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(54) Title: SEPARATION METHOD

(57) Abstract

The present invention relates to a method of separating a particulate solid catalyst from a chemical reaction mixture which further comprises at least one other solid component, either during the reaction or after the reaction.

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SEPARATION METHOD

TECHNICAL FIELD

The present invention relates to a method of separating a particulate solid catalyst from a chemical reaction mixture which further comprises at least one other solid component, either during the reaction or after the reaction.

BACKGROUND OF THE INVENTION

Separation of a precipitate from a liquid is a well known process which can be carried out by decantation, filtration or centrifugation. However, if a reaction mixture contains two or more different solid components, there is no universal method of separating the solid components from each other. Filtration followed by mechanical separation of the solid components by sorting the particles is out of the question for most practical purposes. In some cases solid components can be separated from each other by mechanical processes like floatation. Other methods are physicochemical methods like extraction or partition, which utilize a difference in solubility of the components e.g. in water, in aqueous acid, in aqueous base or in organic solvents. A prerequisite of the applicability of these methods is that conditions can be found under which the components to be separated are stable.

In an industrial process involving the use of a catalyst, the price of the catalyst is often a very important parameter in the overall economy of the process. Therefore, it is an advantage of major importance if the catalyst can be reused without significant loss of catalytic activity. When the catalyst is present in a reaction mixture together with another solid component which is either formed during the reaction, such as a by-product or the desired product, or present during the whole process, e.g. a solid starting

material added in excess, the isolation and reuse of the catalyst is hampered.

In such cases the catalyst can sometimes be isolated by extracting the other solid(s) with organic solvents and/or with acids or bases which will dissolve the solid(s) except for the catalyst. However, the activity of catalysts, including enzymes, is very sensitive to the presence of so-called catalyst poisons. Catalyst poisons exert their activity e.g. by binding very strongly to the catalyst or by decomposing it. Thus, strong acids and bases often have an adverse effect on the activity of catalysts and particularly enzymes generally suffer irreversible damage on exposure to high concentrations of acids or bases. This imposes certain limitations on the use of acids and bases in the work up of reaction mixtures from enzymatic reactions when the enzyme is to be recycled without significant loss of activity. Other limitations on the work up conditions may of course be imposed by the nature of the desired product which may itself be a labile compound.

The prior art does not indicate a satisfactory solution to the separation problems outlined above.

SUMMARY OF THE INVENTION

As an alternative to dissolving the solid component(s) of the reaction mixture after the reaction except for the solid catalyst and separating the catalyst by filtration, the catalyst can, according to the present invention, be separated almost quantitatively by sieving or filtering the reaction mixture after the reaction is considered to be finished or, optionally, in a continuous way. In a particularly preferred mode of this embodiment, catalyst particles of a well defined particle size range are used and the other solid component(s) of the reaction mixture has (have) a particle size smaller than the lower limit of the apparent particle size range of the catalyst. The separation of the

catalyst is then carried out by letting the slurry which constitutes the reaction mixture pass through a sieve or a filter which will retain the catalyst particles and let the remainder of the mixture pass through. This can be done either in a batchwise or in a continuous way.

As the skilled person will know, there are several possibilities to influence the size and the shape of the particles to be separated from the catalyst. Thus, when the particles are crystals, which will most frequently be the case, vigorous stirring of the reaction mixture during their formation tends to result in smaller crystals than moderate stirring. Other parameters which influence the crystal growth are: choice of solvent or solvent mixture, temperature, temperature gradient, pH value of the reaction mixture, seeding and ageing of the crystals in the solvent. The parameters which influence the growth of crystals have no influence or only a very minor influence on the particle size of the catalyst which can be regarded as constant during the reaction. Industrial processes are usually run under well-defined conditions and therefore yield products having well-defined properties including e.g. the particle size of crystals. Therefore, in practical use of the method according to the present invention it may in some cases turn out that by using a catalyst having a suitable particle size range, adjustment of the particle size of the component(s) to be separated from the catalyst becomes unnecessary.

The separation of the solid components may be facilitated if the filter plate or sieve is vibrated during the separation or if the slurry on the filter plate is stirred. After the catalyst has been separated the remainder of the reaction mixture can be filtered on a filter which will retain the remaining solid component(s). The relative amounts of the desired product found in the filter cake and in the filtrate depends on the solubility of the desired product in the reaction medium. The filtrate and the filter cake can be worked up separately, some components optionally being recirculated in the process together with the catalyst.

Thus, in its broadest aspect the present invention relates to a method of separating a particulate solid catalyst from a reaction mixture which further comprises at least one other particulate solid component and a liquid by giving one of the particulate solid components an apparent particle size which is outside the apparent particle size range of the other solid component(s) whereupon the reaction mixture is filtered or centrifuged using equipment which will retain the component(s) having the larger particles and let the remainder of the mixture pass through.

In a first preferred embodiment of the invention the solid component(s) to be separated from the catalyst has (have) an apparent particle size smaller than the lower limit of the apparent particle size range of the catalyst.

