celite-adsorbed lipase is also immobilized with 0.5 g of PU-3 in the absence of water.

Reaction Conditions. For the esterification of menthol, choice of an acyl donor influences significantly both the reactivity and stereoselectivity of the enzyme. Longer chain fatty acids, such as stearic and oleic acids, were good substrates for the esterification reaction. Use of middle chain fatty acids, such as *n*-valeric, *n*-heptanoic, and *n*-nonanoic acids, resulted in a low product yield and shorter chain fatty acids, such as acetic acid, showed no reactivity. In contrast, stereoselective esterification was hardly observed using longer chain fatty acids, while *l*-menthol was selectively esterified using middle chain fatty acids. 5-Phenylvaleric acid was appreciably reactive, yielding optically active product. Therefore, this acid was employed as the acyl donor.

Addition of an organic solvent to the reaction system was essential in order to obtain a homogeneous reaction system and to shift the reaction equilibrium favoring ester formation. Both water-miscible and water-immiscible organic solvents were examined. It was found that enzyme activity in nonpolar solvents, such as cyclohexane and isooctane, was high. The activity of the entrapped enzyme was, however, moderate in *n*-hexane, and low in benzene and carbon tetrachloride. No activity was observed in polar solvents, such as acetone, chloroform, dioxane, methanol, and methyl isobutyl ketone (Table II).

Based on these results, lipase preparations (36,000 units) are suspended in 10 ml of water-saturated isooctane or cyclohexane containing 130 m*M* *dl*-menthol and 100 m*M* 5-phenylvaleric acid. Enzymatic esterification is carried out at 30° by shaking the reaction mixtures (220 strokes/min using test tubes or 120 strokes/min using 100-ml flasks).

Analytical Methods. An aliquot of the reaction mixture (50 μl) is added to 50 μl of methanol containing a known amount of progesterone as an internal standard. After removal of the solvent by evaporation at 4°, the residues are dissolved in 0.2 ml of a mixture of methanol and acetic acid (100:0.5, v/v), and the solution is filtered through a Teflon membrane filter (pore size, 0.22 μm). Amounts of the product and of the substrates are determined by high-performance liquid chromatography using an ALC/GPC 204 instrument (Waters Co., USA) equipped with a data module (type 730; Waters) and a differential refractometer R401

TABLE II
EFFECT OF ORGANIC SOLVENTS ON ESTERIFICATION OF *dl*-MENTHOL BY *Candida cylindracea* LIPASE[a,b]

Organic solvent	Conversion (%)
Acetone	nil
Benzene	1
Carbon tetrachloride	8
Chloroform	nil
Cyclohexane	56
Dioxane	nil
n-Hexane	18
Isooctane	43
Methanol	nil
Methyl isobutyl ketone	nil

[a] From Koshiro *et al.*[11]
[b] The enzymatic reaction was carried out for 62–69 hr with PU-3-entrapped lipase as described in Fig. 5.

model (Waters). Separations are carried out on a Radial-Pak liquid chromatography cartridge C_{18} column (8 mm I.D. × 10 cm; d = 10 μm) using methanol–acetic acid (100 : 0.5, v/v) as the mobile phase (flow rate, 2 ml/min) at a pressure of 70 kg/cm^2.

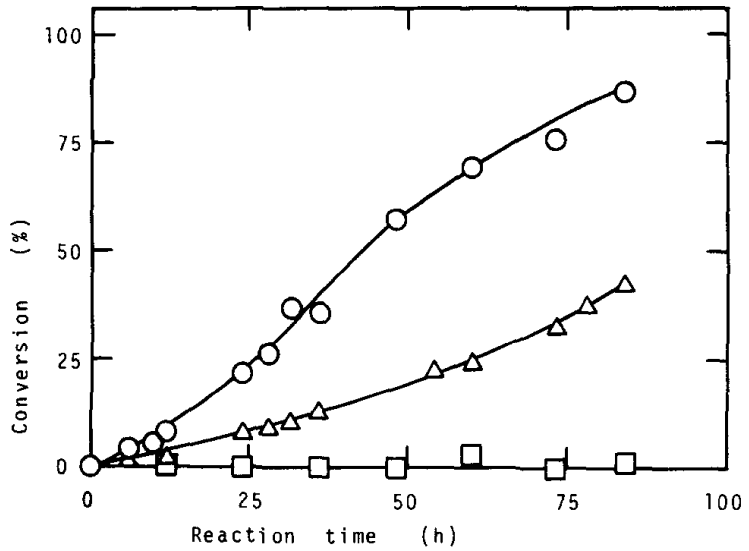

FIG. 5. Stereoselective esterification of menthol by entrapped lipase. Lipase OF 360 (36,000 units) was entrapped with 0.5 g of PU-3 and the immobilized enzyme was incubated at 30° in 10 ml of water-saturated isooctane containing 130 m*M* menthol and 100 m*M* 5-phenylvaleric acid. (○) *l*-Menthol; (△) *dl*-menthol; (□) *d*-menthol. From Koshiro *et al.*[11]

Properties of Entrapped Lipase

Stereoselectivity of the reaction using 5-phenylvaleric acid as an acyl donor was tested by comparing different alcohol substrates, such as *d*-, *l*-, and *dl*-menthol, in the esterification reaction (Fig. 5). Stereoselectivity of the reaction was also analyzed by measuring optical rotation of the menthyl ester isolated from the reaction mixture containing *dl*-menthol. The purification was performed on a column packed with Silica gel 60 (70-230 mesh; solvent, *n*-hexane–diethyl ether–acetic acid, 80:20:1, v/v). The optical purity of the product was about 100%.

Based on the obtained results, we have found that about 80% of the used racemic alcohol substrate was transformed into *l*-menthyl ester after 70–80 hr of incubation under the conditions employed. The entrapped enzyme was more stable at 30° but rather unstable above 35° in repeated batch reactions. The decrease in enzyme activity of Celite-adsorbed lipase was more rapid than the loss of activity of PU-3-entrapped Celite-adsorbed enzyme. In addition, the production of *l*-menthyl 5-phenylvalerate by the entrapped enzyme after 10 batches of reaction (operational period, 930 hr) was 1.5 times greater than that of the Celite-adsorbed enzyme under identical conditions.

It is noteworthy to add that other terpenoids, such as *dl*-borneol, could also be resolved optically by using essentially the same procedure as the one described here for lipase OF 360 from *C. cylindracea*.

[28] Electroenzymatic and Electromicrobial Reduction: Preparation of Chiral Compounds

By IORDANIS THANOS, JOHANN BADER, HELMUT GÜNTHER, STEFAN NEUMANN, FRIEDRICH KRAUSS, and HELMUT SIMON

Principles

Reductases partially or completely purified or in the form of whole microbial cells are often used in the preparation of chiral compounds via stereoselective reduction of suitably substituted unsaturated substrates. Whole cells are usually applied together with sugars as the electron source. So far, the use of isolated enzymes has been restricted to pyridine nucleotide-dependent reductases. However, reductions on a preparative scale cannot be performed using stoichiometric amounts of reduced pyridine nucleotides because of their high cost. At present only enzymatic

[34] Industrial Operation of Immobilized Enzymes

By M. J. DANIELS

The immobilization of enzymes is relatively easy and there is a wealth of papers and patents to testify to this fact. Unfortunately, there are no criteria by which to judge these products on the basis of practical usefulness and economic process viability, but the lack of commercial exploitation suggests that most products are only of academic interest. In an attempt to rectify the situation this article will suggest some guidelines, based on experience gained from manufacturing and marketing immobilized enzymes produced at British Charcoals and MacDonalds, Greenock, Scotland. The enzymes considered will be hydrolases, which are relatively inexpensive, but are used in large quantities. While the conclusions may be more valid for low-cost large-volume enzymes, it is probable that the general picture will be the same for most immobilized enzyme systems.

The comparison of immobilized and liquid enzyme economics for existing manufacturing processes is the final test, but first I would like to consider immobilized enzyme manufacturing costs and the productivity of several fixed enzyme systems.

Manufacturing Cost

The chemistry used for enzyme fixing must be applicable on a large scale, be safe for use and produce a safe, stable product, preferably from a range of enzymes. There are inexpensive and expensive supports, but even the cheapest would require some processing if consistent product quality is to be achieved, and the reuse of expensive ones would involve additional costs and losses. Consider two cases: (1) one utilizing inexpensive enzyme and support with simple processing and (2) the other using relatively expensive raw materials with a more complex process. In the following tabulation I would suggest the following general manufacturing costs per liter of product.

It should be remembered that import duties, technical back-up, and point of operation running costs will need to be added and it is probably realistic to load these prices by 20%. Thus, it appears that immobilized enzymes produced from commercially available liquid enzymes at realistic loading efficiency would cost the user between $35 and $150 per liter to purchase and operate.

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Cost factors	Immobilized enzyme 1	Immobilized enzyme 2
Raw materials and waste disposal	$ 8	$ 80
Factory overheads, services, packing, and distribution	8	10
Quality control, salaries, and administration	8	10
Profit	6	20
Selling price	US$30	US$120

Activity/Productivity

Liquid enzyme processes usually involve the treatment of substrates at 20 to 70% dry solids content at temperatures between 40 and 70°. The residence times are relatively long and the liquid enzyme dose would be 0.1 to 2 g enzyme per kilogram of dry solids treated. Immobilized enzymes must treat substrates at similar concentrations and temperatures to produce the same end result, albeit with a considerably shorter residence time and their value to the factory operation would be proportional to the total product solids produced.

