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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
INVENTARIO

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SUPERINTENDENCIA

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DIVISIÓN DE NUEVAS CREACIONES

08-115385

**SOLICITUD DE PATENTE DE
INVENCION**

**TITULO: MODIFICACIÓN ENZIMÁTICA EN UNA COLUMNA DE LECHO
EMPACADO CONTINUAMENTE REGENERADO**

SOLICITANTE: CARGILL, INCORPORATED

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APODERADO: CARLOS R. OLARTE

BOGOTÁ 28 de Octubre 2008

OLARTE RAISBECK



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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ENTRADA EN FASE NACIONAL 31 oct. 2008 P2008/000175

SOLICITUD DE:

1



Patente de Invención.



Patente de Modelo de Utilidad.



Capítulo I.



Capítulo II.

2

**SOLICITANTE
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Número 79.782.747Bta
TP 74.295

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**Título (54) MODIFICACIÓN ENZIMÁTICA EN UNA COLUMNA DE LECHO
EMPACADO CONTINUAMENTE REGENERADO**

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9

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ANEXOS



Comprobante de pago de la tasa de presentación de la solicitud.



Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.



Poderes, si fuere el caso. Adjunto copia simple ver expediente No.



Documento de cesión del inventor al solicitante o a su causante.



Declaraciones.



Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos).



Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos).



Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA).



Traducción del examen preliminar internacional efectuado por la IPEA.



De ser el caso, copia del contrato de acceso.



De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.



De ser el caso, certificado de depósito del material biológico.



Arte final 12 x 12.



De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.



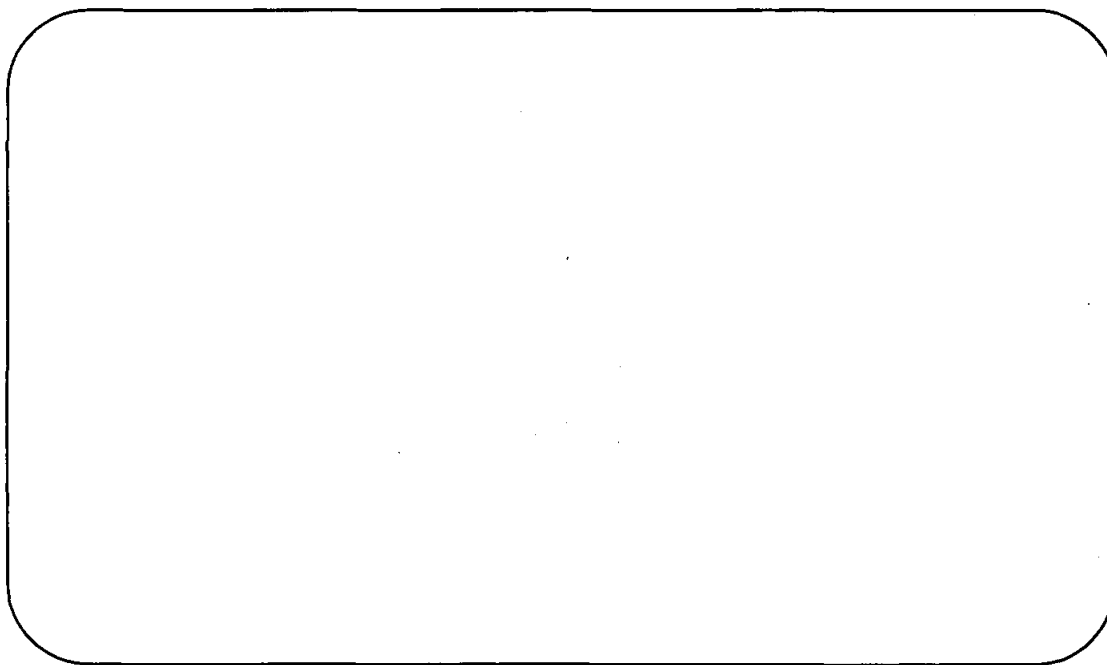
De ser el caso, modificaciones efectuadas a la solicitud internacional.



Otros. Búsqueda Internacional

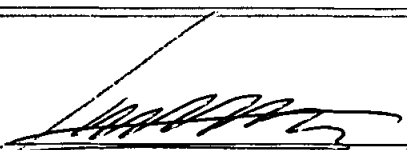


FIGURA CARACTERÍSTICA:



Solicito la concesión de la patente,

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***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01	SOLICITUDES	
		1 TRAMITES DE SOL. DE PATENTE DE IN	501,000.00
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RESPONSABLE :

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(54) Title: ENZYMATIC MODIFICATION IN A CONTINUOUSLY REGENERATED PACKED BED COLUMN

(57) Abstract: A process for the modification of a substrate comprising passing the substrate through a packed bed column of a specific volume of immobilized enzyme wherein the substrate enters the column at or near one end of the column (the 'inlet end') and the modified substrate exits at or near the opposite end of the column (the 'outlet end'), a portion of the volume of immobilized enzyme is periodically removed at or near to the inlet end of the column, and an equivalent portion of immobilized enzyme is periodically added at or near to the outlet end of the column.



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Enzymatic Modification in a Continuously Regenerated Packed Bed Column

Enzymatic reactions are increasingly used for processing materials on an industrial scale. An important commercial step was the development of immobilized enzymes, i.e. enzymes that are physically attached to a solid support - either by adsorption or chemical bonds - to facilitate the separation of the enzyme from the reaction solution. Immobilization also enables multiple or repetitive use of the enzymes and often increases the stability of the enzymes.

Several types of reactors are used in conjunction with immobilized enzymes: packed bed reactors (also referred to as fixed bed reactors), fluidized bed reactors, stirred batch reactors, continuously stirred tank reactors, and membrane reactors. In large-scale production, packed bed reactors are commonly used for particular applications. In such reactors, the immobilized enzyme is packed in a column or flat bed while substrate and product streams are pumped into and out of the reactor, respectively. The main advantages of this type of reactor are an easy adaptation to larger scales, high efficiency, low costs, ease of operation and also an enhanced surface area per unit volume compared to membrane reactor systems (cf. W.M. Willis and A.G. Marangoni, Enzymatic Interesterification, in: Food Lipids – Chemistry, Nutrition, and Biotechnology, edited by C.C. Akoh and D.B. Min, pages 839-875).