In a further preferred embodiment of the invention the ratio between the apparent diameter of the larger particles and the apparent diameter of the smaller particles to be separated is at least 2.

In a further preferred embodiment of the invention the apparent particle diameter of the catalyst is in the range of from 25 to 10,000 μm, preferably from 50 to 750 μm, more preferred from 50 to 300 μm.

In a further preferred embodiment of the invention the solid catalyst is an immobilized enzyme.

In a further preferred embodiment of the invention the solid catalyst is an immobilized protease.

In a further preferred embodiment of the invention the solid catalyst is an immobilized metalloprotease.

In a further preferred embodiment of the invention the solid catalyst is an immobilized serine protease.

In a further preferred embodiment of the invention the solid catalyst is immobilized thermolysin.

In a further preferred embodiment of the invention the solid catalyst is an immobilized amidase.

In a further preferred embodiment of the invention the solid catalyst is an immobilized esterase.

In a further preferred embodiment of the invention the solid catalyst is an immobilized acylase.

In a further preferred embodiment of the invention the solid catalyst is an immobilized enzyme which is able to deacylate the 6-amino group of penicillin G.

In a further preferred embodiment of the invention the solid catalyst is an immobilized enzyme which is able to deacylate the 6-amino group of ampicillin.

In a further preferred embodiment of the invention the immobilized enzyme is a penicillin G acylase.

In a further preferred embodiment of the invention the immobilized enzyme is an ampicillin hydrolase.

In a further preferred embodiment of the invention the solid catalyst is an immobilized whole cell preparation.

In a further preferred embodiment of the invention the solid catalyst is an immobilized cell homogenate preparation.

In a further preferred embodiment of the invention a solid product produced in a process in which the starting material(s) is (are) fully dissolved in the reaction mixture is separated continuously from the catalyst during the reaction by leading the filtrate from the filter which retains the catalyst only to a filter which retains the solid product synthesized and recirculating the filtrate from this filter to the catalyst.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of a β-lactam antibiotic nucleus with the acid corresponding to the side chain or a derivative of this acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 6-amino group in 6-aminopenicillanic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used

for catalyzing acylation of the 7-amino group in 7-amino-cephalosporanic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 7-amino group in 7-amino-7-methoxycephalosporanic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 7-amino group in 7-amino-3-methoxy-3-cephem-4-carboxylic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 7-amino group in 7-amino-desacetoxycephalosporanic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 7-amino group in 3-chloro-7-amino-3-cephem-4-carboxylic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 7-amino group in 7-amino-3-(1,2,3-triazol-4(5)-ylthiomethyl)-3-cephem-4-carboxylic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of the 7-amino group in 7-amino-3-[2-(5-methyl-1,3,4-thiadiazolyl)thiomethyl]-3-cephem-4-carboxylic acid from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-phenylglycine as the acylating agent from the re-

mainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with a derivative of D-phenylglycine as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-phenylglycine amide as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-phenylglycine methyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-phenylglycine ethyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-phenylglycine propyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-phenylglycine isopropyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-4-hydroxyphenylglycine as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with a derivative of D-4-hydroxyphenylglycine as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-4-hydroxyphenylglycine amide as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-4-hydroxyphenylglycine methyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-4-hydroxyphenylglycine ethyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-4-hydroxyphenylglycine propyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with D-4-hydroxyphenylglycine isopropyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with 2-thiopheneacetic acid as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used

for catalyzing the acylation of a β-lactam antibiotic nucleus with a derivative of 2-thiopheneacetic acid as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with 2-thiopheneacetic acid amide as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with 2-thiopheneacetic acid methyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with 2-thiopheneacetic acid ethyl ester as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with 3-thiophenemalonic acid as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the acylation of a β-lactam antibiotic nucleus with a derivative of 3-thiophenemalonic acid as the acylating agent from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of L-phenylalanine methyl ester with a L-aspartic acid derivative in which the amino group is protected from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing acylation of D,L-phenylalanine methyl ester with a L-aspartic acid derivative in which the amino group is

protected from the remainder of the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the hydrolysis of an amide or an ester of an amino carboxylic acid to provide the corresponding free acid or a salt thereof from the remainder of the reaction mixture when the hydrolysis is conducted at such conditions that the product formed or a part thereof precipitates from the reaction mixture.

In a further preferred embodiment, the invention relates to a method of separating an immobilized enzyme used for catalyzing the conversion of fumaric acid or a salt thereof to malic acid or a salt thereof from the solid product obtained when the reaction is conducted at such conditions that the product formed or a part thereof precipitates from the reaction mixture.

DETAILED DESCRIPTION OF THE INVENTION

The separation method according to the present invention is particularly useful when a heterogeneous catalyst, e.g. a solid, particulate, immobilized enzyme, is to be separated from a reaction mixture which further contains at least one other particulate, solid component. This other particulate, solid component can either be a product synthesized under the influence of the catalyst or it can be unreacted starting material e.g. a starting material added in excess. In the present specification the word product when not further specified can mean either the desired product or a by-product resulting from a reaction.