For practical reasons, the lifetime of the enzyme charge would probably not exceed three half-lives and the average productivity would be close to 50% of the day 1 productivity, so if the day 1 activity is described as bed volumes per hour of substrate, the dry weight productivity of 1 liter immobilized enzyme is simple to calculate. The total process cost of liquid enzyme treatments ranges from $6 to $120 per metric ton dry solids (DS), and for the two manufactured enzymes discussed earlier we can calculate the required lifetime productivities to provide equivalent process costs of liquid enzyme treatment.

	Production cost/ton	Productivity (tons dry solids)
Enzyme 1	$6–$20	7–1.8
Enzyme 2	$30–$120	5–0.8

Thus, it appears that an immobilized enzyme formulation must produce 1–7 tons dry solids product during the factory lifetime to provide economics equivalent to liquid enzyme treatment.

Assuming 20% dry solids and 55 days half-life which would provide some 4000 hr of operation, the average flow rates for this range of productivities, neglecting specific gravity, would be tabulated as shown below:

	Tons product/liter	Tons solution at 20°, DS	Average liters/hr	Day 1 bed vol/hr
Enzyme 1	1	5	1.25	2.5
Enzyme 2	7	35	8.75	17.5

These calculated day 1 flows using 20% solids substrate of 2–14 bed vol/hr can also be considered as 3–24 min of empty column residence time, again for cost equivalence to liquid enzyme treatment.

Finally, using a very simplistic empirical comparison, one can calculate very approximately the quantity of *enzyme activity* which must be loaded per liter immobilized enzyme and per gram of support; in this case the unit of activity is 1 g of commercially available product.

	Liquid case		Immobilized case	
	Time (hr)	Enzyme (g/kg DS product)	kg DS/hr	Enzyme (g/liter immobilized enzyme)
1	50	0.5	3.5	88
2	50	2.0	0.5	50

Assuming a bulk density for the support of 1.5 g/cm^3 this corresponds to 75–130 mg of enzyme activity per gram of support.

Below is a summary of these calculated criteria for minimum commercial viability for an immobilized product.

Total user cost	Total productivity	Day 1 activity at 20% DS	Enzyme activity/g support
$35–$150/liter	1–7 tons/liter	2–18 bed vol/hr	75–130 mg

The fact that the majority of the systems described in the literature fall so far short of these targets suggests that their practical usefulness must be questioned.

In the next sections, an economic comparison of liquid and immobilized treatments using specific enzyme systems will serve to demonstrate that financial advantage can be provided by an immobilized system.

Liquid Enzyme Hydrolysis

The time course of a typical hydrolysis is shown in Fig. 1. In the case of glucoamylase the maximum dextrose content is required, but with invertase or lactase, intermediate degrees of conversion may be required. Units on the x axis are enzyme hours, defined as kilograms enzyme at specific activity per ton DS substrate $\times$ time in hours. This concept is very useful, since within fairly broad limits, the enzyme dose can be reduced to increase the saccharification time quantitatively and vice versa.

The total saccharification cost can vary widely even within the same factory, since an increased quantity of enzyme is required for rapid production when there is sudden or heavy demand and a reduced enzyme dose is often used during period of low throughput. The tank volume occupied by the syrup incurs fixed overhead and running costs which could be considered as the "time cost," defined as the running costs per hour for the volume occupied by 1 ton of substrate solids. The total costs for saccharification are, therefore, the sum of the enzyme cost and the time cost generated during the complete saccharification period. The enzyme dose and the saccharification time are inversely proportional, and so it follows that the minimum saccharification cost is when the enzyme cost equals the time cost.

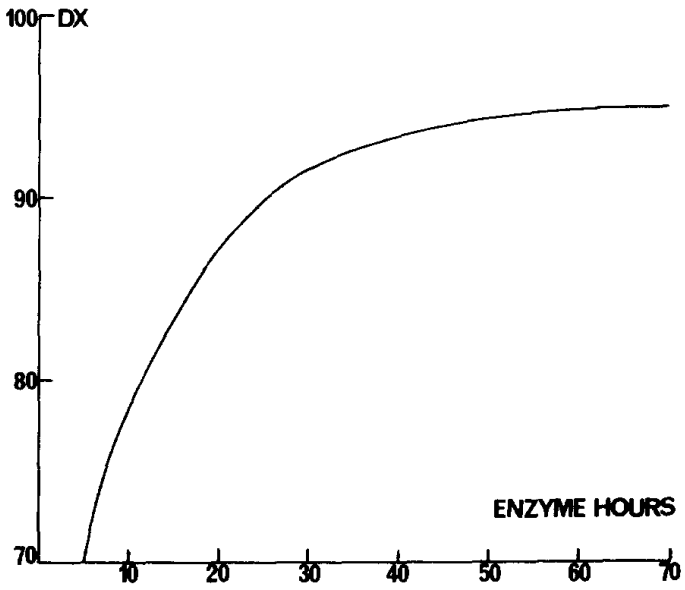


FIG. 1. Time course of a typical hydrolysis. DX, Dextrose content (%). Liquid glucoamylase saccharification. Enzyme-thinned substrate, 55°, pH 4.5, 30% dry solids. See text for description of enzyme hours.

Saccharification Tank System

Some high-fructose corn syrup (HFCS) factories employ a continuous cascade system which is efficient in terms of tank utilization, the number of pumps needed, and the labor requirement, but it is more common to use batch saccharification. Each batch tank is operated independently and is fitted with pumps for filling and emptying so each tank is individually piped. The labor requirement is relatively high, and since the enzyme is added to the tank the fill and empty times are not entirely part of the saccharification time. Each tank is lagged and traced with low-temperature steam and would contain a stirrer in continuous operation.

The running costs are obviously dependent on the size and the age of the plant, but could be estimated as follows (p.a. = per annum):

Capital charges, repairs, maintenance	\$350/m³ p.a.
Labor	100/m³ p.a.
Energy	100/m³ p.a.
Downtime and miscellaneous	50/m³ p.a.
Total	US\$600/m³ p.a.

The time cost would therefore be \$0.069 per cubic meter of tank volume per hour for 360-day, 24-hr operation.

The following terms are defined: EH, enzyme hours to achieve desired conversion; DS, dry solids content of syrup as percentage; C, enzyme cost per kilogram; T, minimum cost saccharification time; D_o, optimum enzyme dose in kilograms of enzyme per ton DS. Thus, neglecting the specific gravity,

$$\mathrm{EH} = D_o T \qquad \therefore \qquad T = \mathrm{EH}/D_o$$

At the minimum cost $D_o C = T(0.069/\mathrm{DS})$ (enzyme cost equals time cost). Then,

$$D_o^2 = \frac{\mathrm{EH}}{C}\,\frac{0.069}{\mathrm{DS}}$$

The enzyme cost would be

$$D_o C = \sqrt{\mathrm{EH}\; C\,\frac{0.069}{\mathrm{DS}}}$$

Thus the total minimum cost is

$$2D_o C = 2\,\sqrt{\mathrm{EH}\; C\,\frac{0.069}{\mathrm{DS}}}$$

Which in other words is the minimum saccharification cost SC:

$$SC = \sqrt{EH\ C\ \frac{0.276}{DS}}$$

It is then possible to calculate the optimum enzyme dose and saccharification time:

$$D_0 = SC/2C \quad \text{and} \quad T = EH/D_0$$

This procedure can be demonstrated using typical glucoamylase, invertase, and lactase hydrolysis processes performed commercially. Three enzymes are commonly used in saccharification. (1) The treatment of α-amylase-treated starch with glucoamylase would be used to produce glucose syrups, alcohol, or high-fructose syrups. Raffinate is the glucose-rich recycle stream from the fructose-enrichment process. Treatment of raffinate with glucoamylase increases the monosaccharide content and ultimately that of the 55% fructose syrups produced. (2) Invertase treatment of sucrose syrups produces a range of table syrups or raw material for sorbitol/mannitol production. (3) Lactase treatment of whey or the permeate from ultrafiltered whey is used to produce glucose/galactose syrups by hydrolyzing the lactose.

Glucoamylase

Mainstream saccharification at 32% DS to 96% dextrose content
Enzyme cost, $4/kg
Enzyme hours required, 72

Raffinate saccharification at 18% DS to 2.4% dextrose content
Enzyme cost, $4/liter
Enzyme hours required, 24

Invertase

60% inversion using 65% solids
Enzyme cost, $5/kg
Enzyme hours required, 8

95% inversion using 50% solids
Enzyme hours required, 72

Lactase

Whey 6% solids—90% hydrolysis
Enzyme cost, $120/kg
Enzyme hours required, 30

Permeate 20% solids—70% hydrolysis
Enzyme cost, $120/kg
Enzyme hours required, 5

The calculated costs are minimum and would be exceeded if the enzyme doses were varied in either direction. The enzyme cost, of course, represents 50% of the total cost.

Operation	Enzyme dose (kg/ton DS)	Saccharification time (hr)	Total cost per ton DS
Glucoamylase mainstream	1.97	36.5	$ 15.8
Glucoamylase raffinate	1.51	15.9	12.1
Invertase 60% conversion	0.41	19.5	4.1
Invertase 95% conversion	1.41	51.1	14.1
Lactase whole whey	0.54	55.9	128.7
Lactase permeate	0.12	41.7	28.8

Immobilized Enzyme Hydrolysis

The concentration of enzyme fixed to the solid support is very high and the contact time of the substrate which is pumped through the bed is therefore very short. Thus the plant size required for a hydrolysis operation will be several hundred times smaller than the tankage required for liquid enzyme treatment. The capital cost of the plant will be considerably less, and in most cases the plant can be movable so that installation costs are minimal.