A type of reaction in which the use of enzymes as catalysts has become increasingly important in recent years in the oils and fats industry is interesterification. Interesterification is the exchange of acyl groups between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis) or between two esters (transesterification). The enzymes capable of interesterification reactions are lipases such as glycerol ester hydrolases (EC 3.1.1.3). These enzymes are predominantly obtained from bacterial, yeast, and fungal sources. These microorganisms secrete lipases into their environment to digest lipid materials for subsequent uptake. In an aqueous environment, lipases catalyse the hydrolysis of triacylglycerides to produce diacylglycerides, monoacylglycerides, glycerol and free fatty acids. However, under water-limiting conditions, the reverse reaction, the synthesis of esters can also be achieved. Therefore, the direction of the reaction can be manipulated by regulating the water activity. At very low water concentrations ester synthesis predominates, above a water content of more than a few percent hydrolysis is the prevailing reaction, and in between, usually at a water content of less than 1% (w/v), transesterification is most effective.

Owusu-Ansar described in 1994 the use of immobilized lipases in a stirred tank reactor for the large-scale production of cocoa butter equivalents by lipase-catalysed interesterification (in: B.S. Carmail and Y. Kakuda (eds.) *Technological Advances in Improved and Alternative Sources of Lipids*, pages 360-389).

A packed bed interesterification process has been disclosed in WO 83/03844 wherein triacylglycerides are dissolved in a polar organic solvent. The lipase was immobilized by precipitation onto kieselguhr, hydroxyapatite or alumina particles.

WO 97/01632 is directed to another process for immobilization of an enzyme, specifically, a lipase or a phospholipase, for the processing of triglyceride oils.

Unilever further disclosed a two-stage process for the production of cocoa butter equivalents and Betapol using packed bed columns (P. Quinlan & S. Moore, *Inform* 4, 580-585 (1993); A. Rozendaal & A.R. Macrae, in: F.D. Gansdone & F.B. Padlee (eds.) *Lipid Technologies and Applications*, 1997, pages 223-263).

X. Xu describes further processes with immobilized lipases, among them a packed bed reactor with a capacity of 10 kg/day (in U.T. Bornscheuer, *Enzymes in Lipid Modification*, 2000, pages 190-215).

The existing packed bed reactors suffer from the problem of a decrease in conversion rate over time. Though some enzymes such as lipases are quite stable, their efficacy inevitably drops over time, which results in lower reaction rates and non-uniform product quality. This lowering of quality can be counteracted by decreasing the flow rate and thereby increasing the residence time of the reactants in the reactor. Such a reduction in the flow rate, however, leads to a non-uniform output rate and while it can reduce the fluctuations in product quality, it cannot avoid them completely. Furthermore, such a process often uses a number of discrete reactors in series which requires a complex flow control in order to run and maintain the system. The current invention solves these and related problems by using a new type of packed bed column process for enzymatic modification of a substrate.

Summary of the invention

The invention relates to a process for the modification of a substrate which comprises passing the substrate through a packed bed column of a specific volume of immobilized enzyme with a flow of substrate in one direction and a periodic exchange of immobilized enzyme in the opposite direction. The substrate enters the column at or near one of its ends (the 'inlet end') and the modified substrate is discharged at or near its other end (the 'outlet end'). A portion of the volume of immobilized enzyme is periodically removed from the inlet end of the column, while an equivalent portion of immobilized enzyme is periodically added at the outlet end, thereby creating a flow of immobilized enzyme in the opposite direction of the substrate flow.

Whenever, in this application, reference is made to "near the inlet end" or "near the outlet end" the term "near" is intended to imply that material (e.g. substrate, modified substrate, immobilised enzyme) is charged to or discharged from the reactor (through an opening, valve or similar) within a reasonable distance from said end, as determined by a skilled person. That could be, for example, 1 to 5%, or 10% of the length of a column measured from the actual end of the column referred to. What is more, expressions such as "at the end" should be understood to include "at or near the end" when used in reference to the column, unless specifically stated otherwise.

Detailed description of the invention

Hereinafter, the terms "substrate" and "substrate solution" are synonymously used to describe a liquid compound or mixture of compounds or a liquid solution of a compound or of a mixture of compounds, which are meant to react in the presence of the immobilized enzyme. Thereby a liquid compound or mixture of compounds or a liquid solution of a compound or of a mixture of compounds is produced, which is referred to as "product" or "modified substrate", synonymously.

The invention relates to a process for the enzymatic modification of a substrate by passing the substrate through a packed bed column of immobilized enzyme. The substrate enters the packed bed column at or near one end of the column (hereinafter referred to as the "inlet end") and passes through the column towards

its other end (hereinafter referred to as the "outlet end"), where the substrate exits the column.

Preferably, the column is a vertical column with its inlet end at the bottom of the column and its outlet end at the top of the column. In this embodiment, the substrate will enter the bed first at or close to its lowermost portion and leave the column at or close to its uppermost portion.

Advantageously, the substrate and modified substrate streams can be essentially continuous. This means that, while the substrate and modified substrate streams may be stopped to allow for the periodic change of a portion of the volume of immobilized enzyme, there is no prolonged downtime necessary. This differentiates the present process from existing methods whereby extended periods of downtime were required amongst other things for changing the whole volume of immobilized enzyme. This not only reduces costs per se but can also prevent cost fluctuations.

According to the present invention, only a defined portion of the immobilized enzyme is periodically removed from the column. This can be achieved, by way of illustration only, with the following procedure. The continuous flow of substrate is stopped. A discharge valve at the inlet end of the column is opened and a defined portion of the slurry containing immobilized enzyme material from the bottom end of the column is released. After a defined amount of this slurry is discharged, the discharge valve is closed and a feeding valve at the outlet end, or in a pipeline feeding towards the outlet end of the reactor is opened, thereby allowing the entry of a fresh slurry of immobilized enzyme material into the column from its supply vessel.

The slurry of immobilized enzyme may be formed by adding a defined amount of the modified substrate into the supply vessel. If necessary, to enhance mixing and to improve the wetting of the pores of the immobilized enzyme, the vessel may be equipped with a stirring device and/or may be operated under vacuum. When a fresh charge of enzyme is to be added to the reactor, the supply vessel can be de-aerated and the slurry, or a portion thereof, emptied into the column. The feeding valve is then closed allowing the fresh slurry to settle at the end of the packed bed. Finally, the valves regulating the substrate and product streams are reopened.

