(12) UK Patent Application (19) GB (11) 2 164 663 A

(43) Application published 26 Mar 1986

(21) Application No 8523279

(22) Date of filing 20 Sep 1985

(30) Priority data

(31) 59/198778 (32) 22 Sep 1984 (33) JP

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(51) INT CL^4
C12M 1/40

(52) Domestic classification
C6F CB
G1B AX

(56) Documents cited
EP A2 0024048
EP A1 0006192
US 4307195
US 4251633

(58) Field of search
C6F
G1B

(54) **Process for carrying out enzymatic or microbial reactions**

(57) An enzymatic or microbial reaction is conducted between at least two reactants by placing an enzyme, a microbe, an immobilized enzyme or an immobilized microbe in an inner space provided between two sheets of porous materials which do not allow the enzyme and the microbe to pass, placing the reactants in the two outer spaces provided ouside the two sheets, respectively, allowing each of the reactants to pass through one sheet to conduct the enzymatic or microbial reaction in the inner space and allowing each product to pass outwardly through one sheet.

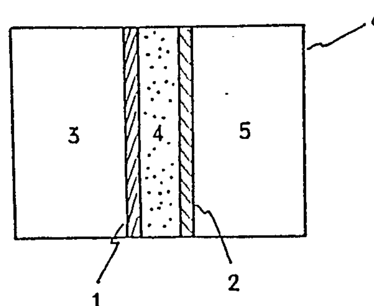

Fig. 1

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Fig. 1

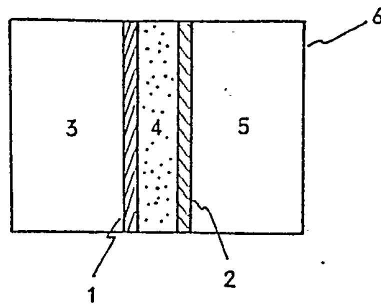


Fig. 2

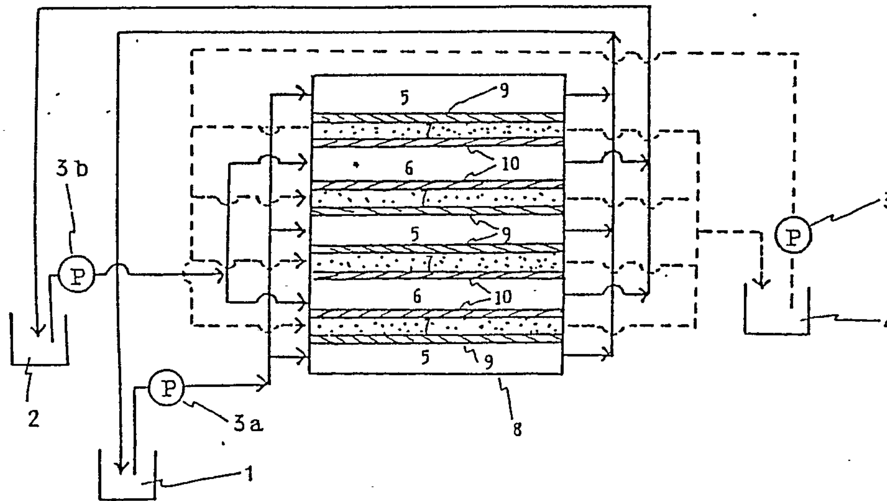


Fig. 3

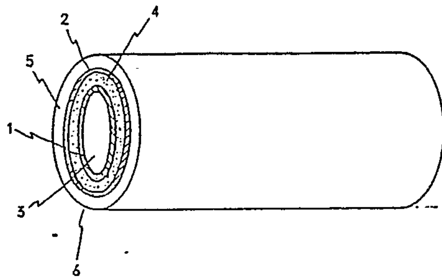
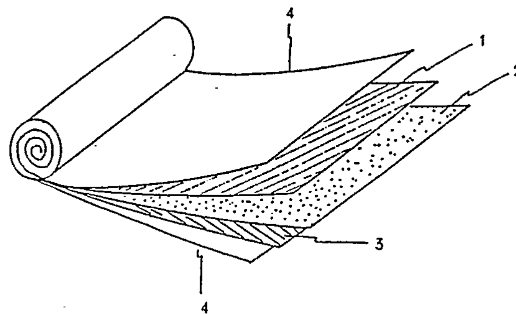