The flow rate is measured in empty column volumes per hour (ECV/HR) and the degree of conversion can be varied by changing the flow rate as shown in Fig. 2. Thus the activity of an immobilized enzyme is best quoted as the flow rate under operating conditions which produces the required degree of conversion. The productivity is also dependent on the rate of activity decay and the factory lifetime of each enzyme charge. The decay of enzyme activity in a factory environment occurs due to thermal denaturation which is exponential with respect to time, and also due to poisoning by trace impurities in the substrate, which is directly proportional to throughput.

There are also practical limitations to the number of "half-lives" which can be utilized due to the acceptable "turn-down" ratio of pumps and ancillary equipment. For these reasons, it is a reasonable assumption that the enzyme will be used for three half-lives and that the average productivity during this period will be 50% of the initial productivity.

The running expenses of an immobilized enzyme rig vary significantly with size, but for the purposes of this cost comparison I will assume overhead costs of $10,000 yearly per cubic meter of column capacity. Thus defining the terms A as initial flow in ECV/HR; H, half-life in days; DS, dry solids as percentage; C, immobilized enzyme costs (in dollars per cubic meter). The total costs are

$$C + 10{,}000\,\frac{3H}{360} = C + 83.3H$$

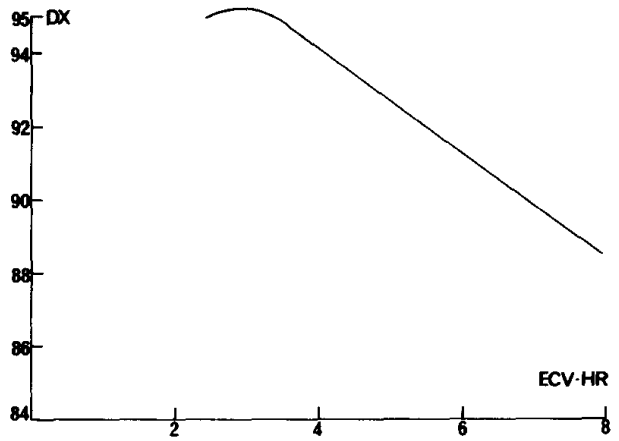


FIG. 2. Flow rate for immobilized glucoamylase saccharification. Enzyme-thinned substrate, 55°, pH 4.5, 30% dry solids. See text for description of empty column volumes per hour (ECV/HR).

and the productivity in tons DS during the enzyme lifetime is

$$A/2\ 3H\ 24\ \text{DS} = 36\ A\ H\ \text{DS}$$

$$\therefore\quad \text{Cost/ton DS} = \frac{C + 83.3H}{36A\ H\ \text{DS}}$$

The equivalent costs for the liquid hydrolysis processes considered earlier are as follows:

Glucoamylase Immobilized enzyme cost \$30,000/m^3
Mainstream: initial activity, 4 ECV/HR; half-life 70 days
Raffinate: initial activity, 15 ECV/HR; half-life 70 days
Invertase Immobilized enzyme cost \$30,000/m^3
60% conversion: 6 ECV/HR; half-life 80 days
95% conversion: 2 ECV/HR; half-life 80 days
Lactase Immobilized enzyme cost \$80,000/m^3
Whey, 90% conversion: 15 ECV/HR; half-life 30 days
Permeate, 70% conversion: 20 ECV/HR; half-life 40 days

Operation	Initial rate	Empty column residence time (min)	Total process cost/ton DS
Glucoamylase mainstream	4	30	\$11.11
Glucoamylase raffinate	15	8	5.3
Invertase 60% conversion	6	20	3.3
Invertase 95% conversion	2	60	12.7
Lactase whole whey	15	8	84.9
Lactase permeate	20	6	14.5

The following tabulation summarizes the comparative economics of immobilized and liquid enzymes, comparing three different enzymes, a range of degrees of conversion, and a range of syrup concentrations.

	Liquid		Immobilized	
Operation	Residence time (hr)	Total cost	Residence time (min)	Total cost
Glucoamylase mainstream	36.5	$ 15.8	30	$11.1
Glucoamylase raffinate	15.9	12.1	8	5.3
Invertase 60% conversion	19.5	4.1	20	3.3
Invertase 95% conversion	51.5	14.1	60	12.7
Lactase whole whey 90%	55.9	128.7	8	84.9
Lactase permeate 70%	41.7	28.8	6	14.5

The minimum saccharification cost for liquid hydrolysis would be obtained if saccharification tanks were run at constant capacity 360 days per year, 24 hr per day. Immobilized enzyme systems can still be more cost effective if the total process costs are considered, although the actual enzyme cost component is greater.

There is clearly some generalization in these cost comparisons and specific cases may show greater or smaller cost advantages than those calculated. The capital cost charges represent some 25% of the liquid enzyme treatment costs and unless the equipment can be utilized for other purposes the incentive to change to an immobilized enzyme system is considerably less.

In conclusion, immobilized enzyme systems using sufficiently active products can offer cost advantages to the user on the basis of total manufacturing costs, in addition to a number of process advantages which are also provided. The cost advantages are not sufficient at the current stage of development to induce all liquid enzyme users to change, but further improvements are probable which could lead to even more favorable process economics.

translates into a significant savings of energy and labor costs. This process can be operated automatically when a computer control system is applied. Also, using minimal instrumentation, fixed-rate control of the fermentation is available. Higher alcohol concentration (more than 10% v/v) can be attained if a suitable strain is used. In this case, still more than 10 times higher productivity, compared with conventional batchwise fermentation, can be obtained. Conversion yield is 90–95% versus the theoretical maximum yield. In total, this process should economize the alcohol fermentation process to a great extent.

At present, a combined process using this immobilized yeast cell process and a vacuum fermentation technique is under investigation at the pilot plant in Hofu Plant as a project of RAPAD. Accordingly, we expect to obtain much higher alcohol fermentation efficiency through higher productivity of alcohol production and lesser amounts of waste liquor.

Acknowledgment

This work has been done as a part of the Biomass Utilization Project of the Research Association for Petroleum Alternatives in Japan.

[37] Regiospecific Interesterification of Triglyceride with Celite-Adsorbed Lipase

By SHIGERU YAMANAKA and TAKASHI TANAKA

Chemical interesterification has been used to modify the physical properties of triglyceride mixtures in the oils and fats industry. In the process, a chemical catalyst is used to promote acyl migration among glyceride molecules. The resulting products consist of glyceride mixtures in which the fatty acid moieties are randomly distributed among the glyceride molecules. It is also well known that during hydrolysis or synthesis of triglycerides lipases (EC 3.1.1.3) show specifities toward the carbon position of the glycerol molecule and the acyl residue with the use of such lipases products not obtainable by chemical interesterification methods may be produced. Interesterification with pancreatic lipase in an aqueous system has been reported for obtaining palmitate-enriched glyceride from glyceryl-1-palmitate 2,3-dioleate and palmitic acid.[1] In this reaction sys-

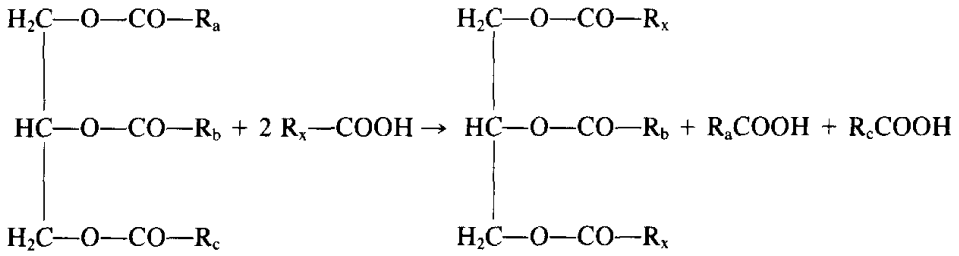
[1] R. W. Stevenson, F. E. Luddy, and H. L. Rothbart, *J. Am. Oil Chem. Soc.* **56,** 659 (1979).

tem the yield of triglyceride is low, probably due to the large amount of buffer solution used.

Recently, interesterification with carrier-adsorbed lipase was successfully attempted in organic solvent systems for the production of cacao butterlike fat from fats and oils containing triglycerides with oleic acid residues at the 2-position, fatty acids, aliphatic alcohol esters of fatty acids, or other fats and oils.[2-6]

In such a reaction system in the presence of a small amount of a trihydric alcohol such as glycerol (more than 0.1% of the raw material), water, or a buffer solution (less than 2 % of the total weight of the reaction mixture), lipase catalyzes the interesterification reaction when a carrier is used as a disperser of the enzyme.

This paper describes a regiospecific interesterification reaction method for triglyceride in *n*-hexane using Celite-adsorbed lipase in the presence of a small amount of glycerol or buffer solution. A simplified reaction scheme is shown below (see also Table I).



We will also describe the isolation method for reformed triglyceride from the reaction mixture, and analytical methods for total fatty acid composition and fatty acid in the 2-position of the triglycerides. (Analysis of fatty acid in the 2-position of triglyceride discloses the position of individual fatty acids within a triglyceride.)

Experiment A: Preparation and Application of Lipase Adsorbed to Celite Coated with Glycerol

Reagents

Olive oil (commercial product of Yoshida Pharmaceuticals Ltd., Japan)

[2] T. Tanaka, E. Ono, M. Ishihara, S. Yamanaka, and K. Takinami, *Agric. Biol. Chem.* **45,** 2387 (1981).

[3] T. Tanaka, M. Ishihara, and E. Ono, *Hakko to Kogyo* **41,** 375 (1983).

[4] T. Tanaka, E. Ono, and K. Takinami, *British Patent* 2,042,579 (1982).

[5] M. H. Coleman and A. R. Macrae, *British Patent* 1,577,933 (1980).

[6] T. Matsuo, N. Sawamura, Y. Hashimoto, and W. Hoshida, *British Patent Application* 2,035,359A (1980).