The reactor may be operated under vacuum. Preferably, however, it will be operated under a certain pressure ("design pressure"). The design pressure should be chosen

to allow for a maximum pressure drop over the length of the column. The pressure drop will depend on the characteristics of the immobilized enzyme particles (e.g. particle size distribution), the substrate flow rate and the height of the bed. By way of example only, a suitable design pressure in a relatively high enzyme bed and with a relatively high substrate flow rate might be about 10 Bar. With a lower bed height and slower flow rate, that value might be closer to 6 Bar. The appropriate design pressure for any given reactor will easily be determined by a skilled person.

To ensure that the design pressure is not exceeded by the feed pressure, the feeding pumps should not have a capacity above the design pressure. What's more, the reactor inlets should be equipped with non-return valves, open/close valves and flow-rate control devices.

Ideally, the discharge of spent enzyme and addition of fresh enzyme will occur concurrently or substantially concurrently. By this, it is meant that the discharge and addition should be performed during the same downtime. It is also preferred that substantially the same quantity of enzyme material is discharged as is added thereby ensuring that the reactor maintains its packed bed characteristics. Exception to both these rules may occur, for instance, after initial start-up of the reactor owing to a certain amount of compaction in the column. It may indeed be necessary in this situation, to add a little more slurry than is discharged or even to add slurry without any discharge.

By repeating this change of a portion of the bed material, a gradual flow of the immobilized enzyme from the top to the bottom of the column is assured. In consequence, the substrate solution will first come in contact with the most exhausted enzyme material and lastly with the freshest enzyme.

Advantageously, the periodic change of a portion of the immobilized enzyme can be largely automated, thereby limiting operator involvement, reducing associated costs, and restricting the probability of any malfunctions.

This system can be compared with multiple separate small basic reactors in series. In comparison to such a series, the complexity of the present invention is greatly reduced, particularly the number of input and output valves. Moreover, the processed solution will not have to be transported between the multiple reactors. This is particularly important when the substrate and/or product solution has to be

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kept at a certain temperature or when they have a high viscosity. In addition, a system of small reactors requires that one or more of the reactors is periodically out of service while spent enzyme is removed and fresh enzyme added. This is an intensive manual operation and during this time the efficiency of the entire system is greatly reduced. Accordingly, the overall capital and operating costs for such a system are not optimal. What is more, the system of the present invention will allow for an almost infinite gradation of enzyme activity from fresh enzyme to spent enzyme. This could not be achieved even with a great number of separate reactors.

The process of the invention allows for an essentially continuous flow rate as basically all substrate encounters the same amount of enzyme activity during its passage through the reactor. As the activity of the immobilized enzyme varies with age and use, the uniform contact of substrate with enzyme at various stages of activity is decisive to ensure a more constant product quality. Therefore, a constant flow rate can be applied.

The frequency with which enzyme material is replaced will depend, amongst other things, on the rate of activity decrease of the enzyme over time and the desired residence time of the substrate solution in the packed enzyme bed. The volume of enzyme material changed and the length of the intervals between these changes can easily be adapted to the needs of the process and/or substrate. In addition, there will be a relation between the frequency with which the enzyme is refreshed and the volume of enzyme that is refreshed. With all other process variables remaining constant, a small amount of enzyme can be refreshed frequently or a larger amount of enzyme refreshed less frequently. Preferably, a fixed percentage of the entire column volume will be refreshed on a daily basis. Ideally, that percentage will be less than 35% of the total volume of immobilized enzyme. Preferably, it will be less than 25%, more preferably less than 10%, even more preferably less than 5%. According to one possible embodiment, between about 0.3% and about 3% of the total volume of immobilized enzyme will be refreshed on a daily basis.

After passing through the packed bed column of immobilized enzyme, a sufficient conversion of the substrate should be reached. At the top of the column, product outlet may be facilitated by small cylindrically shaped filter screens. These regulate the outflow of the product while the immobilized enzyme material is kept inside the reactor. There are two types of screens: vent screens and standard screens. While the standard screens are used for the actual outflow of product, the vent screens are

higher up in the column and facilitate the flow of air or gas during draining and refilling.

The pumps regulating the input and output of the enzyme material slurries should be designed so that they do not break up the immobilized enzyme creating additional fines. This will assist an even flow and prevent fines from passing subsequent filtering devices.

The discharged enzyme will be a slurry of used (or "spent") immobilized enzyme and substrate solution. This slurry can be separated and the substrate solution can be re-introduced into the tank which provides the input of substrate to the reaction column. The used enzyme can be disposed of. Preferably, however, it will be processed for further use.

The enzyme removed from the column can indeed be subject to recycling. For instance, in some cases the enzyme removed from the column still has some residual activity, which may be accessed if the immobilize material is broken into smaller particles, thereby revealing active sites of the enzyme that were previously not at all or just restrictedly accessible to the substrate (this is known as "reactivation"). Alternatively, the enzyme may be regenerated and reused. Thus, in a preferred embodiment of the process according to the invention, the enzyme material discharged from the column is regenerated or reactivated and reused, e.g. for pre-treating the substrate prior to it entering the column or by being added to the tank which supplies the input of immobilised enzyme. It may also be re-used in other systems like batch-modification.

The process of the present invention may be operated in a single column or by passing the substrate through two or more columns of immobilized enzyme disposed in parallel. The term "immobilized enzyme" takes its normal meaning in the art, i.e. that of an enzyme which is physically attached to a solid support. Preferred supports include silica and resin supports. The immobilized enzyme may be present in the form of granules, agglomerated particles or any other form deemed suitable by a skilled person.

The choice of enzyme will of course depend on the type of reaction to be carried out, i.e. on the nature of the substrate and its desired modification. It could be any oxidoreductase, transferase, hydrolase, lysase, isomerase or ligase available in the

art. By way of illustration, suitable hydrolases could include carboxylic ester hydrolases (EC-3.1.1) such as carboxylesterases, triacylglycerol lipases, phospholipase A2, sterol esterases, acylglycerol lipases, phospholipase A1, and wax-ester hydrolases; and phosphoric diester hydrolases (EC-3.1.4) such as phospholipase C and phospholipase D. These enzymes can be used, for example, to catalyse hydrolysis, alcoholysis or the corresponding reverse conversion (condensation). Other suitable enzymes will be known to the skilled person depending on the specific modification to be performed.