Fig. 4



SPECIFICATION

Process for carrying out enzymatic or microbial reactions

The present invention relates to a novel method of performing an enzymatic or microbial reaction, and more particularly to a method of performing an enzymatic or microbial reaction wherein at least two substances participate, which comprises using a bioreactor which is constructed in such a manner that an enzyme or a microbe is placed in an inner space (an enzyme chamber) defined by two sheets of porous materials, such as membranes, which do not allow the enzyme or the microbe to freely pass therethrough, and the substances participating in the reaction are placed in outer spaces in contact with the two porous materials, so that these substances are allowed to pass through the porous materials into the inner space to thereby undergo the reaction in the chamber and reaction products are allowed to pass through the porous materials into the outer spaces of the chamber, whereby the enzymatic or the microbial reaction as well as the separation of the reaction products can be efficiently carried out.

Hitherto it has been known that in most enzymatic and microbial reactions, two substrates and additionally an activator other than said substrates are usually necessary to carry out the reaction, and it is rare that only a single substance participates in the reaction. Likewise at least two reaction products are usually formed, it is unusual that only one reaction product is formed. Accordingly, it is usual that at least two reaction substrates and at least two reaction products are involved in the enzymatic or microbial reaction. Thus, two or more steps are usually necessary to separate these reaction products after the completion of the reaction to obtain the desired products. Furthermore, in the case of a reaction requiring at least two substrates, one of them may sometimes act as an inhibiting or deactivating factor against the enzyme or microbe. A reaction product itself may sometimes act as an inhibitor. Thus, enzymatic and the microbial reactions may be more efficiently carried out, if the quantities of these inhibitors to be introduced into the enzymatic or microbial reaction system can be controlled, or if such inhibitors can be rapidly discharged from the process line. Furthermore, it will be possible to dispense with a stage of product separation, if it is possible to use a bioreactor which serves to separate reaction products as well. Enzymes are generally so expensive that their reuse is indispensable for the reduction of manufacturing costs. Therefore, it is of great importance to bioreactor design that enzymes or microbes can be easily taken out of the reaction system for reuse.

A bioreactor capable of meeting all these requirements has not been developed as yet. For example, although the separation of an enzyme or a microbe from a reaction system involving an immobilized enzyme or mircobe, or the reuse thereof may be possible, the requirements for the simultaneous operation of the reaction, the separation of products and for the control of the inhibitor have not been achieved. An attempt has been made in recent years to impart a product-separating function (adsorptivity) to the support when using immobilized enzymes or microbes. However, this method has various disadvantages. For example, when a substance acting as an inhibitor or deactivator is required in the reaction, it is difficult to control its concentration by this method. When the adsorption properties of the support are to be utilized, a very large quantity of the support is necessary to adsorb products and this has hindered industrialization of such bioreactors.

The inventors have made studies on a bioreactor for use in an enzymatic or microbial reaction and have developed a bioreactor capable of meeting the requirements described below.

Accordingly, the present invention provides a method of performing an enzymatic or microbial reaction wherein at least two substances (the "reactants") participate, which is characterised in that sheets of porous materials which do not allow an enzyme or a microbe to pass therethrough are used in pairs; enzymes, microbes, immobilised enzymes or immobilised microbes are placed in an inner space provided between these porous materials; the reactants participating in the reaction are placed in each of two outer spaces provided outside the porous materials; and the above reactants are allowed to pass through the porous materials into the inner space to undergo the reaction in that space.