Stearic acid
Glycerol [water content in glycerol was less than 1.5% (Karl Fischer titration)]
Lipase (Seikagaku-kogyo Co., Japan, *Rhizopus delemar,* fine grade 200 units/mg solid) (one unit is defined as that liberating 1 μmol equivalent of fatty acid from oil or fat per minute at 25°)
Celite (Johns-Manville Sales Co., United States, No. 535)
n-Hexane

Procedure. Two grams of Celite is washed three times with about 20 ml of deionized water and dried *in vacuo* for 2 days at 50°. About 10 ml of methanol containing less than 0.1% water and 0.2 ml of glycerol is added to the dried Celite. After thoroughly mixing, methanol is evaporated at 50° *in vacuo*. The Celite coated with glycerol is transferred into 40 ml of *n*-hexane in a 100-ml flask and mixed thoroughly to obtain a suspension. Ten grams of olive oil and 10 g of stearic acid are added to the above suspension. Then finally 40 mg of lipase is added to the suspension. In this way lipase is adsorbed to the glycerol-coated Celite. This reaction mixture is shaken in a rubber-stoppered flask on a reciprocal shaker at 37° for 24 hr (120 strokes/min).

Isolation of Triglyceride. When the enzyme reaction is completed, Celite is eliminated by decanting. The reaction mixture containing 0.2–0.5 g of triglyceride is loaded on a Florisil column. (Preparation of column: 30 g of 60–80 mesh Florisil containing 7% water is dispersed in 100 ml of *n*-hexane, and the suspension is packed in a 2 × 25 cm column. Then the column is washed with 5 column-volumes of *n*-hexane.) The column is then eluted with the solvent system composed of diethyl ether and *n*-hexane (1 : 4, v/v). The eluted fractions (40–80 ml) are collected, and concentrated to about 1 ml. After checking that this concentrate is free from diglycerides, monoglycerides, and fatty acids by the conventional analytical TLC method, it is subjected to the following analyses.

Analysis of the Total Fatty Acid Composition. One hundred and fifty milligrams of triglyceride is hydrolyzed with 3 ml of 0.5 *N* NaOH in methanol at 80° for 10 min in a test tube equipped with a Liebig condenser. Then the fatty acid in the reaction mixture is subjected to methyl ester preparation for further analyses by gas chromatography.

Preparation of fatty acid methyl esters is basically carried out using the American Oil Chemists Society method (Ce 2-66). To the hydrolyzate is added 3.5 ml of 7% BF_3–methanol reagent and the reaction mixture is boiled for 2 min. After the addition of 2.5 ml of *n*-hexane the reaction mixture is boiled for an additional minute. After cooling to room temperature, 15–20 ml of saturated NaCl aqueous solution is added to the mix-

ture. One milliliter of the *n*-hexane layer containing methyl esters of fatty acid is removed with a pipette and dehydrated on anhydrous sodium sulfate. This dehydrated *n*-hexane layer is used as the sample for analyses. The sample (0.5–5 μl) is injected directly in a gas chromatograph. Analysis is carried out by the conventional method.

Analysis of the Fatty Acid in the 2-Position of a Triglyceride. The method is modified from that described by Usui *et al.*[7]

One-tenth gram of triglyceride is added to 7.5 ml of 1% poly(vinyl alcohol) (average degree of polymerization 200), and the suspension is homogenized twice for 3 min using a homogenizer. To 7 ml of the homogenizate, 2 ml of 0.5 *M* phosphate buffer (pH 8.0) and 0.5 mg of lipase (Sigma Type IV porcine pancreas) are added. The reaction mixture is then kept at 40° for 1 hr. To stop the reaction, 2.5 ml of an acetone–ethanol mixture (1 : 1, v/v) is added to the reaction mixture, which is then acidified by the addition of 2.5 ml of 1 *N* HCl. The acidified reaction mixture is subjected to extraction with diethyl ether 3 times. After the ether layer is washed with water twice, the washed ether layer is dehydrated with sodium sulfate. Monoglyceride isolated by conventional preparative TLC from the dehydrated ether layer is used as a sample for further analyses.

Finally, the fatty acid composition of monoglyceride is analyzed by the same method used for total fatty acid composition analyses.

Experiment B: Preparation and Application of Lipase Adsorbed to Celite Coated with Buffer Solution

Reagents

Oleic safflower oil (product of the United States, extracted from safflower species having oil rich in oleic acid)
Palmitic acid
0.3 *M* TES [*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid] buffer (pH 6.5)
Lipase I (Seikagaku-kogyo Co., Japan, *Rhizopus delemar,* fine grade 200 units/mg solid)
Lipase II (Meito-sangyo Co., Japan, *Candida cylindracea,* Lipase MY, 42 units/mg solid)
Celite (Johns-Manville Sales Co., United States, No. 535)
n-Hexane

Procedure. TES buffer (0.1 ml) is mixed with 1 g of Celite in a test tube and the mixture is shaken until Celite is coated with buffer solution as

[7] H. Usui, H. Kuwayama, and M. Nagakura, *Yukagaku* **20,** 284 (1971).

uniformly as possible. Celite coated with buffer solution is transferred to a 100-ml flask containing 40 ml of *n*-hexane. The suspension is mixed thoroughly. Then 10 g of oleic safflower oil and 10 g of palmitic acid are added to this suspension. Finally 20 mg of lipase I or 100 mg of lipase II is added to this suspension, respectively. (In this way the enzyme is adsorbed onto the buffer-coated Celite.) The reaction mixture is shaken at 30° for 3 days on a reciprocal shaker (120 strokes/min).

Isolation of Triglyceride. Same as in the case of Experiment A.

General Considerations

Hydrolysis and resynthesis of glycerides occur because lipase reactions are reversible. When the water content in the reaction system is restricted, the hydrolysis of the oil or fat is minimized, permitting lipase-catalyzed interesterification to occur. Free fatty acid exchanges with the fatty acid moieties of the triglycerides to produce novel triglycerides, incorporating the added fatty acid. When 1,3-specific lipase is used, the reaction occurs only at the 1- and 3-positions of the glycerides, whereas if nonspecific lipase is used, the reactions occur at all three positions.[2,8]

An interesterification reaction using olive oil and stearic acid is described as the first experiment (A). In this experiment, lipase is considered to be activated by a small amount of glycerol which coats the Celite. Activation in this case means that the lipase is put into an active form. In this way it is possible to convert olive oil to a new reformed fat where the oleic acid moieties at the 1- and 3-positions are replaced by stearic acid (25–30%).

In the second experiment (B) an interesterification reaction is described in which a small amount of TES buffer (pH 6.5) is used as an activator. Such Celite-adsorbed lipase from *Rhizopus delemar* converted safflower oil to a reformed fat, where oleic acid moieties at the 1- and 3-positions were replaced by palmitic acid moieties. The analytical data on the total fatty acid composition and the 2-position fatty acid composition of the fat are shown in Table I.

The oleic safflower oil, composed of about 75% oleic acid and 20% linoleic acid, was converted to an interesterified fat composed of about 50% oleic acid and 40% palmitic acid.

When the lipase from *Candida cylindracea* was used, palmitic acid exchanged with oleic acid at random on all three positions of the glyceride (Table I).

[8] A. R. Macrae, *J. Am. Oil Chem. Soc.* **60**, 291 (1983).

TABLE I
FATTY ACID COMPOSITION OF OLEIC SAFFLOWER OIL INTERESTERIFIED TRIGLYCERIDE[a]

Composition and reaction	Oleic safflower oil		Interesterified triglyceride			
			Lipase I		Lipase II	
	Total (%)	2-Position (%)	Total (%)	2-Position (%)	Total (%)	2-Position (%)
Palmitic acid	6.2	0.2	40.1	2.1	47.0	34.0
Stearic acid	2.2	0.5	0.8	0.1	2.0	0.1
Oleic acid	75.5	77.2	48.4	75.0	42.2	54.1
Linoleic acid	17.0	23.2	11.1	23.0	9.1	13.2
Main reaction	O,L + O + O,L	O,L + L + O,L (+ Pal)	P,O,L + O + P,O,L	P,O,L + L + P,O,L	P,O,L + O + P,O,L	P,O,L + L + P,O,L
						P,O,L + P + P,O,L

[a] Lipase I, lipase of *R. delemar;* lipase II, lipase of *C. cylindracea.* O, oleoyl; L, linoleoyl; P, palmitoyl; and Pal, palmitic acid.

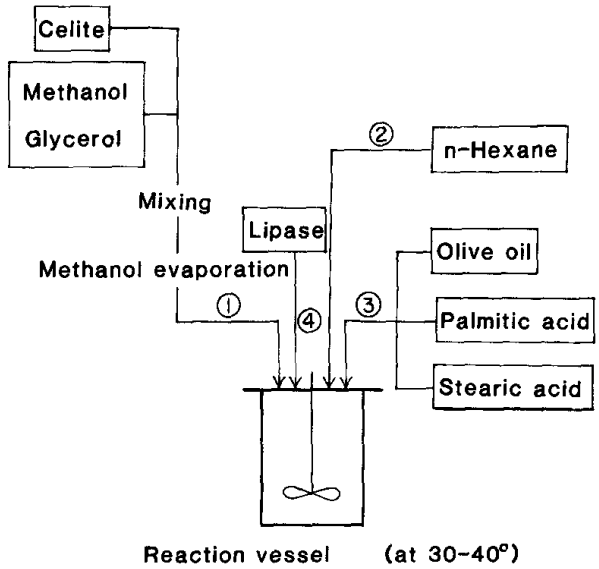


FIG. 1. The lipase-catalyzed interesterification setup; A flowchart of a semiscale enzyme process is given. Addition of carrier, reactants, and catalyst is carried out according to the numbers designated.