When the catalyst is the only solid component present in the reaction mixture at the beginning of the reaction and the product is sparingly soluble in the liquid part of the reaction mixture the catalyst can be separated from the reaction mixture by giving the solid product particles an apparent particle size which is outside the apparent particle

size range of the solid catalyst particles whereupon the reaction mixture is filtered or centrifuged using a filter or a centrifuge which will retain the component having the larger particles and let the remainder of the reaction mixture pass through. In the following the liquid part of the reaction mixture is designated "the reaction liquid" or just "the liquid". The reaction liquid thus comprises the solvent (or solvent mixture) in which the reaction is conducted and the dissolved part of starting materials and products be they solids or liquids. When a solid product is produced in a process in which the starting materials are fully dissolved and the product particles are smaller than the catalyst particles the separation of the catalyst from the remainder of the reaction mixture can be performed in a continuous way by leading the filtrate from the filter which retains the catalyst only to a filter which retains the solid product synthesized and recirculating the filtrate from this filter to the catalyst. In this way the equilibrium of the reaction is influenced towards a higher yield.

One or more starting materials may be sparingly soluble in the solvent in which the reaction is performed. In this case the starting material(s) may be present in solid form in the reaction mixture and the reaction liquid will then be saturated with respect to the pertinent component(s). If the product resulting from the reaction is freely soluble in the reaction liquid the problem to be solved by the present invention during the working up is to separate the catalyst from the reaction liquid containing solid, unreacted starting material. If the product is also sparingly soluble in the reaction liquid the product and the unreacted starting material will have to be separated from each other after the catalyst has been separated from the remainder of the reaction mixture.

The true dimension of particles like crystals can be determined e.g. by using a microscope equipped with a suitable scale. Particles come in many different shapes. Thus crystals can e.g. be needle-like, plate-like or cubic. The

important feature in the present context is not the true dimension of the particles but rather the apparent dimension e.g. stated as the apparent diameter. In the present specification the designation "apparent dimension" or "apparent diameter" is used to reflect how a particle behaves on a sieve or a filter. Thus, the apparent diameter of a particle corresponds to the diameter of a hole or a pore which in practical use will just allow the particle to pass through it. In a preferred embodiment of the invention the particle size of the catalyst is reduced as much as the separation procedure allows. This helps to ensure a high activity of the catalyst and helps to eliminate diffusion problems.

It is well known that many processes conducted under the influence of a catalyst are very specific and that they run in a very high yield, particularly so if the catalyst is an enzyme. One of the advantages of the present method is that the catalyst can be reused. Due to the very mild conditions utilized for separating the catalyst from the remainder of the reaction mixture the loss of catalytic activity is usually very small. A contribution to a high throughput is offered by the fact that the present method is well suited for use with reactions conducted with such large amounts of starting material present per volume unit of the reaction mixture that part of it may initially be in solid form.

The solid catalyst to be used according to the present method may exist in the form of a particulate immobilized enzyme preparation and may have a density higher or lower than that of the reaction liquid. In this preparation, the enzyme may be adsorbed, absorbed, covalently bound, entrapped or bound by ionic forces. Methods for immobilizing enzymes are known in the art. The known art also provides methods for preparing particles for carrying immobilized catalysts e.g. enzymes and for isolating various fractions of particulate solids according to their particle size distribution. The specific catalyst to be used in each case depends on the process to be conducted.

The process of this invention is generally carried out in water. Optionally, organic solvents can be added. Organic solvents are preferably selected among water-miscible solvents such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 1,4-butanediol, acetone, acetonitrile, N,N-dimethylformamide and dimethylsulfoxide.

As mentioned earlier, the temperature is one of the parameters which influences the size of the particles when a solid product is formed. This can be because the growth rate of the crystals depends on the temperature or in some cases because different crystal modifications occur at different temperatures. The freezing point of the reaction liquid forms the absolute lower temperature limit for carrying out the method of the present invention. If the catalyst used is an immobilized enzyme the absolute upper temperature limit for carrying out the method will usually depend on the enzyme.

Examples of specific areas within which the present invention leads to essential improvements are: 1) Preparation of semisynthetic penicillins and cephalosporins, 2) Hydrolysis of amides and esters and 3) Synthesis of peptides.

Examples of β-lactam antibiotics (penicillins and cephalosporins) which can be prepared with advantage using the method of the present invention are ampicillin, amoxicillin, ticarcillin, cefaclor, cefatrizine, cefaparol, cephalexin, cefadroxil, cephaloglycin and cephalothin.

At present, semisynthetic β-lactams are prepared in industry by chemical methods. Penicillins for example, are prepared by reacting 6-APA, usually having its carboxyl group protected, with an activated side chain derivative, followed by removal of the protecting groups by hydrolysis. Thus, ampicillin can be prepared by reacting 6-APA, having a suitably protected carboxylic acid group, with D-phenylglycyl chloride, followed by hydrolytic removal of the group which protects the carboxylic acid group. These reactions typically involve costly steps such as the use of temperatures below

0°C (in certain cases even below -25°C), silylation reagents and organic solvents like methylene chloride, which must be handled with care since they are injurious to health and harmful to the environment.