The reaction method according to the present invention is characterised in that sheets of porous materials such as membranes, which do not allow an enzyme or a microbe to freely pass therethrough are used in pairs; enzymes or microbes are placed in an inner space defined by the porous materials; the reactants participating in the reaction are allowed to pass through the porous materials into the inner space to undergo an enzymatic or microbial reaction; and reaction products are allowed to pass through the porous materials after the reaction to remove them from the enzymatic or microbial reaction system.

Brief description of the drawings:-

Figure 1 shows diagrammatically an embodiment of a device used in the present invention wherein reference numerals 1 and 2 represent porous materials, 3 and 5 represent reactant chambers, 4 represents an enzyme chamber and 6 represents an outer wall of the bioreactor;

Figure 2 shows diagrammatically an embodiment of a planar membrane type reactor according to the present invention;

Figure 3 shows diagrammatically an embodiment of a tubular type reactor;

Figure 4 shows diagrammatically an embodiment of a spiral type reactor.

Now, the present invention will be described in more detail by referring to the accompanying drawings showing preferred embodiments according to the present invention.

A lipolytic reaction using lipase will be given as

an example of the enzymatic reaction which can be represented by the formula:
A+B → C+D (wherein A and B are substances participating in the reaction, and C and D are products; for example, in the reaction using lipase, A is oil, B is water, C is fatty acid and D is glycerol). Referring to Figure 1, an enzyme is placed in an inner space 4 (an enzyme chamber) defined by porous materials 1 and 2, a substrate A is placed in an outer space 3 (a reactant chamber) and a substrate B in another outer space 5 (a reactant chamber). The porous materials 1 and 2 are constructed in such a manner that enzymes are not allowed to pass therethrough, the substrate A is allowed to pass through the porous material 1 and the substrate B is allowed to pass through the porous material 2. In the bioreactor of Figure 1, the substrates A and B placed in the respective reactant chambers 3 and 5 are allowed to pass through the respective porous materials into the enzyme chamber 4 where they undergo an enzymatic reaction in the presence of the enzyme to form products C and D. A preferred porous material 1 which can be used is one which allows the substrate A to pass therethrough but does not allow the substrate B to pass therethrough and which allows only one (for example, product C) of the reaction products C and D to pass therethrough. Alternatively, a preferred porous material 2 is one which allows the substrate B to pass therethrough, but does not allow the substrate A to pass therethrough and which allows only the reaction product D to pass therethrough. Porous materials having such properties can be obtained by suitably selecting on the basis of quality and the pore size, of the porous material. In the reaction using lipase, a hydrophobic material such as polypropylene or polyethylene may be used as the porous material which allows only the oil as the substrate and the fatty acid as the reaction product to pass therethrough, and a hydrophobic material such as cellulose material (e.g. cellulose acetate) or an acrylonitrile polymer may be used as the porous material which allows only water and glycerol to pass therethrough. The pore size of the porous materials must be such that the enzyme or microbe is not allowed to pass therethrough. Since the size of enzymes is thought to be from several tens to several hundred Angstroms, the porous materials must have a pore size of less than the above order. In some cases, however, such a fine pore size is not necessary depending on reaction systems. For example, in the lipolysis with lipase in the space 4 defined by the porous materials, an oil/water emulsion is formed and the enzyme is suspended on the surfaces of the emulsion. Therefore, the porous materials may have a pore size such that the emulsion is not allowed to pass therethrough and a pore size ranging from 0.1 to several μm will suffice to inhibit the passage of the enzyme through the porous materials. When an immobilised enzyme or microbe is used, no fine pore size is necessary.

Accordingly, the porous materials may be chosen according to the purpose and use without particular limitations to the quality or pore size, so long as they can meet the above requirements.

An enzyme or a microbe to be placed in the space 4 defined by the porous materials may be in the form of a powder, or a solution, or a suspension in a solvent which does not deactivate the enzyme nor microbe. In the practice of the present invention, it is preferred that a spacer is provided within the space 4 and the enzyme or microbe is spread or immobilised over the spacer to prevent localization of the enzyme or microbe. Alternatively, a solution of the enzyme or microbe may be circulated in the space 4 (the enzyme chamber 4) by means of a pump as will be described in more detail hereinafter. The spacer to be provided within the space 4 may be used without particular limitation to material, shape, etc. For example, if a material capable of adsorbing an enzyme, such as an ion exchange fiber, is used as the spacer, the enzyme can be immobilized thereon. Furthermore, other features can be imparted to the spacer.