Production of a cacao butterlike fat using 1,3-specific lipases is very interesting to the oils and fats industry. An illustration of an enzyme-catalyzed reaction in a semiscale reactor is presented in Fig. 1. The enzyme reaction is carried out in a stirred tank fermenter, equipped with temperature recording and control at 30–40°, under agitation for 24–72 hr. The reaction mixture is agitated at a speed giving a uniform dispersion. After the enzyme reaction, the Celite particles are separated from the reaction mixture, and the solvent is removed by evaporation. From this oils and fats fraction, the interesterified triglycerides (or cacao butterlike fat fraction) is concentrated and purified, using ethanol in an ordinary fractionation procedure, accompanied by differential scanning calorimetric analyses.

In order to make this process practical from an industrial point of view, repeated use of adsorbed lipase as well as utilization of cheap raw material (like the midfraction of palm oil) and efficient fractionation of the products are indispensable.

[38] Large-Scale Production and Application of Immobilized Lactase

By J. L. BARET

Whey is a major by-product of the dairy industry. Most of it is spray-dried or processed and used in a variety of applications as a food, a feed, or a fermentation substrate. Ultrafiltration techniques have also been developing for the past decade and are now becoming a well-established technology to recover whey proteins and also to process milk in cheese making. Secondary by-products from the ultrafiltration, known as whey or milk permeates, are now produced in a significant amount. A continuous effort for better utilization of wheys and permeates is being made by the dairy industry. The hydrolysis of lactose into glucose and galactose appears to be an interesting approach to widen the profitable uses of wheys and permeates.

Hydrolyzed lactose is sweeter and more soluble than lactose; it also presents several additional advantages which allow producers to obtain new attractive products. This report presents some aspects of the immo-

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Of these factors, the specific activity and the half-life ($t_{1/2}$) are affected most by the immobilization method. The specific activity may also be affected by the initial activity of the microorganism. The initial activity is not as important as the retained activity and the catalyst half-life ($t_{1/2}$), however, due to the labor costs associated with the packing of columns. The support resin is the most expensive factor in the above equation.

Thus ratios above 1:1:1 *E. coli* are clearly preferred for phenylalanine production and with half-lives in excess of 8 months,[2] the economic viability of this process is quite high. A 600 metric ton/year plant has been constructed. Catalyst columns of heights exceeding 10 ft and diameters as large as 4 ft have been used without restriction of flow rate or deterioration of flow distribution during operation. Other support resins have also been used with excellent success.

[46] Production of Aspartame by Immobilized Thermoase

By KIYOTAKA OYAMA, SHIGEAKI IRINO, and NORIO HAGI

Aspartame (α-L-Asp-L-Phe-OMe) is the methyl ester of the terminal dipeptide of the digestive hormone gastrin. It is about 200 times sweeter than sucrose and has a pleasant sweetness without a bitter aftertaste.[1] In the preparation of aspartame by conventional organic synthetic methods, it is necessary to protect the side-chain carboxylate of aspartic acid (Asp) in order to avoid the formation of the unwanted β-peptide linkage. However, in order to reduce production cost it is better to omit protection and deprotection of the side-chain carboxylate. Therefore, all of the chemical synthetic methods with industrial potential do not involve protection of the side-chain carboxylate and thus ordinarily produce 20–40% of the β isomer, which has a bitter taste, along with the desired α isomer.[2–4]

In recent years, the enzymatic synthesis of peptide by the reverse of the hydrolysis of the peptide bond has been receiving increasing attention.[5,6] We investigated whether the enzymatic method could be applied to the synthesis of aspartame, and found that some proteinases can cata-

[1] R. H. Mazur, J. M. Schlatter, and A. H. Goldkamp, *J. Am. Chem. Soc.* **91,** 2684 (1969).
[2] *U.S. Patent* 3,786,039 (1974).
[3] *U.S. Patent* 3,833,553 (1974).
[4] *U.S. Patent* 3,933,781 (1976).
[5] J. S. Fruton, *Adv. Enzymol.* **53,** 239 (1982).
[6] K. Oyama and K. Kihara, *Jpn. Chem. Rev.* **35,** 195 (1982); *CHEMTECH* **Feb.,** 100 (1984).

lyze the condensation reaction between *N*-protected Asp and phenylalanine methyl ester (Phe-OMe) in high yield.[7] The reaction is specific only on the α-carboxylate of Asp, even though the side-chain carboxylate is unprotected, and moreover, only the L-amino acids participate in the reaction when the racemic amino acids are used. In addition, the enzymatic reaction can be done under mild reaction conditions. From these findings we thought that the enzymatic method might become an economical way to produce the sweetener, and therefore have carried out research on the development of an industrial technology based on this novel method.

Selection of the Enzymes

Extensive screening of the commercially available proteinases revealed that some enzymes that cleave the peptide bond of proteins at the amine site of a hydrophobic amino acid residue can also catalyze the condensation between *N*-protected Asp and Phe-OMe. Thus the enzymes belonging to the group of metalloproteinases (EC 3.4.24._) are found to give the product in a high yield. Thiolproteinases (EC 3.4.22._) such as papain can also effect the reaction, but due to their concurrent esterase activity hydrolyses of the ester linkages of Phe-OMe in the reactant as well as in the product occur as side reactions, resulting in a lower yield of the desired product. Among the enzymes investigated, thermolysin, which is obtained from the strain found in a Japanese hot spring (Bacillus thermoproteolyticus Rokko), shows a high catalytic activity and a marked stability against heat, organic solvents, and extreme pH, and does not show esterase activity.[8] These properties are desirable in a catalyst for industrial use.

A crude enzyme preparation is often contaminated with other enzymes that cause unwanted side reactions. However, it was found that thermoase, a crude preparation of thermolysin, which is commercially available and is much cheaper than thermolysin, does not show any side reactions, so it was used for the present study.

Selection of the Protecting Group and the Raw Materials

The enzymes that can catalyze the condensation between the two amino acids are endoproteinases, so it is necessary to protect the amino group of Asp and the carboxylic group of phenylalanine (Phe) in order for the reaction to take place. Several protecting groups that are commonly

[7] Y. Isowa, M. Ohmori, T. Ichikawa, K. Mori, Y. Nonaka, K. Kihara, K. Oyama, H. Satoh, and S. Nishimura, *Tetrahedron Lett.* 2611 (1979).

[8] S. Endo, *J. Ferment. Technol.* **40,** 346 (1962).

employed in organic peptide synthesis were found to be effective for the present reaction, and we selected the carbobenzoxyl group (Z) for the protection of Asp, since it is rather cheap and versatile, and gives a high reaction yield. Since the C-terminus of aspartame is the methyl ester, phenylalanine methyl ester was used directly.

As raw materials, we selected L-Asp and DL-Phe for the following reasons: (1) L-Asp is available cheaply, whereas L-Phe is much more expensive than the racemic mixture; in fact the high price of L-Phe is currently the bottleneck in industrial production by chemical methods; (2) our kinetic study showed that Z-D-Asp acts as a competitive inhibitor in the enzymatic condensation, but D-Phe-OMe does not interfere with the reaction at all[9]; (3) the racemization of unreacted Z-D-Asp without destroying the Z protecting group, which is much more expensive protection group than the methyl ester protection group of Phe, is difficult, whereas the racemization of D-Phe to DL-Phe can be easily done.

The entire process of the enzymatic production of aspartame, consisting of six reaction steps, is shown in the following scheme:

N-Protection

$$\text{L-Asp} + \text{Z-Cl} \rightarrow \text{Z-L-Asp} \quad (1)$$

Esterification

$$\text{DL-Phe} + \text{MeOH} \rightarrow \text{DL-Phe-OMe} \quad (2)$$

Condensation

$$\text{Z-L-Asp} + \text{DL-Phe-OMe} \rightarrow \text{Z-L-Asp-L-Phe-OMe} \cdot \text{D-Phe-OMe} \quad (3)$$

Separation

$$\text{Z-L-Asp-L-Phe-OMe} \cdot \text{D-Phe-OMe} \rightarrow \text{Z-L-Asp-L-Phe-OMe} + \text{D-Phe-OMe} \quad (4)$$

Hydrogenolysis

$$\text{Z-L-Asp-L-Phe-OMe} \rightarrow \text{L-Asp-L-Phe-OMe} \quad (5)$$

Racemization

$$\text{D-Phe-OMe} \rightarrow \text{DL-Phe} \quad (6)$$

where Z-Cl is carbobenzoxychloride. Of the above reactions the key step is the enzymatic condensation. From the viewpoint of industrial production, the establishment of a technology for repeated use of the enzyme is of great importance.

Use of Immobilized Thermoase for the Condensation between Z-Asp and Phe-OMe

The synthesis of Z-L-Asp-L-Phe-OMe from Z-L-Asp and DL-Phe-OMe and the hydrolysis back to the reactants catalyzed by thermoase are in

[9] K. Oyama, K. Kihara, and Y. Nonaka, *J. Chem. Soc. Perkin Trans.*, 356 (1981).

equilibrium, with the equilibrium lying in the hydrolysis direction (K = 1.5 M^{-1}).[10] Therefore in order to obtain a high yield, it is necessary to shift the equilibrium toward the synthesis side. One approach is to make the product sparingly soluble in the reaction medium. In aqueous solution, the reaction between Z-L-Asp and DL-Phe-OMe gives Z-L-Asp-L-Phe-OMe, which deposits as an extremely insoluble salt with D-Phe-OMe, resulting in almost quantitative yield under reasonable concentration of the reactants (~1 M).[6]

Another approach that may enhance the yield of the product is to add a water-miscible organic cosolvent, which can shift the equilibrium toward the synthesis side.[11] A third approach is the addition of a water-immiscible organic cosolvent, resulting in a two-phase system. In a system where the reactants are soluble in the aqueous layer and the product in the organic layer, transfer of these compounds through the layers may overcome the unfavorable equilibrium. From the viewpoint of the application of an immobilized enzyme to the present system, the first approach is impractical, since separation of the catalyst from the deposited product is very difficult. Therefore only the latter two approaches may be of practical value.