Enzymatic production of semisyntetic β-lactam antibiotics by acylation of a β-lactam nucleus with a derivative (such as a lower alkyl ester) of D-phenylglycine or D-4-hydroxyphenylglycine is known e.g. from DE patent application No. 2,163,792, AT patent No. 243,986, NL patent application No. 70-09138, DE patent application No. 2,621,618 and EP patent application publication No. 339,751. Processes described in the prior art have typically been conducted at concentrations below 300 mM of the D-phenylglycine derivative and below 25 mM of the β-lactam nucleus.

This rather low concentration of the starting materials is a potential drawback of these known methods for enzymatic production of semisynthetic β-lactam antibiotics (none have yet been used on an industrial scale) since it makes the isolation of the β-lactam antibiotics formed more difficult and thus more costly. Furthermore, the yields reported are low, typically less than 85%, and a process for recycling the unreacted β-lactam nucleus is required, which leads to more and costly unit operations.

One of the problems encountered by increasing the concentration of the starting materials in the enzymatic synthesis of semisynthetic β-lactam antibiotics is that the solubility of some of the starting materials involved and of the products formed during the reaction is rather low. Hence, in order to run the process under economically more favourable conditions, it may be necessary to have so much starting material present at the beginning of the reaction that it cannot be fully dissolved in the reaction liquid. Also the product formed may separate in solid form because the large amount produced per volume unit may not be fully soluble in the reaction liquid. The need then arises to separate the catalyst from the reaction mixture containing solid products and/or unreacted starting materials, before

the remainder of the reaction mixture is further worked up as the working up may involve conditions harmful to the enzyme activity, for example, dissolution of products and unreacted starting materials at a low pH value (e.g. at a pH value of 0.5 - 2.0).

The acylating agent used for introducing the sidechain in a β-lactam antibiotic nucleus i.e. for introducing the acyl group in the 6-amino group of the penicillins or in the 7-amino group of the cephalosporins can be the corresponding acid or a derivative thereof, e.g. a lower alkyl (methyl, ethyl, propyl or isopropyl) ester or a primary, secondary or tertiary amide thereof. The methyl ester, the ethyl ester, and the amide are preferred. The derivative may be used in the free form or in the form of a salt, for example, the HCl salt or the H_2SO_4 salt.

The enzyme to be used may be any enzyme catalyzing the reaction in question. Such enzymes are usually termed penicillin amidases, penicillin acylases, or ampicillin hydrolases. A number of microbial enzymes known to have this activity, are derived from, for example, Acetobacter, Xanthomonas, Mycoplana, Protaminobacter, Aeromonas DE patent application No. 2,163,792) Pseudomonas (AT patent No. 243,986), Flavobacterium (NL patent application No. 70-09138), Aphanocladium, Cephalosporium (DE patent application No. 2,621,618), Acetobacter pasteurianus (DE patent application No. 2,163,792 A), Acetobacter turbidans (Takahashi et al., Biochem.J. **137** (1974), 497 - 503), Pseudomonas melanogenum (Kim & Byun, Biochim.Biophys. Acta, **1040** (1990), 12 - 18), Xanthomonas citrii (EP patent application publication No. 339,751), Kluyvera citrophila (Okachi et al., Agr.Biol.Chem., **37** (1973), 2797 - 2804), Escherichia coli (DE patent application No. 2930794), and Bacillus megaterium (Chiang & Bennett, J.Bacteriol., **93** (1967), 302).

After being separated from the remainder of the reaction mixture the catalyst can be reused, optionally after having gone through a washing procedure. Products and optional unreacted starting material can be separated and worked

up separately or recycled respectively.

An example of a peptide which can be prepared with advantage using the method of the present invention is N-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester, in the following designated ZAPM. ZAPM is a key intermediate in the production of the sweetener aspartame. In the production of aspartame (L-α-aspartyl-L-phenylalanine methyl ester), one of the critical steps involves an enzyme catalyzed coupling of an aspartic acid derivative and phenylalanine methyl ester hydrochloride (for example coupling N-benzyloxycarbonyl-L-aspartic acid and L-phenylalanine methyl ester hydrochloride to form ZAPM. The ZAPM forms a very sparingly soluble addition compound with L-phenylalanine methyl ester (or D-phenylalanine methyl ester, if present) and therefore precipitates during the synthesis. The equilibrium of the reaction is hereby shifted towards condensation.