The substrates in the reactant chambers 3 and 5 are diffused into the enzyme chamber 4 along diffusion gradients, and the reaction products are diffused from the enzyme chamber 4 into the reactant chamber 3 or 5 also along diffusion gradients. However, when the diffusion rate, due to only the concentration gradient, is too low and a sufficient reaction rate can not be obtained, the diffusion can be enhanced by for example applying pressure or temperature to each chamber. Alternatively, it is possible to enchance diffusion by inducing a difference in osmotic pressure between the chambers by adding to each chamber a third material which does not interfere with the enzymatic reaction, nor the microbial reaction, nor passes through the porous material.

The bioreactor used in the present invention can be applied to various enzymatic and microbial reactions. Examples of such enzymatic reactions wherein at least two substances participate include the aforementioned lipolysis with lipase, triglyceride synthesis with lipase, an ester exchange reaction of triglyceride in the presence of lipase, hydrolysis of phospholipid with phospholipase, a series of reactions with a transferase as represented by the phosphorolysis of polysaccharide in the presence of phosphorylase, malic acid synthesis using fumarase, a series of reactions in the presence of a hydrolase such as phosphatase or nucleotidase, and a series of reactions in the presence of an oxido-reductase such as alcohol dehydrogenase or amino acid oxido-reductase. Furthermore, the bioreactor of the present invention can be applied to growing up most microbes, since many growth factors in addition to the growth substrates are required for the growth of microbes. For example, glutamic acid can be produced from the substrate glucose in the glutamic acid fermentation process using a strain of the microorganism *Corynebacterium glutamicum*. However, biotin is required for the growth of the strain and the yield of glutamic acid varies depending on the concentration of biotin present. Microbial reactions frequently require other growth factors such as biotin, including vitamins, inorganic salts, amino

acids, etc. in addition to the growth substrates. The bioreactor of the present invention can thus be applied to most of the microbial reactions.

Enzymes and microbes used in the present invention are not always highly purified, and may be in the form of an extract, a partially purified product or a fermentation liquor.

In order to carry out efficiently the reaction with the bioreactor of the present invention, the bioreactor is constructed, as shown in Figure 2, such that there are provided a plurality of reaction chambers wherein each chamber is formed by interposing an enzyme chamber 7 between porous materials 9 and 10 in such a manner that the porous materials of the same kind are opposed to each other, a reactant chamber 5 is formed between two porous materials 9 and 9, or between a porous material 9 and the outer wall 8 of the bioreactor, and a reactant chamber 6 is formed between other two porous materials 10 and 10. Each of reactants is fed to each of the reactant chambers 5 and 6 by means of each of pumps 3a and 3b. In the bioreactor of Figure 2, enzymes or microbes can also be fed to the enzyme chamber 7 by means of a pump 3. In Figure 2, reference numerals 1 and 2 represent each a reactant reservior, 4 represents an enzyme or microbe reservoir. Alternatively, the enzymes or the microbes may be placed on a spacer by providing the spacer within the enzyme chamber 7 and spreading or immobilizing the enzymes or the microbes thereon as described above.

The bioreactor used in the present invention is not limited to a planar membrane type as shown in Figures 1 and 2, but may be in any form, for example, a tube as shown in Figure 3 or a spiral as shown in Figure 4. In the tubular reactor of Figure 3, an enzyme chamber 4 is formed between porous materials 1 and 2, a reactant chamber 3 is provided inside the tubular membrane 1 of the porous material, a reactant chamber 5 is provided outside the tubular membrane 2 of the porous material and 6 represents an outer wall of the bioreactor. In the spiral type reactor of Figure 4, a spacer 2 having enzymes or microbes spread or immobilized thereon is inserted into an enzyme chamber 2 formed between membranes 1 and 3, composed of a porous material. The membranes 1 and 3 are put between two sheets of outer walls 4 and 4, and they are rolled up into a spiral form. A reactant chamber is formed between one outer wall and the membrane 1, and another reactant chamber is formed between the other outer wall and the membrane 3.