The substrate will be any material or substance capable of being acted upon by an enzyme. It may include, for instance, one or more lipids, one or more proteins, one or more carbohydrates or a mixture thereof. The term "lipid" as used herein refers not only to crude or processed oils and fractions thereof, but also to triacylglycerides, diacylglycerides, monoacylglycerides, phospholipids, free fatty acids, fatty acid esters and mixtures thereof. The term "protein" may refer, amongst others, to both complex and simple polypeptides, whether in their folded or denatured state, to short peptide sequences and to single amino-acids. The term "carbohydrate" refers, for instance, to single monosaccharide units, disaccharides, oligosaccharides and polysaccharides such as starch, cellulose and so on. The substrate may be mixed with other reagents (such as an alcohol or water), or with a solvent if necessary. If desired, the substrate may be refined before entering the column. Refining refers to the removal of impurities such as residues and by-products and will contribute to extending the life of the immobilized enzymes.

As stated above, the process of the present invention may be used to perform any type of enzymatic reaction. Nonetheless, a number of possible embodiments will now be described in further detail by way of illustration. These should not be interpreted in any way as limiting the scope of the invention. Rather, their teachings should be understood, where appropriate, to apply to the invention as a whole.

Interesterification

This embodiment concerns the interesterification of oils. Interesterification changes the acyl-glycerol profile of the oil or oil mixture thereby modifying its physical properties.

According to this embodiment, the enzyme of choice is a lipase. It will ideally be immobilized on a silica support. A suitable immobilized enzyme is the Lypozyme TL IM lipase from Novozymes.

The substrate, which may be used in a refined or unrefined state depending on requirements, will typically comprise an acylglyceride such as a triacylglyceride, a diacylglyceride, a monoacylglyceride or a mixture of two or more thereof. It may be derived from a plant, animal, fish, algae or single-cell source. Preferably, it will be derived from a plant source. Suitable substrates will therefore include, but are not limited to, palm kernel oil, palm oil, coconut oil, olive oil, rapeseed oil, canola oil, linseed oil, ground nut oil, soybean oil, cotton seed oil, sunflower seed oil, pumpkin seed oil, corn oil, castor oil, walnut oil and mixtures of two or more thereof. The substrate may include whole oils and/or oil fractions such as stearins or oleins.

According to one embodiment, the substrate will comprise palm stearin, coconut oil, palm kernel oil or a mixture of two or more thereof. Preferably, the substrate will comprise a mixture of palm stearin plus coconut oil or a mixture of palm stearin plus palm kernel oil. Even more preferably, the mixture will include 50-80 % by weight palm stearin and 50-20% by weight coconut oil and/or palm kernel oil, based on total substrate weight. Ideally, the mixture will include 65-75% by weight palm stearin and 35-25% by weight coconut oil or 60-70% by weight palm stearin and 40-30% by weight palm kernel oil. According to a most preferred embodiment, the substrate of the present invention will consist of a such a mixture.

The substrate will be pumped through one or more columns in parallel filled with the immobilized enzyme. The substrate and immobilized enzyme material will flow in opposite directions with the oil input at or near the bottom of the column and the fresh enzyme material being added at or near the top. Periodically, a part of the immobilized enzyme will be exchanged with fresh enzyme material. Therefore, the enzyme in the column will have a nearly constant quality profile. The immobilized enzyme at the top of the column will be most fresh with nearly full activity and the immobilized enzyme near the bottom of the column will be nearly exhausted of activity. Accordingly, there will be a gradation of activity along the length of the column.

The interesterification reaction should be carried out at a defined temperature. This temperature will be chosen with a number of factors in mind:

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- as most of the interesterified oils and fats produced according to this process are intended for nutritional products, the process will preferably be free of organic solvents. As such, the process should be performed at a temperature which guarantees that the oils/fats remain in a liquid state;
- the temperature should be high enough to allow a high reaction rate which, in turn, will allow for shorter residence times;
- the temperature should be low enough to not affect the stability and activity of the enzyme. Microbial lipases are quite thermostable and immobilization was found to further improve the stability of these enzymes so that the optimal temperatures for many lipases range from 30°C to 62°C (W.M. Willis and A.G. Marangoni, in *Enzymatic Interesterification, Food Lipids – Chemistry, Nutrition, and Biotechnology*, edited by C.C. Akoh and D.B. Min). Some lipases even allow temperatures of 60°C to 75°C (WO 97/01632). Future enzyme development could include even more temperature resistant varieties, e.g. resistant to 80°C, 90°C, 100°C or above. At these temperatures, most triacylglycerides, even fully saturated ones, will be in a liquid state.

Taking these factors into account, the skilled person will be able to determine the *ideal reaction temperature*.

By way of example, interesterification of palm stearin with coconut oil by Lypozyme TL IM (Novozymes) will preferably be performed at about 70°C.

After heating the oils to the desired temperature and mixing, if necessary, they are directed to the input valve at or near the inlet end of the reactor. The reactor will preferably be designed to operate under vacuum and up to a suitable pressure.

Fresh immobilized enzyme is supplied at the top of the column at regular intervals. A defined amount of interesterified product oil may be pumped into the supply vessel and mixed with the immobilized enzyme to produce a free-flowing slurry. To enhance mixing and to improve the wetting of the pores of the immobilized enzyme, a vacuum can be applied to the vessel. Advantageously, the vessel will be flushed with nitrogen to prevent oxidation of the oil.

When a fresh charge of enzyme is to be added to the column, the supply vessel is de-aerated, if applicable, and the slurry or a portion thereof emptied into the reactor. If required, the vessel and pipelines can be flushed with a certain volume of product oil to ensure complete emptying of the vessel. Preferably, the supply vessel will be situated above the reactor to facilitate enzyme loading.

The process of this embodiment will preferably be essentially continuous. The only downtime will be the few moments that it takes to exchange a portion of the enzyme from the column. This downtime is only a very small fraction of the overall running time of the process.

Alcoholysis

Unless otherwise stated, the details of this embodiment are the same as for the interesterification reaction described above.

The substrate will be a suitably agitated mixture of one or more oils and one or more alcohols. Suitable alcohols include but are not limited to methanol, ethanol, glycerol, monopropylene glycol, ethylene glycol, butane diol, erythritol, pentaerythritol, sorbitol, sterols, stanols, tocopherols, polyphenols, esters of such alcohols and mixtures of two or more thereof. The immobilized enzyme will preferably be lipase.

The reaction will preferably be carried out at 50-70°C. After being collected at the outlet of the reactor, the products (fatty acid esters and glycerol) can be isolated. Isolation can be performed by cooling to a temperature where phase separation occurs followed by isolation of the fatty acid ester phase and a drying step. The alcohol can be recovered by evaporation directly from the mixture or after isolating the fatty acid esters.