Selection of the Organic Cosolvent

In selecting the organic cosolvent, one must consider the effect of cosolvent on the following aspects of the reaction: (1) the activity and the stability of the enzyme, (2) the equilibrium, (3) the solubility of the product, and (4) the partition of the reactants between the aqueous phase and the organic phase when a water-immiscible cosolvent is used. We investigated a number of organic cosolvents and found that, in the presence of a water-miscible cosolvent, the activity as well as the stability of the enzyme were markedly impaired, although some cosolvents could shift the equilibrium toward the synthesis side. (For example, in a 50% methanol–H_2O solution, the equilibrium constant was found to be increased approximately 3-fold as compared with pure H_2O.[12])

On the other hand, with a water-immiscible cosolvent, the impairing effects were found to be generally smaller than with a water-miscible cosolvent, since in the former case, the enzyme could stay in the aqueous layer. Among these solvents, however, aliphatic and aromatic hydrocar-

[10] K. Oyama, S. Irino, T. Harada, and N. Hagi, *in* "Enzyme Engineering VII" (A. I. Laskin, G. T. Tsao, L. B. Wingard, Jr., eds.), p. 95. The New York Academy of Science, New York, 1984.

[11] G. A. Homandberg, J. A. Mattis, and M. Laskowski, Jr., *Biochemistry* **17**, 5220 (1978).

[12] K. Oyama and S. Irino, unpublished results.

bons are unsatisfactory, since they hardly dissolve the product. Solvents such as chloroform, dichloroethane, ethyl and isopropyl acetates, and methyl isobutyl ketone can satisfy the requirements to some extent, but we selected ethyl acetate for the following reasons: (1) the impairing effects on the enzyme are smaller than with the others, especially chlorinated hydrocarbons, which adversely effect the enzyme; (2) the solubility of the product in ethyl acetate is high, and the partitions of the reactants into the organic layer are rather low (the partition constants between the organic layer and the aqueous layer for Z-Asp and Phe-OMe were found to be 0.13 and 0.30, respectively[12]). These facts indicate that most of the reactants can stay in the aqueous layer where the enzymatic reaction takes place, and the product can transfer to the organic layer, thus shifting the equilibrium toward the less favorable synthesis side.

Immobilization of Thermoase

From the industrial point of view, the reaction rate needs to be sufficiently fast in order to minimize the capital investment. In the present case, however, the rate of condensation is much slower than in pure water system due to the presence of an organic cosolvent. In order to overcome such a handicap, it is preferable to immobilize the enzyme on a support with as high volumetric activity as possible. We investigated various immobilization methods, which include physical adsorption, ionic binding, and covalent binding.

Physical adsorption was studied using Amberlite XAD-7 and XAD-8. The resins have a high adsorptive power to proteins owing to hydrophobic interactions, and are often used for the recovery of enzymes from a fermentation broth.[13] In fact, Amberlite XAD-7 could immobilize the largest amount of thermoase per weight of the supporting material (Table I). In addition, the procedure for the immobilization is very simple as shown later, rendering the method very attractive.

Ionic binding was studied using Amberlite IRA-94 and IRC-50, representative anionic and cationic ion-exchange resins, respectively. The method is as simple as physical adsorption, but only a small amount of the enzyme could be immobilized on the resins under various binding conditions.

Covalent binding was studied using Toyopearl gel as a supporting material. Toyopearl is a hydrophilic polymer gel which was originally developed for use in high-performance gel filtration chromatography.[14] It

[13] H. Y. Ton, R. D. Hughes, D. B. A. Silk, and R. Williams, *J. Biomed. Mater. Res.* **13,** 407 (1979).

[14] J. Germeshausen and J. D. Karkas, *Biochem. Biophys. Res. Commun.* **99,** 1020 (1981).

has good mechanical strength, even when heated or in the presence of organic solvents, and carries many hydroxyl groups available for immobilization. These properties are attractive in a support for immobilization of enzymes. Thermoase was immobilized on Toyopearl gel via several routes as shown in Fig. 1.

Thermolysin is known to have a large number of tyrosine residues in the molecule,[15] and so immobilization via the diazo coupling method appeared promising. However, such attempts were not successful, since the activity of immobilized thermoase was very small, although good yields of immobilization were attained (Table I). The low activity of immobilized thermoase is presumably due to the destruction of its rigid three-dimensional structure, which derives from the hydrogen bondings of the tyrosine residues and which is known to be the key of the unusual stability of thermolysin.[16]

As noted in Table I, the binding by glutaraldehyde or cyanuric chloride via treatment with ethylenediamine appears to be advantageous, since these gave an immobilized enzyme with a large quantity of active thermoase. The immobilization via treatment with CNBr, followed with ε-aminocaproic acid, and then with the water-soluble carbodiimide [*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride] was unsatisfactory because the yield was very poor.

The experimental procedures for immobilization by physical adsorption on Amberlite XAD-7 and by covalent bindings to Toyopearl by glutaraldehyde and cyanuric chloride are described below.

Materials

Thermoase: Daiwa Kasei Co. (Osaka, Japan), activity of 1.6×10^6 protease units per gram (determined by Anson's method[17] modified by Hagihara[18]).

Amberlite XAD-7, XAD-8, IRA-94, and IRC-50: Rohm & Haas Co.

Toyopearl: Toyo Soda Manufacturing Co. (Tokyo, Japan).

Calcium buffer: 10 m*M*, pH 7.5.

L-Asp and DL-Phe: Nippon Rikagaku Yakuhin Co. (Tokyo, Japan), reagent grades.

Carbobenzoxychloride (Z-Cl): Kokusan Kagaku Co. (Tokyo, Japan), purity greater than 95%.

Physical Adsorption to Amberlite XAD-7. Ten grams of a water-wet Amberlite XAD-7 is stirred in 50 ml of the calcium buffer containing 3 g of

[15] Y. Ohta, Y. Ogura, and A. Wada, *J. Biol. Chem.* **241,** 5919 (1966).
[16] Y. Ohta, *J. Biol. Chem.* **242,** 509 (1967).
[17] M. L. Anson, *J. Gen. Physiol.* **22,** 79 (1934).
[18] B. Hagihara, *Annu. Rep. Sci. Works, Fac. Sci. Osaka Univ.* **2,** 35 (1954).

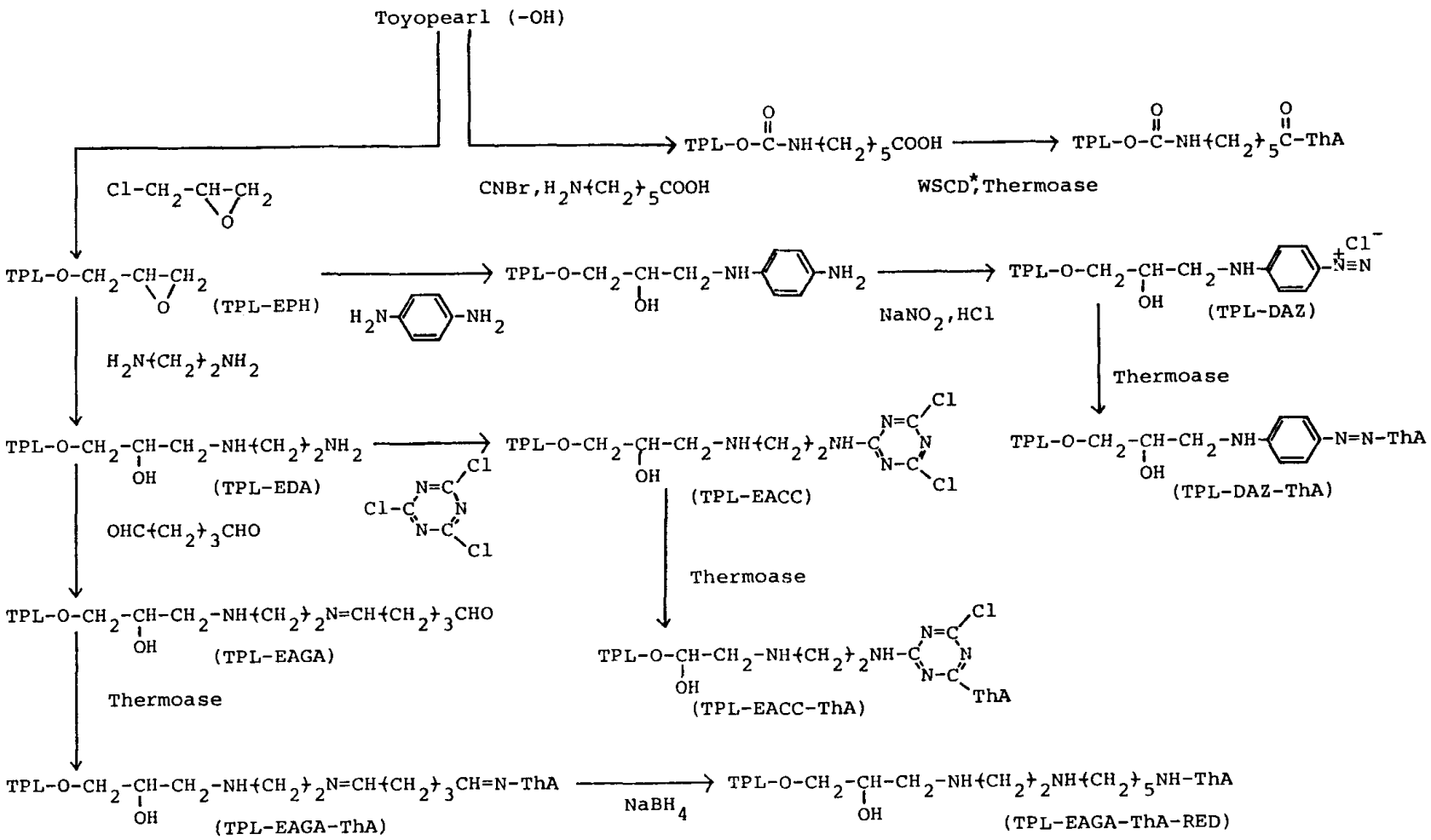

FIG. 1. Reaction scheme of the immobilization of thermoase on Toyopearl by covalent binding methods. *WSCD, Water-soluble carbodiimide.