One of the features of the present invention is that the enzyme or microbe can be easily removed from the reaction system, providing the enzymes or microbes are not immobilized on the porous materials. Thus it is possible to exchange only the enzymes or the microbes, when they are deactivated, without the necessity of exchanging the porous materials. Thus, the expensive porous materials can be preserved. Furthermore, when there are inhibitors in the enzymatic or the microbial reaction, the concentrations of these inhibitors added to the reaction system can be controlled by selecting the materials of the membranes or controlling the pore size thereof, as appropriate. When a reaction product acts as an inhibitor, the reaction product can be rapidly removed from the reaction system. Hence, the enzymes or the microbes can be prevented from being deactivated and can be reused. For example, water, as the substrate, acts as a factor of lipase deactivation in the lipolysis with lipase. Therefore, when the quantity of water is excessively increased, the enzymes are greatly deactivated. When the quantity of water passing through the porous material can be controlled, only a required quantity of water can be fed to the reaction system and lipase can be prevented from being deactivated.

The quantities of substrates and reaction products in an enzymatic reaction system of the present invention, as described above, are frequently larger than those in conventional enzymatic reactions. Furthermore, the enzyme concentration in the reaction system is generally higher than in conventional cases. Thus the environment of the enzyme in the reaction system according to the present invention is different from those of conventional systems, which might bring about various advantages. For example, the lipolytic reaction using lipase as cited above when performed in a bioreactor of the present invention, resulted in an increase in the thermostability of the enzymes. Hence the use of the reactor of the present invention makes it possible to perform the lipolytic reaction with non-thermostable lipase at 40 to 60°C with little inactivation, although this lipase is generally regarded to be unsuitable for hydrolysis of oils having a high melting point, such as beef tallow, since it is usually thermostable and should generally be used at a temperature lower than 40°C. Therefore the process of the present invention sometimes alters properties of the enzyme, such as thermostability or optimum, pH value, depending on the conditions of the reaction system.

Another feature of the present invention is that the reaction and the separation of reaction products can be simultaneously conducted. For example, the separation of fatty acids and glycerol, formed as the reaction products, can be simultaneously conducted in the lipolysis with lipase.

The reaction rate in the bioreactor of the invention greatly depends on the transfer rate of a reactant through the porous membrane of the bioreactor and a surface area of the membrane. It is therefore important to choose a porous material which allows the substrates to pass therethrough at a high speed in order to increase the reaction rate. Furthermore, since the reaction rate greatly depends on the surface area of the membranes, the reaction time can be greatly shortened by increasing the surface area of the membranes.

As described above, the bioreactor of the present invention provides a method of containing an enzyme or a microbe between the porous materials, wherein the enzyme or the microbe can be easily removed from the reaction system. Furthermore it makes possible the reuse of the enzyme or the mi-

crobe and allows the inhibition of deactivation, as well as the simultaneous reaction and separation of reaction products. Thus the bioreactor of the present invention provides a quite new reaction method having various advantages which the conventional methods employing an immobilized enzyme do not have. Accordingly, the method of the present invention is industrially very useful.

Examples

The following examples will further illustrate the present invention. These examples, however, should not be regarded as limiting the present invention.

Example 1

10 sheets of porous ultrafiltration membranes with a fractionation molecular weight of 20000 Daltons), composed of polyacrylonitrile and having a surface area of 0.02m² and 10 sheets of porous membranes (pore size of 0.1μ) composed of "TEFLON" (registered trademark) were prepared. Two grams of a lipolytic enzyme, lipase the activity of which was 320,000 units per gram, was suspended in a small quantity of soybean oil. About 1/10 of the suspension was spread on each of the several spacers, and the spacers were put between both the membranes, thus preparing a planar membrane type reactor as shown in Figure 2. In the method of this example, some of the enzyme was immobilized on the spacer while the rest of the enzyme in solution was circulated by means of a pump in the method shown in Figure 2.