Glycerolysis

Glycerolysis is a specific type of alcoholysis, as described above. The substrate is a mixture of one or more oils and glycerol. The enzyme is a lipase immobilized on a carrier which is inert against glycerol.

The substrate is prepared by mixing or dissolving glycerol into the oil(s) at reaction temperature or slightly above. The amount of glycerol mixed with the oil can be

increased by adding an emulsifier. Depending on the glycerol-to-oil ratio, the enzyme closest to the inlet end of the reactor will act both as a substrate purifier and to adjust the equilibrium concentration of glycerol in the substrate.

The reaction will preferably be carried out at 50-70°C. The product recovered after modification will depend the glycerol-to-oil ratio of the substrate.

Hydrolysis

In this embodiment - which follows the interesterification embodiment described above unless indicated to the contrary - the substrate is a pumpable lecithin or lecithin solution. A lecithin solution may be obtained by mixing lecithin with a solvent such as hexane and water.

The enzyme will be a lipase or phospholipase. Depending on the enzyme, different products will be obtained. Thus, if a lipase is used, the product will be a mixture of phospholipids and mono- and/or diglycerides. Using phospholipase A or B, at a preferred reaction temperature of 20-70°C, will lead to the production of lysophospholipids. Using phospholipase C, at a preferred reaction temperature of 20-50°C, will lead to the production of di- and/or triglycerides. Using phospholipase D, at a preferred reaction temperature of 20-70°C, will lead to the production of phosphatidic acid. *If necessary, a mixture of different enzymes can be used to obtain the desired product.*

After conversion, the product mixtures can be used as such or after further purification steps (e.g. washing, drying, evaporation, fractionation, etc.).

Isomerisation

Another possible reaction which could be performed according to the process of the present invention is isomerisation. A typical example of isomerisation is the conversion of glucose to fructose. Thus, according to one embodiment, the substrate will preferably be glucose (or a glucose syrup) and the immobilised enzyme will preferably be a glucose isomerase (EC 5.3.1.5). The reaction should ideally be carried out between 55-75°C. The product will be a syrup containing both glucose and fructose. If desired, the fructose content can be further increased by purification or by splitting the glucose and fructose into separate products.

Claims

1. A process for the modification of a substrate comprising passing the substrate through a packed bed column of a specific volume of immobilized enzyme wherein:
 - a) the substrate enters the column at or near one end of the column (the 'inlet end') and the modified substrate exits at or near the opposite end of the column (the 'outlet end'),
 - b) a portion of the volume of immobilized enzyme is periodically removed at or near to the inlet end of the column, and
 - c) a portion of immobilized enzyme is periodically added at or near to the outlet end of the column.
2. A process according to claim 1 wherein the column is substantially vertical with its inlet end at or near the bottom of the column and its outlet end at or near the top of the column.
3. A process according to claim 1 or claim 2, wherein the process is essentially a continuous process.
4. A process according to any of the preceding claims wherein the portion of immobilized enzyme which is removed from the column is less than 35% of the total volume.
5. A process according to any one of the preceding claims wherein the portion of immobilized enzyme which is removed from the column is substantially equivalent in volume to the portion of immobilized enzyme which is added.
6. A process according to any one of the preceding claims wherein steps of removing immobilized enzyme from and of adding immobilized enzyme to the column occur substantially concurrently.
7. A process according to any one of the preceding claims wherein the removed portion of immobilized enzyme is used to pre-treat the substrate before it enters the column.

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8. A process according to claim 7 wherein the removed portion of immobilized enzyme is regenerated or reactivated before being used to pre-treat the substrate.
9. A process according to any one of the preceding claims wherein the modification is a condensation or includes condensation steps.
10. A process according to any one of claims 1 to 8 wherein the modification is an interesterification.
11. A process according to any one of claims 1 to 8 wherein the modification is an isomerisation.
12. A process according to any one of claims 1 to 10 wherein the enzyme is a hydrolase.
13. A process according to claim 12 wherein the enzyme is a lipase or a lipid-modifying enzyme and the substrate comprises one or more crude oils, one or more processed oils or a mixture thereof.
14. A process according to claim 12 wherein the substrate comprises one or more triacylglycerides, diacylglycerides, monoacylglycerides, phospholipids, free fatty acids, fatty acid esters or mixtures of two or more thereof.
15. A process according to claim 14 wherein the substrate further comprises an alcohol.
16. A process according to claim 15 wherein the alcohol is selected from: methanol, ethanol, glycerol, monopropylene glycol, ethylene glycol, butane diol, erythritol, pentaerythritol, sorbitol, sterols, stanols, tocopherols, polyphenols, esters of such alcohols and mixtures of two or more thereof.
17. A process according to any one of claims 14-16 wherein the substrate further comprises water.
18. A process according to claim 13 wherein the substrate comprises one or more plant based oils or plant based oil fractions.

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19. A process according to claim 18 wherein the substrate comprises palm stearin, coconut oil, palm kernel oil or a mixture of two or more thereof.
20. A process according to claim 19 wherein the substrate comprises 50% to 80% by weight palm stearin and 50% to 20% by weight coconut oil.
21. A process according to claim 20 wherein the substrate comprises 65 to 75% by weight palm stearin and 35 to 25% by weight coconut oil.
22. A process according to claim 19 wherein the substrate comprises 50% to 80% by weight palm stearin and 50% to 20% by weight palm kernel oil.
23. A process according to claim 22 wherein the substrate comprises 60 to 70% by weight palm stearin and 40 to 30% by weight palm kernel oil.

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(54) Título: MODIFICACION ENZIMÁTICA EN UNA COLUMNA DE LECHO EMPACADO
CONTINUAMENTE REGENERADO

(57) Resumen: La presente invención se refiere a un proceso para la modificación de un sustrato que comprende, pasar el sustrato a través de una columna de lecho empacado de un volumen específico de enzima inmovilizada, en donde el sustrato entra a la columna en o cerca de un extremo de la columna (el "extremo de entrada") y el sustrato modificado sale en o cerca del extremo opuesto de la columna (el "extremo de salida"), una porción del volumen de la enzima modificada es periódicamente removida en o cerca del extremo de entrada de la columna, y una porción equivalente de enzima inmovilizada es periódicamente agregada en o cerca del extremo de salida de la columna.