TABLE I
AMOUNT OF THERMOASE IMMOBILIZED ON THE SUPPORTS AND RELATIVE ACTIVITY AS COMPARED WITH NATIVE THERMOASE

Supporting material	Amount of thermoase[a]	Relative activity[b]
Physical adsorption		
Amberlite XAD-7	2.31	1.0
Amberlite XAD-8	2.10	1.0
Ionic binding		
Amberlite IRA-94	0.53	0.8
Amberlite IRC-50	0.25	0.8
Covalent binding		
TPL-EAGA[c]	1.52	1.1
TPL-EAGA-ThA-RED[d]	1.38	0.9
TPL-EACC[e]	0.76	0.7
TPL-DAZ[f]	1.79	0.3

[a] Grams per 10 g of the wet immobilized thermoase.
[b] The relative activity is determined on the basis of the same amount of the immobilized and the native enzymes.
[c] Toyopearl activated with ethylenediamine–glutaraldehyde.
[d] Immobilized thermoase on TPL-EAGA reduced with $NaBH_4$.
[e] Toyopearl activated with ethylenediamine–cyanuric chloride.
[f] Toyopearl activated with phenylenediamine followed by diazo coupling.

thermoase at 10° for 3 hr. Immobilized thermoase is obtained by filtration followed by washing with 50 ml of the calcium buffer.

Activation of Toyopearl. Toyopearl gel is washed on a glass filter with distilled water, and dried *in vacuo* at 60°. Fifty grams of the dry gel, 200 ml of 1 *N* NaOH, and 11 ml of epichlorohydrin are placed in a 500-ml flask and stirred for 3 hr at 30°. The contents are filtered by a glass filter and washed with cold distilled water. The gel thus obtained, designated as TPL-EPH, is added to a 500 ml flask containing 63 ml of ethylenediamine and 35 ml of distilled water. The mixture is stirred for 1.5 hr at 80°, and then filtered by a glass filter. The gel is washed with acetone several times to remove unreacted ethylenediamine, and dried *in vacuo* at 60° overnight. This gel is designated as TPL-EDA.

Immobilization by Glutaraldehyde. Fifty grams of dry TPL-EDA is added into a 1-liter flask containing 500 ml of 5% glutaraldehyde. After the

pH of the mixture is adjusted to 7.0 by 5 *N* NaOH, it is stirred overnight at 20°, and then filtered by a glass filter. The gel is washed successively with distilled water and with acetone to remove unreacted glutaraldehyde, and then dried *in vacuo* at 60°. This gel is designated as TPL-EAGA. The immobilization is carried out by stirring the mixture of 13.3 g of TPL-EAGA and 250 ml of the calcium buffer containing 5% of thermoase in a 500-ml flask at 4° for 24 hr. Immobilized thermoase, designated as TPL-EAGA-ThA, is obtained after filtration of the mixture and washing with the calcium buffer. The reduction of the C=N double bonds of TPL-EAGA-ThA is done as follows: 10 g of TPL-EAGA-ThA in 100 ml of the calcium buffer is treated with 0.5 g of $NaBH_4$ at 4°. The stirring is continued at the same temperature for 24 hr, and then the immobilized thermoase, designated as TPL-EAGA-ThA-RED, is obtained after filtration followed with washing by the calcium buffer.

Immobilization by Cyanuric Chloride. A mixture of 10 g of dry TPL-EDA, 130 ml of acetone, and 22 g of cyanuric chloride in a 500-ml flask is stirred at −10 to −5° for 30 min, followed by the dropwise addition of 10 g $NaHCO_3$ in 60 ml of distilled water at the same temperature. With the progress of the reaction the pH of the mixture decreases, so 5 *N* NaOH is added to keep pH at the constant value of 7.5. After 3 hr at 0°, the decrease of pH is no longer observed. The gel is filtered by a glass filter and washed with cold acetone to remove unreacted cyanuric chloride, and then dried *in vacuo* at 20° overnight. This gel is designated as TPL-EACC. The immobilization is carried out by stirring the mixture of 3.3 g of TPL-EACC and 50 ml of the calcium buffer containing 5% of thermoase in a 100-ml flask at 4° for 24 hr. Immobilized thermoase, designated as TPL-EACC-ThA, is obtained after filtration of the mixture followed by washing with the calcium buffer.

Determination of the Amounts of Thermoase Immobilized on the Supporting Materials

The amounts of thermoase can be determined by hydrolyzing the enzyme into the constituent amino acids, followed by high-performance liquid chromatography (HPLC) analysis of the hydrolyzate, since the peak areas of the amino acids in the hydrolyzate are found to correlate very well with the amount of thermoase, as shown in Fig. 2. Thus approximately 50 mg of an accurately weighed, dry immobilized thermoase and 10 ml of 6 *N* HCl are taken into a test tube, which is then sealed under vacuum. The sealed tube is heated at 110° for 24 hr in an oil bath. The contents are evaporated to dryness on a rotary evaporator, and the residue is treated with 0.67 *M* citric acid–sodium citrate buffer (pH 2.2). After

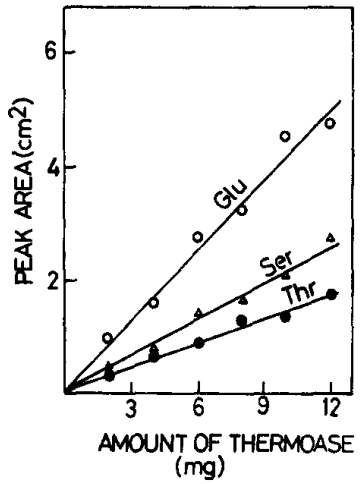

FIG. 2. Correlation between the amount of thermoase and the peak area of amino acids of the hydrolysate of thermoase determined by the amino acid analyzer.

the insoluble materials are removed by centrifugation, the supernatant solution is analyzed by an amino acid analyzer (TSK-HLC-805, Toyo Soda Manufacturing Co.) equipped with a 75 × 7.5 mm (I.D.) column packed with strong cation-exchange resin (TSK-GEL IEX-215SC, Toyo Soda Manufacturing Co.), a 10 m × 0.4 mm (I.D.) reaction coil, an *o*-phthalaldehyde supply, and a fluorescence spectrofluorometer with the excitation at 360 nm and the emission at 450 nm. The amount of thermoase is determined from standard calibration by the peak area of glutamic acid. The operating conditions of the amino acid analyzer are as follows: temperature of the column and the reaction coil, 50°; eluent, a mixture of citric acid (28.0 g), sodium citrate (19.6 g), β-thiodiglycol (2.5 ml), *n*-capric acid (0.1 ml), ethyl alcohol (80 ml), 20% Brij-35 (polyoxyethylene lauryl ether, 2.5 ml), in 800 ml deionized water, is adjusted to pH 3.3 by concentrated HCl, which is then made up to 1 liter by deionized water; *o*-phthaldehyde solution, a mixture of *o*-phthaldehyde (1 g) in 100 ml ethyl alcohol, 1 liter of the aqueous solution containing boric acid (49.4 g) with pH adjusted at 9.7 by 10 *N* KOH, a few drops of 2-mercaptoethanol, and 25% Brij-35 (2 ml); pressure and the flow rate of the eluent, 60–80 kg/cm^2 and 1.0 ml/min, respectively; pressure and the flow rate of the *o*-phthaldehyde solution, 15 kg/cm^2 and 1.0 ml/min, respectively.

Enzyme Assay

An Erlenmyer flask containing accurately weighed wet immobilized enzyme (1–1.5 g) and 50 ml of McIlvaine buffer (pH 6.0) is thermally preequilibrated at 40° for 5 min. The substrates stock solution (5 ml)

containing 2 mmol of Z-L-Asp and 4 mmol each of DL-Phe-OMe · HCl and NaOH is added, and then the flask is stoppered and placed in a waterbath shaker thermostated at 40°. After 30 min, 10 ml of 0.1 *M* EDTA is added to quench the reaction, and the amount of Z-L-Asp-L-Phe-OMe produced is measured by HPLC. The activity of immobilized thermoase is determined as a relative activity by comparison with the parallel control experiment using the standard sample solution of thermolysin. The HPLC analysis is done with a TSK-HLC-802 (Toyo Soda Manufacturing Co.) equipped with a 600 × 7.5 mm (I.D.) column packed with TSK-LS-170 (cross-linked polysaccharide gel, particle size of 5 μm, Toyo Soda Manufacturing Co.), and a UV detector (254 nm). The eluent is 0.5 *M* aqueous CH_3COOH–NaOH buffer (pH 6.0) with the flow rate of 9.2 ml/min (pressure 20 kg/cm^2).