400g of soybean oil and 400 g of water were circulated in such a manner that soybean oil was brought into contact with the Teflon membrane and water was brought into contact with the polyacrylonitrile membrane. The temperature was kept at 30°C in a constant temperature bath to hydrolyze the soybean oil in the presence of lipase.

After 24 hr, 70% of the soybean oil was hydrolyzed and the glycerol concentration in water was 7%. The glycerol content of the oil and the fatty acid content of water were each 0.1% or below.

It is evident from the above results that soybean oil and water are able to pass through the porous membranes into the central enzyme chamber where they are reacted and, after the reaction, the fatty acids are able to pass through one type of the porous membrane and diffuse into the oil chamber, while glycerol is able to pass through the other type of porous membrane and diffuse into the water chamber. In this way, the reaction and the separation of the reaction products can be simultaneously conducted.

Example 2

The enzyme used in Example 1 was left as such in the reactor, and the fatty acid solution and an aqueous solution of glycerol were discharged after the reaction of Example 1. 400g of fresh soybean oil and 400g of fresh water were then fed to the reactor in a similar manner to that described in Example 1. After 24hr, 70% of the soybean oil was hydrolysed which was the same value as that of Example 1.

Later, the enzyme was left as such in the reactor, and 400g of fresh soybean oil and 400g of fresh water were fed to the reactor. After 24 hr, the third experiment showed that 70% of the soybean oil was hydrolysed.

Accordingly, no deactivation of the enzyme occurred in the bioreactor of the present invention.

The reaction was efficiently carried out by leaving the enzyme as such in the bioreactor, discharging only the solutions after the reaction, and feeding fresh oil and fresh water.

It is clear that a step of separating the enzyme from the process line is unnecessary.

Example 3

This test was carried out under the same conditions as those of Example 1 except that 10 sheets of polyvinyl chloride membranes (surface area of 0.02m² and pore size of 2.5μ) were used as the porous membranes to be brought into contact with the oil. The porous membranes to be brought into contact with water were polyacrylonitrile membranes as in Example 1. Under similar conditions to those of Example 1, 400g of soybean oil and 400g of water were fed to the reactor by circulation to carry out the lipolysis. When the polyvinyl chloride membranes were used, the diffusion of water into the oil chamber was noted and thus a pressure difference of about 0.03 kg/cm² was applied to the oil chamber side to prevent water from diffusing into the oil chamber.

After 24 hrs. 80% of the soybean oil was hydrolysed and the glycerol concentration in water was 8.1%. After 48 hrs. 90% of the soybean oil was hydrolysed and the glycerol concentration in water was 9%.

It was found that the reaction rate varied depending on the material of the membrane and the pore size thereof when compared with the results of Example 1.

Example 4

The same porous membranes as those of Example 1 were used except that the number of the membrane used was five. 400g of soybean oil and 400g of water was fed to the reactor. After 24 hrs. 44% of the soybean oil was hydrolysed.

Thus, it was found that the reaction rate greatly depended on the surface area of the membrane.

Example 5

Five sheets of cellulose acetate membranes (reverse osmosis membranes of 10% in common salt exclusion ratio) having a surface area of 0.02 m² and five sheets of porous polyethylene membranes (pore size of 0.1 u) were prepared. These membranes were placed in the manner as shown in Figure 2 to thereby form a porous structure. An enzyme suspension comprising 2.4g of lipase and 300g of soybean oil was circulated with a pump between the cellulose acetate and polyethylene membranes while 300g of soybean oil and 300g of water were circulated with a pump in such a manner that the former and the latter were respectively

brought into contact with the polyethylene membrane and with the cellulose acetate membrane. The reaction system was kept at 50°C in a thermostatic chamber to facilitate the hydrolysis of the soybean oil by lipase. 92% of the soybean oil after 48 hours was hydrolysed.