**MODIFICACION ENZIMATICA EN UNA COLUMNA DE LECHO EMPACADO
CONTINUAMENTE REGENERADO**

CAMPO DE LA INVENCIÓN

5 Las reacciones enzimáticas son cada vez más usadas para procesamiento de materiales en una escala industrial. Una etapa comercial importante es el desarrollo de enzimas inmovilizadas, es decir, enzimas que están físicamente unidas a un soporte sólido, ya sea por adsorción o enlaces químicos, para facilitar la separación de la enzima a partir de la solución de reacción. La inmovilización también permite el uso múltiple o repetitivo de las enzimas y a menudo incrementa la estabilidad de las enzimas.

15 **ANTECEDENTES DE LA INVENCIÓN**

Se usan varios tipos de reactores junto con enzimas inmovilizadas: reactores de lecho empacado (también referidos como reactores de lecho fijo), reactores de lecho fluidizado, reactores de lote agitado, reactores de tanque continuamente agitado y reactores de membrana. En producción a gran escala, los reactores de lecho empacado son comúnmente usados para aplicaciones particulares. En tales reactores, la enzima inmovilizada es empacada en una columna o lecho plano mientras el sustrato y corrientes del producto son bombeados en y fuera del reactor, respectivamente. Las ventajas

principales de este tipo de reactor son una adaptación fácil a gran escala, gran eficiencia, bajos costos, facilidad de operación y también una superficie intensificada por volumen de unidad comparada con sistemas de reactor de membrana (cotéjese, W.M. Willis and A.G. Marangoni, Enzymatic Interesterification, in: Food Lipids - Chemistry, Nutrition and Biotechnology, editado por C.C. Akoh and D.B. Min, páginas 839-875).

Un tipo de reacción en el cual el uso de enzimas como catalizadores ha llegado a ser cada vez más importante en años recientes en la industria de aceites y grasas es la interesterificación. La interesterificación es el intercambio de grupos acilo entre un éster y un ácido (acidólisis), un éster y un alcohol (alcoholisis) o entre dos ésteres (transesterificación). Las enzimas capaces de reacciones de interesterificación son lipasas tal como glicerol éster hidrolasas (EC 3.1.1.3). Estas enzimas son predominantemente obtenidas de fuentes bacterianas, de levadura y fúngicas. Estos microorganismos secretan lipasa en su ambiente para digerir materiales lípidos para retención subsecuente. En un ambiente acuoso, las lipasas catalizan la hidrólisis de triacilglicéridos para producir diacilglicéridos, monoacilglicéridos, glicerol y ácido grasos libres. Sin embargo, bajo condiciones limitantes acuosas, la reacción inversa, puede lograrse la síntesis de ésteres. Por lo tanto,

la dirección de la reacción puede ser manipulada regulando la actividad acuosa. A concentraciones acuosas muy bajas predomina la síntesis de éster, arriba de un contenido de agua de más de algún porcentaje, la hidrólisis es la reacción dominante, y entre, usualmente a un contenido acuoso de menos del 1% (p/v), la transesterificación es más efectiva.

Owusu-Ansar describió en 1994, el uso de lipasas inmovilizadas en un reactor de tanque agitado para la producción a gran escala de equivalentes de manteca de cacao por interesterificación catalizada por lipasa (en: B.S. Carmail and Y. Kakuda (eds) Technological Advances in Improved and Alternative Sources of Lipids, páginas 360-389).

Se ha descrito un proceso de interesterificación de lecho empacado en el documento WO 83/03844, en donde se disuelven triacilglicéridos en un solvente orgánico polar. La lipasa se inmoviliza por precipitación en partículas de diatomita, hidroxapatito o alúmina.

El documento WO 97/01632 se dirige a otro proceso para inmovilización de una enzima, específicamente, una lipasa o una fosfolipasa, para el procesamiento de aceites de triglicérido.

Unilever además, describe un proceso de dos etapas para la producción de equivalentes de manteca de cacao y Betapol usando columnas de lecho empacado (P. Quinlan & S. Moore, Inform 4, 580-585 (1993)); A. Rozendaal & A.R. Macrae;

in: F.D. Gansdone & F.B. Padlee (eds.) Lipid Technologies and Applications, 1997, páginas 223-263).

X. Xu describe procesos adicionales con lipasa inmovilizada, entre estos un reactor de lecho empacado con una capacidad de 10 kg/día (en U.T. Bornscheuer, Enzymes in Lipid Modification, 2000, páginas 190-215).

Los reactores de lecho empacado existentes sufren del problema de una disminución en la relación de conversión en tiempo. Sin embargo, algunas enzimas tales como lipasas son muy estables, su eficacia inevitablemente cae con el tiempo, lo cual resulta en velocidades de reacción bajas y calidad de producción no uniforme. Esto disminuye la calidad que es contrarrestada por la disminución de velocidad de flujo y con ello incrementa el tiempo de residencia de los reactivos en el reactor. Tal reducción en la velocidad de flujo, conduce a una velocidad de salida no uniforme y mientras puede reducir las fluctuaciones en calidad del producto, no puede evitarse completamente. Además, tal proceso a menudo usa un número de reactores en serie discretos lo cual requiere un control de flujo completo para correr y mantener el sistema. La invención actual resuelve estos problemas y los relacionados usando un nuevo tipo de proceso de columna empacada para modificación enzimática de un sustrato.

BREVE DESCRIPCIÓN DE LA INVENCION

La invención se refiere a un proceso para la modificación de un sustrato, el cual comprende, pasar el sustrato a través de una columna de lecho empacado de un volumen específico de enzima inmovilizada con un flujo de sustrato en una dirección y un intercambio periódico de enzima inmovilizada en la dirección opuesta. El sustrato entra a la columna en o cerca de uno de sus extremos (el "extremo de entrada") y el sustrato modificado es descargado en o cerca de su otro extremo (el "extremo de salida"). Una porción del volumen de la enzima modificada es periódicamente removida del extremo de entrada de la columna, mientras una porción equivalente de enzima inmovilizada es periódicamente agregada en el extremo de salida, con ello se crea un flujo de enzima inmovilizada en la dirección opuesta del flujo del sustrato.

Cuando, en esta solicitud, se hace referencia a "cerca del extremo de entrada" o "cerca del extremo de salida" el término "cerca", se pretende para implicar que tal material (por ejemplo, sustrato, sustrato modificado, enzima inmovilizada) se carga a o descarga del reactor (a través de una abertura, válvula o similar) dentro de una distancia razonable del extremo, como se determina por la persona experta. Esta puede ser, por ejemplo, 1 a 5%, o 10% de longitud de una columna medida a partir del extremo actual de

la columna referenciada. Lo que es más, las expresiones tales como "en el extremo" se deben entender por incluir "en o cerca del extremo" cuando se usan en referencia a la columna, a menos que específicamente se declare de otra forma.