According to the prior art a semi-purified, soluble enzyme preparation (e.g. thermolysin) can be used as catalyst in the condensation step. In order to separate the enzyme from the reaction product, the reaction product is dissolved and the enzyme is precipitated by addition of an organic solvent (for example acetone) and removed, for example, by filtration. However, from 14% up to at least 60% of the catalytic activity of the enzyme is lost during the process (Nonaka et al., US patent No. 4,212,945, and Meijer et al. in "Biocatalysts in Organic Syntheses" (Eds.: Tramper, van der Plas and Linko), pp. 135 - 156 (1985)). The major loss of activity occurs during the precipitation and isolation of the enzyme.

Using the process according to the present invention with an immobilized thermolysin preparation in the above mentioned process for preparing ZAPM, simplifies the separation step and reduces the loss of enzyme activity in the separation step to about 1%.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features dis-

closed in the foregoing description and in the following examples and claims may, both seperately and in any combination thereof, be material for realising the invention in diverse forms thereof.

EXAMPLE 1

Preparation of a catalyst suitable i.a. for the synthesis of β-lactams.

Immobilized penicillin G acylase from E. coli (250 g, Eupergit®-PcA, obtained from Röhm Pharma) was sieved on a 300 μm and then on a 180 μm screen by flushing with water. The material retained on the 180 μm screen was used as the enzyme catalyst in the examples 2-5 given below. When assayed by the 4-dimethylaminobenzaldehyde method as described by Balasingham et al., Biochim. Biophys. Acta, **276** (1972) 250-256 the activity of the catalyst was 115 penicillin G acylase Units (U) per g of moist catalyst.

EXAMPLE 2

Enzymatic synthesis of ampicillin.

D-phenylglycine methyl ester (in the following designated D-PGM) and 6-aminopenicillanic acid (in the following designated 6-APA) was suspended in 50 mM phosphate buffer having a pH value of 6.0, the final concentrations of D-PGM and 6-APA being 450 mM and 100 mM, respectively. The temperature of the suspension was brought to 35 °C and 10.0 g of moist immobilized enzyme (according to Example 1) was added, the total volume being 100 ml. The reaction was allowed to proceed with efficient stirring at 35 °C, the pH value being kept at 6.0 by titration with 2 M H_2SO_4.

After approximately 0.5 hours, D-phenylglycine (in the following designated D-PG) started to precipitate from the reaction mixture and after about 3 hours, the

ampicillin concentration reached a maximum of 77 mM, corresponding to 85% conversion of the 6-APA. At this point, the contents of the reaction vessel were transferred to a filter unit having a 100 µm pore size screen as the bottom and a rotating propeller placed immediately above the screen. The immobilized enzyme was retained by the screen while the remaining part of the slurry passed through. The precipitate in this filtrate consisted of D-phenylglycine crystals containing some ampicillin and the liquid contained i.a. dissolved product and unreacted starting materials. The ampicillin crystals formed had a particle size of less than 50 µm. The filtrate (i.e. the slurry comprising the crystals and the reaction liquid) was centrifuged and the clear centrifugate was used to wash off crystals remaining on the catalyst. By adjusting the flow of the filtrate from the separation unit and the flow of the clear supernatant to the separation unit the catalyst was kept in suspension at all times during the separation. When no crystals could be seen in the catalyst fraction the tank was drained completely, leaving only the catalyst on the screen.

After the separation, 97% of the ampicillin and 95% of the D-PG formed during the synthesis was in the filtrate. The ampicillin was isolated and further purified by known methods.

The catalyst retained in the separation unit was washed with 50 mM phosphate buffer (pH value: 6.0) and its activity was then assayed according to the method mentioned in Example 1. As indicated in Table 1, there was no significant loss of catalyst activity after the synthesis, and the catalyst was thus suitable for reuse.

Table 1

	Amount of catalyst, g	Activity, U/g
Before use	10.00	115.0
After one use	9.85	114.5

HPLC analysis of reaction components

Column: RP LC-18, (250 x 4.6 mm; 5 µm),
Eluent A: 25 mM phosphate buffer, pH value 6.5.
Eluent B: acetonitrile.
Elution was performed with mixtures of eluents A and B according to Table 2.

Table 2

Time, minutes	% A
0→10	99→80
10→20	80
20→21	80→99
21→35	99

Flow rate: 1 ml/minute
UV Detection at 215 nm
Retention time in minutes: D-PG: 4.1, 6-APA: 8.1, ampicillin: 13.9, D-PGM: 18.

EXAMPLE 3

Enzymatic synthesis of cephalexin.

D-phenylglycine methyl ester, HCl-salt, (1.6526 g) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) (0.4278 g) were dissolved in 50 mM phosphate buffer (pH value: 6.5) and thermostated to 35°C. Enzyme catalyst according to

Example 1 (2 g) was added and the volume of the reaction mixture was adjusted to 20 ml with buffer. The reaction was allowed to proceed under efficient mixing, keeping the pH value and temperature constant.

Approximately 45 minutes after the addition of the enzyme catalyst a precipitate was formed and after further 20 minutes the stirring was stopped and the contents of the reaction vessel were transferred to the filter unit described in Example 2 and the catalyst was separated from the precipitate formed and reaction liquid. From the precipitate and reaction liquid which contained, i.a., cephalexin, D-phenylglycine, D-phenylglycine methylester and 7-amino-desacetoxycephaloranic acid, cephalexin was isolated and purified by known methods.