Subsequently the fatty acid solution and the aqueous solution of glycerol were discharged while leaving the enzyme as it was and 300g portions of fresh soybean oil and water were fed to the bioreactor in the same manner as described above. 92% of the soybean oil after 48 hours was hydrolysed. Then 300g portions of additional fresh soybean oil and water were fed to the bioreactor with the remaining enzyme as it was. As a result of the third reaction, the same degree of hydrolysis of the soybean oil as after 48 hours (i.e. 92%) was obtained. Thus the bioreactor of the present invention made it possible to recover the product and to reuse the enzyme while sustaining the enzymatic activity at a high temperature (i.e. 50°C) without altering the properties of the enzyme itself nor pretreating the same, by for instance immobilization.

Comparative Example

The procedure of Example 5 was followed except that the substrate (i.e. soybean oil), water and lipase were mixed under stirring without using the membrane reactor to thereby reuse the enzyme. 300g of soybean oil, 300g of water and 2.4g of the enzyme were mixed under stirring, at 50°C as in Example 5. 92% of the soybean oil, after 48 hours, was hydrolyzed. After 48 hours, the reaction mixture was centrifuged at 800 rpm for five minutes at 50°C. Thus the reaction mixture was divided into three layers, i.e. an oil layer (top), an emulsion layer (medium) and an aqueous layer (bottom). Approximately 90% of the enzyme was distributed in the emulsion layer while the residual 10% thereof was in the aqueous phase. Thus the emulsion phase was taken out for the subsequent enzymatic reaction while the aqueous phase was subjected to ultrafiltration to thereby recover the enzyme contained therein. To achieve this, the aqueous phase was ultrafiltered through a polyacrylonitrile membrane which retains molecules of greater than 20000 Daltons) under 1 kg/cm^2 pressure. Then the enzyme retained by the membrane was recovered and reused in the subsequent enzymatic reaction. The concentrated enzyme solution, recovered from the emulsion layer and the membrane, was then added to 300g portions of soybean oil and water stirred for 48 hours, at 50°C as in the first reaction. 45% of the soybean oil was hydrolysed during the second reaction. The enzyme was recovered by the same separation procedure and subjected to a third reaction. 6% of the soybean oil was hydrolysed during the second reaction.

Thus the activity of the enzyme significantly dropped in a process wherein lipase was mixed with an oil and water, under stirring, without the membrane bioreactor of the present invention, at a high temperature (50°C). As the number of reactions was increased it became impossible to reuse the enzyme.

Example 6

The procedure of Example 5 was followed except that the soybean oil was substituted by beef tallow which was solid at room temperature. After performing the reaction at 50°C for 48 hours, 90% of the beef tallow was hydrolysed. The second and third reactions also gave a hydrolysis ratio after 48 hours of 90% each.

Thus solid fats such as beef tallow might by hydrolysed, with little inactivity, by using the reactor of the present invention.

CLAIMS

1. A process for effecting an enzymatic or microbial reaction between at least two reactants, which comprises the steps of placing an enzyme, a microbe, an immobilized enzyme or an immobilized microbe in an inner space provided between two sheets of porous materials which do not allow the enzyme and the microbe to pass, placing the reactants in the two outer spaces provided outside the two sheets, respectively, allowing each of the reactants to pass through one sheet to conduct the enzymatic or microbial reaction in the inner space and allowing each product to pass outwardly through one sheet.

2. A process as claimed in Claim 1, in which one of the sheets easily allows only one of the reactants and only one of products to pass therethrough, and the other easily allows the other reactant and the other product to pass therethrough.

3. A process as claimed in Claim 1, in which a plurality of pairs of the two sheets is provided and aligned so that two sheets of the same porous material may be opposed to each other.

4. A process as claimed in Claim 1, in which the hydrolysis of oils and fats is effected, using water and oil as the reactants and lipase as the enzyme.

Printed in the UK for HMSO, D8818935, 2/86, 7102.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.