5

DESCRIPCIÓN DETALLADA DE LA INVENCION

Posteriormente, los términos "sustrato" y "solución de sustrato" son usados con el mismo significado para describir un compuesto líquido o mezcla de compuestos o una
10 solución líquida de un compuesto o de una mezcla de compuestos, los cuales significan que reaccionan en la presencia de la enzima inmovilizada. De esta forma, se produce un compuesto líquido o mezcla de compuestos o una
solución líquida de un compuesto o de una mezcla de
15 compuestos, el cual es referido como "producto" o "sustrato modificado", con el mismo significado.

La invención se refiere a un proceso para la modificación enzimática de un sustrato pasando el sustrato a través de una columna de lecho empacado de enzima
20 inmovilizada. El sustrato entra a la columna de lecho empacado en o cerca de un extremo de la columna (en adelante referido como el "extremo de entrada") y pasa a través de la columna hacia su otro extremo (en adelante referido como el "extremo de salida"), en donde el sustrato sale en la
25 columna.

Preferiblemente, la columna es una columna vertical con su extremo de entrada en el fondo de la columna y su extremo de salida en la parte superior de la columna. En esta modalidad, el sustrato entrará al primer lecho en o cerca a su porción más baja y deja la columna en o cerca de su porción más alta.

Ventajosamente, el sustrato y corrientes de sustrato modificados pueden ser esencialmente continuos. Esto significa que, mientras el sustrato y corrientes del sustrato modificado pueden ser detenidos para permitir el cambio periódico de una porción del volumen de la enzima inmovilizada, no hay necesariamente tiempo de reposo prolongado. Esto distingue el presente proceso de los métodos existentes por los cuales se extienden los periodos de tiempo de reposo, en donde se requieren entre otras cosas, cambiar el volumen completo de enzima inmovilizada. Esto no solamente reduce los costos per se, sino también puede prevenir las fluctuaciones de costos.

De conformidad con la presente invención, solamente una porción definida de la enzima inmovilizada es periódicamente removida de la columna. Esto se puede lograr, por medio de ilustración solamente, con el siguiente procedimiento. El flujo continuo de sustrato es detenido. Se abre una válvula de descarga en el extremo de entrada y una porción definida de la suspensión que contiene material

enzimático inmovilizado del extremo inferior de la columna es liberada. Después que una cantidad definida de esta suspensión es descargada, la válvula de descarga es cerrada y se abre una válvula de alimentación en el extremo de salida, o en una tubería de alimentación hacia el extremo de salida del reactor, de esta forma se permite el ingreso de una suspensión fresca de material enzimático inmovilizado en la columna del recipiente de suministro.

La suspensión de la enzima inmovilizada puede ser formada agregando una cantidad definida del sustrato modificado en el recipiente de suministro. Si es necesario, para intensificar el mezclado y para mejorar la humedad de los poros de la enzima inmovilizada, el recipiente puede ser equipado con un dispositivo de agitación y/o puede ser operado bajo vacío. Cuando una carga fresca de enzima es agregada al reactor, el recipiente de suministro puede ser des-aireado y la suspensión, o una porción de la misma, vaciada en la columna. La válvula de alimentación es después cerrada permitiendo a la suspensión fresca asentarse en el extremo del lecho empacado. Finalmente, las válvulas que regulan el sustrato y las corrientes del producto son reabiertas.

El reactor puede ser operado bajo vacío. Preferiblemente, sin embargo, será operado bajo una cierta presión ("presión designada"). La presión designada debe ser

elegida para permitir una máxima caída de presión sobre la longitud de la columna. La caída de presión dependerá de las características de las partículas de enzima inmovilizada (por ejemplo, distribución de tamaño de partícula), la velocidad
5 de flujo del sustrato y la longitud del lecho. Por medio del ejemplo solamente, una presión designada adecuada en un lecho de enzima relativamente alto y con una velocidad de flujo del sustrato relativamente alta puede ser de aproximadamente 10 Bar. Con una longitud de lecho más baja, y velocidad de flujo
10 más lenta, tal valor podría ser cercano a 6 Bar. La presión designada apropiada para cualquier reactor proporcionado será fácilmente determinada por una persona experta.

Para asegurar que la presión designada no exceda la presión alimentada, las bombas de alimentación no tienen una
15 capacidad arriba de la presión designada. Y sobre todo, las entradas del reactor deben ser equipadas con válvulas sin regreso, válvulas de abrir/cerrar y dispositivos de control de velocidad de flujo.

Idealmente, la descarga de la enzima consumida y
20 adición de enzima fresca puede ocurrir concurrentemente o sustancialmente concurrentemente. Por esto, significa que la descarga y adición serán realizadas durante el mismo tiempo de reposo. También se prefiere que sustancialmente la misma cantidad de material enzimático sea descargada conforme sea
25 agregada, con esto se asegura que el reactor mantenga sus

características de lecho empacado. A excepción que ambas reglas puedan ocurrir, por ejemplo, después del encendido inicial del reactor debe haber una cierta cantidad de compactación en la columna. Puede realmente ser necesario en
5 esta situación, agregar una suspensión más pequeña que es descargada o aún agregar suspensión sin alguna descarga.

Repitiendo este cambio de una porción del material de lecho, se garantiza un flujo gradual de la enzima inmovilizada de la parte superior del fondo de la columna. En
10 consecuencia, la solución de sustrato primero llegará a estar en contacto con el material enzimático agotado y finalmente con la enzima más fresca.

Ventajosamente, el cambio periódico de una porción de la enzima inmovilizada puede ser principalmente
15 automatizado, de esta forma limitando la participación del operador, reduciendo costos asociados, y restringiendo la probabilidad de algún mal funcionamiento.

Este sistema puede ser comparado con múltiples reactores básicos pequeños separados en serie. En comparación
20 a tales series, la complejidad de la presente invención es muy reducida, particularmente el número de válvulas de entrada y salida. Sin embargo, la solución procesada no tendrá que ser transportada entre los múltiples reactores. Esto es particularmente importante cuando el sustrato y/o
25 solución del producto tiene que ser mantenido a una cierta

temperatura o cuando tiene una alta viscosidad. Además, un sistema de reactores pequeños requiere que uno o más de los reactores sean periódicamente sacados de servicio mientras la enzima agotada se remueve y se agrega enzima fresca. Esta es una operación manual intensiva y durante este tiempo la eficiencia del sistema completo es muy reducida. Por lo tanto, el capital total y costos de operación para tal sistema no son óptimos. Lo que es más, el sistema de la presente invención permitirá una gradación casi infinita de actividad enzimática a partir de la enzima fresca a la enzima agotada. Esto puede no ser logrado, aún con un número mayor de reactores separados.