More than 99% of the catalytic activity was retained in the catalyst after the separation step and the catalyst was thus suitable for reuse. A rinsing step may be introduced before the catalyst is reused.

The reaction was followed by HPLC using the same conditions as described in Example 2. The retention time for 7-ADCA and cephalexin was found to be 6.3 and 13.4 minutes, respectively.

EXAMPLE 4

Enzymatic synthesis of amoxicillin.

A slurry of D-4-hydroxyphenylglycine amide (44.94 g, in the following designated HPGA) and 6-APA (14.30 g) was prepared in a 50 mM phosphate buffer (pH value: 6.5) and thermostated to 25 °C in a reaction vessel supplied with a 100 μm pore size screen as the bottom and a rotating propeller placed immediately above the screen. The reaction was started by adding 40 g of catalyst (according to Example 1) (final volume: 400 ml), and the pH value was kept constant during the reaction by titration with 2 M H_2SO_4. The volume below the screen was less than 15 ml and by the aid of a pump

the filtrate (containing reaction liquid and crystals of starting materials and products), was returned continuously to the top of the reaction vessel during the whole process.

After 10 hours, the concentration of amoxicillin reached a maximum corresponding to 85% conversion and the reaction mixture contained, i.a., crystals of amoxicillin, D-4-hydroxyphenylglycine (in the following designated HPG) and unreacted HPGA. The recirculation of the filtrate to the reaction vessel was stopped and instead the filtrate was led to a centrifuge. As described in the previous two examples the clear supernatant was used for washing off the last crystals from the catalyst.

95% of the amoxicillin produced and 98% of the HPG produced were recovered from the filtrate. The amoxicillin was further purified by methods known in the art.

The catalyst used was suitable for reuse since more than 99% of its catalytic activity was retained.

The reaction was followed using the same HPLC system as described in Example 2 except that 5% acetonitrile in 95% 25 mM phosphate buffer (pH value: 6.0) was used for an isocratic elution (1 ml/min) of the compounds. These conditions gave the following retention times (in minutes) for the compounds of interest: D-4-hydroxyphenylglycine: 2.5, D-HPGA: 3.3, 6-APA: 6.0 and amoxicillin: 15.0.

EXAMPLE 5

Enzymatic hydrolysis of D-4-hydroxyphenylglycine amide (HPGA).

To a mixture of HPGA (8.35 g) in water, adjusted to a pH value of 6.0, moist catalyst (2.25 g, according to Example 1) was added, the total volume being adjusted to 100 ml. The hydrolysis was allowed to proceed at 35°C keeping the pH value constant at 6.0 by titration with 2 M sulfuric acid, while maintaining an efficient stirring.

After 3 hours, the HPGA was fully hydrolyzed to the free acid, HPG. Due to the catalyst and the content of partly precipitated HPG the reaction mixture appeared as a slurry.

The contents of the reaction vessel were transferred to the separation unit and the catalyst was separated from the reaction mixture as described in Example 2. The filtrate from this separation comprised the precipitated HPG and the supernatant which contained, i.a., salts and dissolved HPG. In total 96% of the HPG formed was found in the filtrate. The HPG was further purified by known methods.

The catalyst in the reaction vessel was washed with 50 mM phosphate buffer (pH value: 6.0) and was reused without a significant loss of catalytic activity (less than 1% of the total activity was lost during the synthesis and subsequent separation).

The HPLC method described in Example 4 was used for monitoring the reaction.

EXAMPLE 6

Preparation of a thermolysin catalyst.

Thermolysin (8 g, Sigma P-1512) was dissolved in 25 mM of phosphate buffer (pH value: 7) to approximately 50 mg protein per ml and approximately 3750 U (vide infra) per ml. Cells from an E. coli fermentation (e.g. A. Gebauer et al., Bioprocess Engineering 2 (1987) 55-58) were harvested (approx. 40 g of dry matter) and heated to 100°C for 10 min. to inactivate most enzyme activity in the cells. The thermolysin was added to the cells which were immobilized as described in Wümpelmann, M. et al. US patent No. 4,892,825 (to Novo Industri A/S). The material carrying the immobilized cells was extruded and dried to approximately 10% water content. The resulting particles were milled and the milled material was sieved. The 75 - 150 µm particle size fraction was used as catalyst in the synthesis of N-benzyloxycarbonyl-

L-aspartyl-L-phenylalanine methyl ester. The activity of the catalyst was approximately 3000 U per g. The activity was measured by the casein digestion method (1 unit (U) will hydrolyze casein to produce color equivalent to 1.0 μmole of tyrosine per minute at a pH value of 7.5 at 35°C (color by Folin-Ciocalteu reagent)).

Synthesis of N-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester.