Preparation of Substrates

Z-L-Asp. Carbobenzoxylation of L-Asp is carried out by the standard Schotten–Baumann method by using carbobenzoxychloride (Z-Cl) and aqueous NaOH.[19] After the reaction, the reaction mixture is made acidic (pH ~2) by the addition of concentrated HCl, and then stirred overnight at 0°. The deposited crystals are filtered, dried, and then recrystallized from ethyl acetate–*n*-hexane to give the white crystals of Z-L-Asp in 80% yield.

DL-Phe-OMe · HCl. Esterification of DL-Phe by CH_3OH and HCl is done by the standard Fisher method.[20] After the reaction, the reaction mixture is concentrated on a rotary evaporator to a slight turbidity, and then kept in a refrigerator overnight. The resulting deposited crystals are collected by filteration, washed with diethyl ether, and recrystallized from methanol–diethyl ether to give the white crystals of DL-Phe-OMe · HCl in a yield of 83%.

Operation

The enzymatic condensation of Z-L-Asp and DL-Phe-OMe was carried out in the biphasic system of water and ethyl acetate. A continuous column operation and a batchwise operation using thermoase immobilized by several methods were investigated.

Continuous Column Operation. Continuous column operation was studied using thermoase immobilized on Amberlite XAD-7 by physical adsorption and on Toyopearl by the covalent binding through ethylenedi-

[19] N. Izumiya, T. Kato, M. Ohno, and H. Aoyagi, "Peptide Synthesis," p. 25. Maruzen, Tokyo, 1975.

[20] M. Bergmann and L. Zervas, *Ber.* **65,** 1192 (1932).

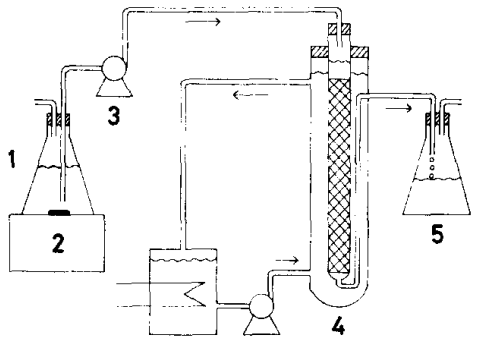

FIG. 3. The apparatus of the continuous column operation: 1, reactants reservoir; 2, magnetic stirrer; 3, feed pump; 4, column reactor; 5, product reservoir.

amine–glutaraldehyde (TPL-EAGA-ThA). The apparatus is shown in Fig. 3. It consists of a column reactor (350 × 30 mm I.D.) packed with the catalyst, a feed pump, and two reservoir vessels of the reactants and the product. The reactor is thermostated at 40° by a water-circulating jacket. The reactants mixture solution, consisting of 0.12 *M* of Z-L-Asp, 0.31 *M* of DL-Phe-OMe, and 0.012 *M* of $Ca(OAc)_2$ in ethyl acetate–water (7 : 3 in weight ratio), was continuously fed by the pump into the reactor from the top, and the product solution mixture exited via the bottom of the reactor. During the feed the mixture solution was magnetically stirred in order to maintain the homogeneity of the solution. Since the activity of the catalyst decreases with time, the space velocity was changed in order to maintain the reaction yield at a high level (80–90%).

The results of the operations are illustrated in Fig. 4, which shows that, although Amberlite XAD-7 can immobilize a large quantity of thermoase (Table I), the decrease of the activity is so rapid that it is far less satisfactory than TPL-EAGA-ThA. Analysis of the product solution by the amino acid analyzer after the hydrolysis at 110° in 6 *N* HCl as described before showed that the decrease was attributable to the leakage of thermoase from the support, largely due to the presence of the organic cosolvent and a high ionic strength by the reactants. Treatment of the catalyst with glutaraldehyde (which is shown as Amberlite XAD-7* in Fig. 4) in the hope of preventing such leakage was unsuccessful. In contrast, although TPL-EAGA-ThA showed a rapid decrease of the activity at the beginning of the operation, it gradually settled down. After 20 days of the operation, the operation was stopped and analysis of the catalyst activity was measured. It showed that 45% of the initial activity was lost during that period.

Batchwise Operation. Batchwise operation was studied using thermoase immobilized on Toyopearl by covalent binding methods, i.e., TPL-EAGA-ThA, TPL-EAGA-ThA-RED, and TPL-EACC-ThA. The reactions were carried out by shaking the flask containing the reaction mixture

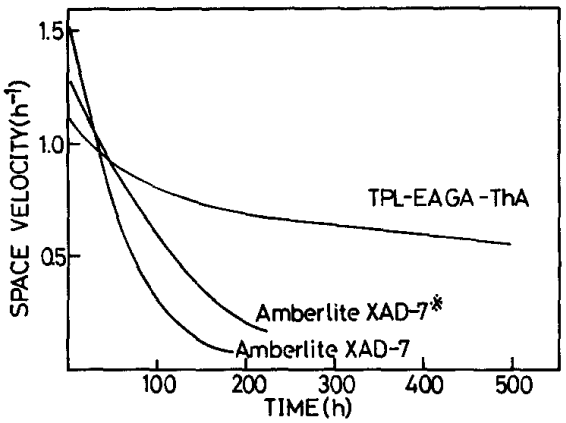

FIG. 4. Time course of continuous column operation of the production of Z-L-Asp-L-Phe-OMe · D-Phe-OMe from Z-L-Asp and DL-Phe-OMe by immobilized thermoase on Toyopearl gel and Amberlite XAD-7. TPL-EAGA-ThA, Thermoase immobilized on Toyopearl activated with ethylenediamine–glutaraldehyde; Amberlite XAD-7*, thermoase immobilized on Amberlite XAD-7 followed by treatment with glutaraldehyde.

at 120 strokes/hr in a waterbath shaker thermostated at 40°. The reaction mixture consisted of 2 g of Z-L-Asp, 3.4 g of DL-Phe-OMe, 13 ml of H_2O, 43 ml of ethyl acetate, 0.1 g of $Ca(OAc)_2$, and immobilized thermoase, the amount of which was chosen to give approximately the same enzyme activity for each catalyst. Therefore 6 g of TPL-EAGA-ThA, 8 g of TPL-EAGA-ThA-RED, and 19 g of TPL-EACC-ThA were used. After 22 hr of the reaction, the contents of the flask were filtered by a glass filter, and the recovered catalyst was washed with the calcium buffer, then used for the next run. The filtrate was analyzed by HPLC as described before to determine the yield of the produced Z-L-Asp-L-Phe-OMe. The results of the repeated runs are shown in Fig. 5.

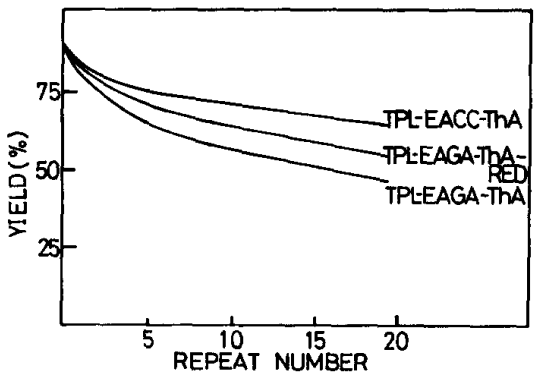

FIG. 5. Results of batchwise operation of the production of Z-L-Asp-L-Phe-OMe · D-Phe-OMe from Z-L-Asp and DL-Phe-OMe by immobilized thermoase on Toyopearl gel. TPL-EACC-ThA, Thermoase immobilized on Toyopearl activated with ethylenediamine–cyanuric chloride; TPL-EAGA-ThA, thermoase immobilized on Toyopearl activated with ethylenediamine–glutaraldehyde; TPL-EAGA-ThA-RED, $NaBH_4$-reduced TPL-EAGA-ThA.

As with the continuous column operation, a rapid decrease in activity was observed for all the catalysts studied during the early stage of the operation. This was then followed by a slower, gradual loss of activity during continued operation. After 20 repeated runs the operation was stopped and the activity of the used catalysts were measured, which showed that the activity loss of TPL-EAGA-ThA, TPL-EAGA-ThA-RED, and TPL-EACC-ThA were 38, 33, and 25%, respectively. The improvement of the stability by the reduction of the C=N double bond of TPL-EAGA-ThA indicates that the hydrolysis of the C=N bonds is occurring during the operation. Although TPL-EACC-ThA is less active than TPL-EAGA-ThA-RED, the rate of the activity loss is much slower in the former than in the latter; thus the former appears to be advantageous.

Summary

The enzymatic method for the production of aspartame has many advantages over chemical methods, e.g., cheap racemic phenylalanine can be used and no β-aspartame is produced. The former leads to a lowering in the cost of raw materials and the latter to a simplified purification procedure. The disadvantages as compared with chemical methods are that the racemization of unreacted D-Phe-OMe is necessary and that an expensive catalyst is used. The racemization of D-Phe-OMe can be done easily as stated before, but the recovery and recycling of the delicate biocatalyst are not easy, although thermoase is a very stable enzyme.

Here we have presented two possibilities for using immobilized thermoase, i.e., continuous column operation and batchwise operation. In both procedures, the use of an organic cosolvent is required, which leads to deactivation of the catalyst. Therefore it is most important to clarify the mechanism of deactivation by organic solvents and to find strategies to overcome it, perhaps by development of more sophisticated immobilization methods.

In an industrial operation using the batchwise mode, deterioration of the immobilized enzyme occurs by the friction caused by stirring. Furthermore, separation of the immobilized enzyme is necessary after batchwise reaction, which is troublesome and costly. In the continuous column operation, channeling of the organic and aqueous layers was observed in the packed column, thus rendering the efficiency of the catalyst low, and in addition, deactivation tended to occur more rapidly than during batchwise operation. Nevertheless, the continuous column operation seems to be more advantageous than the batchwise operation from the viewpoint of industrial production needs.