N-benzyloxycarbonyl-L-aspartic acid (0.1 mole) and L-phenylalanine methyl ester hydrochloride (0.25 mole) was dissolved in water and adjusted to a pH value of 6.5 and a final volume of 350 ml. The solution was thermostated at 40°C and catalyst prepared as described above (15 g) was added. The catalyst was swelled in water before use, whereby the particle size increased to approximately 150-400 μm. The reaction was allowed to proceed at 40°C keeping the pH value constant at 6.5 and maintaining an efficient low shear stirring. The condesation product, N-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester (ZAPM) forms an addition compound with unreacted L-phenylalanine methyl ester which has a very poor solubility in water. Accordingly, the product precipitated almost quantitatively as this addition compound gradually as it was formed. After 20 hours, the reaction mixture was cooled to approximately 5°C and transferred to the separation unit described in Example 2. The catalyst was separated from the product and the reaction liquid as described in Example 2. The catalyst in the reaction vessel can be reused as more than 99% of the total catalytic activity was retained after the separation step. ZAPM can be further processed to aspartame by methods known per se (removal of the N-protection group of the aspartic moiety, crystallization etc.). The HPLC method of Oyama et al., J.C.S. Perkin II, (1981) 356, was used to follow the formation of ZAPM.

EXAMPLE 7

Enzymatic synthesis of cephalexin in the presence of 2-naphthol using an immobilized enzyme which can be recycled.

E. coli having Penicillin G acylase activity was fermented according to Gebauer, A. et al. Bioprocess Engineering 2 (1987) 55-58. Immobilization was performed according to Wümpelmann, M. et al. US Patent No. 4,892,825 (to Novo Industri A/S). The substance containing the immobilized enzyme was extruded and dried until the residual water content was approximately 10 % (w/w). The dried material was milled and a fraction having a particle size distribution of 100-200 µm was obtained from the milled product by the use of appropriate sieves. The enzyme activity in this fraction was found to be approximately 200 Penicillin G acylase Units/g. After swelling in water, the particle size distribution was approximately 200-500 µm.

The pH value of a mixture (slurry) of D-PGA·½H_2SO_4 (74.7 g, 0.375 mol), 7-ADCA (21.4 g, 0.10 mol), and 2-naphthol (10.8 g, 0.075 mol, particle size < 100 µm) in approximately 300 ml of water was adjusted to 6.7 by addition of 4 M ammonium hydroxide. Water was added to 400 ml followed by immobilized E. coli Penicillin G acylase prepared as described above (50 g on dry basis suspended in water to a total of 100 ml), and the mixture was stirred at 25 °C.

After 3 hours, the reaction mixture was poured on to a 100 µm pore screen, which retained the particles carrying the enzyme while the remaining part of the reaction mixture, still a slurry, passed through. The slurry passing the screen was filtered on a sintered glass filter which retained the solid material and some of the mother liquor was used to wash the solid material remaining on the 100 µm screen in order to free the enzyme particles from any adhering fine slurry containing the synthesized product. Also the washings were filtered through the sintered glass filter. The product collected on the glass filter was washed with butyl acetate (200 ml) and then suspended in a mixture of

water (150 ml) and butyl acetate (150 ml). The pH value of the water phase was adjusted to 1.5 by addition of 3 M sulfuric acid and stirring was continued for 10 minutes. The water phase was then separated from the butyl acetate phase and washed with further butyl acetate (2x20 ml). The volume of the water phase was reduced to 75 ml by evaporation, 2-propanol (75 ml) was added and the pH value was adjusted to 4.7 by addition of 4 M ammonium hydroxide. The slurry obtained was cooled to 5 °C for 15 minutes, whereupon the solid material was collected on a sintered glass filter and washed with water/2-propanol (1:1, 25 ml). After drying in a vacuum oven at 30 °C for 12 hours, 33.6 g (92.4% of the theoretical yield) of cephalexin monohydrate was obtained as a white powder (purity by HPLC: 99.9%).

After use, the enzyme particles left on the 100 µm screen were washed with water (3x100ml), drained, and finally dried to a water content of approximately 10 %. The weight was approximately 50 g, and the enzyme activity was found to be approximately 200 Penicillin G acylase Units/g, indicating that practically no loss of activity had occurred. Thus, the immobilized enzyme was suitable for recycling, e.g. in a process as described above.

CLAIMS

1. A method of separating a particulate solid catalyst from a reaction mixture which further comprises another particulate solid component and a liquid, **characterized in** that one of the particulate solid components is given an apparent particle size which is outside the apparent particle size range of the other solid component(s) whereupon the reaction mixture is filtered or centrifuged using equipment which will retain the component(s) having the larger particles and let the remainder of the mixture pass through.

2. A method according to claim 1, **characterized in** that the solid component(s) to be separated from the catalyst has (have) an apparent particle size smaller than the lower limit of the apparent particle size range of the catalyst.

3. A method according to claim 1 or 2, **characterized in** that the ratio between the apparent diameter of the larger particles and the apparent diameter of the smaller particles is at least 2.

4. A method according to claim 2 or 3, **characterized in** that the apparent particle diameter of the catalyst is in the range of from 25 to 10,000 μm, preferably from 50 to 750 μm, more preferred from 50 to 300 μm.

5. A method according to any one of the claims 1 to 4, **characterized in** that the solid catalyst is an immobilized enzyme.

6. A method according to claim 5, **characterized in** that the immobilized enzyme is a protease, a metalloprotease, a serine protease, thermolysin, an amidase, an esterase or an acylase.

7. A method according to claim 5, **characterized in** that the immobilized enzyme is able to deacylate the 6-amino group of penicillin G or the 6-amino group of ampicillin.

8. A method according to any one of the claims 1 to 4, **characterized in** that the solid catalyst is an immobilized whole cell or cell homogenate preparation.

9. A method according to any one of the claims 2 to 8, **characterized in** that a solid product produced in a process in which the starting material(s) is (are) fully dissolved in the reaction mixture is separated continuously from the catalyst during the reaction by leading the filtrate from the filter which retains the catalyst only to a filter which retains the solid product synthesized and recirculating the filtrate from this filter to the catalyst.

10. A method according to any one of the preceding claims for separating an immobilized enzyme used for catalyzing acylation of 6-aminopenicillanic acid, 7-aminocephalosporanic acid, 7-amino-7-methoxycephalosporanic acid, 7-amino-3-methoxy-3-cephem-4-carboxylic acid, 7-aminodesacetoxycephalosporanic acid, 3-chloro-7-amino-3-cephem-4-carboxylic acid, 7-amino-3-(1,2,3-triazol-4(5)-ylthiomethyl)-3-cephem-4-carboxylic acid or 7-amino-3-[2-(5-methyl-1,3,4-thiadiazolyl)thiomethyl]-3-cephem-4-carboxylic acid with D-phenylglycine, D-4-hydroxyphenylglycine, 2-thiopheneacetic acid or 3-thiophenemalonic acid or the amide, the methyl ester, the ethyl ester, the propyl ester or the isopropyl ester of D-phenylglycine, D-4-hydroxyphenylglycine, 2-thiopheneacetic acid or 3-thiophenemalonic acid from the remainder of the reaction mixture when solid material other than the catalyst is present in the reaction mixture when the working up is initiated.

11. A method according to any one of the claims 1 to 6 and 8 to 9 for separating an immobilized enzyme used for

catalyzing acylation of L-phenylalanine methyl ester or D,L-phenylalanine methyl ester with a N-protected L-aspartic acid from the solid acylation product obtained when the reaction is conducted at such conditions that the product formed or a part thereof precipitates from the reaction mixture.

12. A method according to any one of the claims 1 to 9 for separating an immobilized enzyme used for catalyzing the hydrolysis of an amide or an ester of an amino carboxylic acid to provide the corresponding free acid or a salt thereof from the solid product obtained when the reaction is conducted at such conditions that the product formed or a part threof precipitates from the reaction mixture.

13. A method according to any one of the claims 1 to 6 and 8 to 9 for separating an immobilized enzyme used for catalyzing the conversion of fumaric acid or a salt thereof to malic acid or a salt thereof from the solid product obtained when the reaction is conducted at such conditions that the product formed or a part thereof precipitates from the reaction mixture.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00159

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: B01J 37/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: B01D, B03B, C12N, C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 5: Biosis, 68-90/May, accession no. 6581148, Biosis accession no. 86047699, Takamatsu S. et al: "recirkulating bioreactor-separator system for simultaneous biotransformation and recovery of product immobilized L aspartate beta-decarboxylase reactor system", Biotechnology Bioeng 32 (2) 1988, 184-191	1-13
Y	--	1-13
Y	Dialog Information Services, File 351: WPI, 81-92, Dialog accession no. 004310425, (KONISHIROKU PHOTO KK), "Analytical vessel for immunoassay use contains buffer liq. for immune reaction and carrier for immobilisation of particular components in fluid sample", JP 60017357, A, 850129, 8523	1-13
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[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search	Date of mailing of the international search report
23 August 1993	25 -08- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Mikael G:son Bergstrand Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 351: WPI 81-92, Dialog accession no. 007453169, (KYOWA HAKKO KOGYO KK), "Prodn. of immobilised cells - involves treating cells with polyamine and dialdehyde", JP 63036785, A, 880217, 8813	1-4,8-9
X	Dialog Information Services, File 351: WPI 81-92, Dialog accession no. 007140942, (MITK) MITSUI TOATSU CHEM INC), "Recycling catalyst comprises sepq, catalyst components as solid from reaction soln. contq. components and subjecting sepd. components to oxidn. treatment", JP 62081350, A, 870414, 8720	1-4
X	Dialog Information Services, File 351, WPI 81-92, Dialog accession no. 008561914, (PETR) VEB PETROCHEM SCHWEDT), "Fines removal from palladium-active carbon catalyst - using sieve with specified mesh width and catalyst vol. flow", DD 283030, A, 901003, 9110	1-4