El proceso de la invención permite una velocidad de flujo esencialmente continua ya que básicamente en todo el sustrato se encuentra la misma cantidad de actividad enzimática durante su paso a través del reactor. Como la actividad de la enzima inmovilizada varía con la edad y uso, el contacto uniforme del sustrato con la enzima en varias etapas de actividad es decisivo para asegurar una calidad de producto más constante. Por lo tanto, se puede aplicar una velocidad de flujo constante.

La frecuencia con la cual el material enzimático es reemplazado dependerá, entre otras cosas, en la velocidad de disminución de actividad de la enzima durante un tiempo y el tiempo de residencia deseado de la solución de sustrato en el

lecho de enzima empacada. El volumen de material enzimático cambiado y la longitud de los intervalos entre estos cambios pueden fácilmente ser adaptados a las necesidades del proceso y/o sustrato. Además, puede existir una relación entre la frecuencia con la cual la enzima es refrescada y el volumen de la enzima que es refrescada. Con todas las otras variables del proceso que permanecen constantes, una cantidad pequeña de enzima puede ser frecuentemente refrescada o una cantidad más grande de enzima refrescada menos frecuente.

10 Preferiblemente, un porcentaje fijado del volumen de columna completa será refrescado en una base diaria. Idealmente, el porcentaje será menos de 35% del volumen total de enzima inmovilizada. Preferiblemente, será menos del 25%, más preferiblemente menos del 10%, aún más preferiblemente menos del 5%.

15 De conformidad a una posible modalidad, entre aproximadamente 0.3% y aproximadamente 3% del volumen total de enzima inmovilizada serán refrescados en una base diaria.

Después de pasar a través de la columna de lecho empacado de la enzima inmovilizada, se alcanzará una conversión suficiente del sustrato. En la parte superior de la columna, la salida del producto puede ser facilitada por tamices de filtro de forma cilíndrica pequeños. Esto regula el flujo de salida del producto mientras el material enzimático inmovilizado es mantenido dentro del reactor.

20 Existe dos tipos de tamices: tamices de abertura y tamices

25

estándares. Mientras se usan los tamices estándares para el flujo de salida actual del producto, los tamices de abertura están más arriba en la columna para facilitar el flujo de aire o gas durante el drenado y rellenado.

5 Las bombas que regulan la entrada y salida de las suspensiones del material enzimático deben ser diseñadas para que no se separe la enzima inmovilizada, creando una tarifa adicional. Esto ayudará incluso a un flujo y previene a los finos de pasar dispositivos de filtrado subsecuentes.

10 La enzima descargada será una suspensión de enzima inmovilizada usada (o "agotada") y solución de sustrato. Esta suspensión puede ser separada y la solución de sustrato puede ser reintroducida en el tanque, el cual proporciona la entrada de sustrato a la columna de reacción. Puede ser
15 dispuesta la enzima usada. Preferiblemente, sin embargo, será procesada para uso adicional.

La enzima removida de la columna puede efectivamente ser sometida a reciclaje. Por ejemplo, en algunos casos la enzima removida de la columna todavía tiene
20 algo de actividad residual, a la cual se puede tener acceso si el material inmovilizado es roto en pequeñas partículas, con ello, revelando los sitios activos de la enzima que no fueron previamente en todo o solamente de manera restringida accesibles al sustrato (esto se conoce como "reactivación").
25 Alternativamente, la enzima puede ser regenerada y

reutilizada. De esta forma, en una modalidad preferida del proceso de conformidad con la invención, el material enzimático descargado de la columna es regenerado o reactivado y reutilizado, por ejemplo, para pretratar el sustrato antes de entrar a la columna o siendo agregado al tanque el cual suministra la entrada de la enzima inmovilizada. Puede también ser reutilizada en otros sistemas similares a modificación de lote.

El proceso de la presente invención puede ser operado en una columna única o pasando el sustrato a través de dos o más columnas de enzimas inmovilizadas dispuestas en paralelo. El término "enzima inmovilizada" toma su significado normal en la técnica, es decir, aquella de una enzima la cual está físicamente unida a un soporte sólido. Soportes preferidos incluyen soportes de sílice y resina. La enzima inmovilizada puede estar presente en la forma de gránulos, partículas aglomeradas o cualquier otra forma considerada adecuada por una persona experta.

La elección de la enzima será por supuesto, dependiendo del tipo de reacción a llevarse a cabo, es decir, en la naturaleza del sustrato y su modificación deseada. Puede ser cualquier oxidoreductasa, transferasa, hidrolasa, lipasa, isomerasa o ligasa disponible en la técnica. Por medio de ilustración, las hidrolasas adecuadas pueden incluir hidrolasas de éster carboxílico (EC-3.1.1) tales como

carboxiesterasas, triacilglicerol lipasas, fosfolipasa A2, esterol esterasas, acilglicerol lipasas, fosfolipasa A1 e cero-éster hidrolasas; y diéster fosfórico hidrolasas (EC-3.1.4) tales como fosfolipasa C y fosfolipasa D. Estas 5 enzimas pueden ser usadas, por ejemplo, para catalizar hidrólisis, alcoholólisis o la correspondiente conversión inversa (condensación). Otras enzimas adecuadas serán conocidas por la persona experta dependiendo de la modificación específica a ser realizada.

10 El sustrato será cualquier material o sustancia capaz de ser actuado por una enzima. Puede incluir, por ejemplo, uno o más lípidos, una o más proteínas, uno o más carbohidratos o una mezcla de los mismos. El término "lípido" como se usa en este documento, se refiere no solamente a 15 aceites crudos o procesados y fracciones del mismo, sino también a triacilglicéridos, diacilglicéridos, monoacilglicéridos, fosfolípidos, ácidos grasos libres, ésteres de ácido graso y mezclas de los mismos. El término "proteína" puede referirse, entre otros, tanto a polipéptidos 20 complejos como simples, ya sea en su estado plegado o desnaturalizado, a secuencias de péptido corto y a aminoácidos únicos. El término "carbohidrato" se refiere, por ejemplo, a unidades de monosacárido único, disacáridos, oligosacáridos y polisacáridos tales como almidón, celulosa 25 y etc. El sustrato puede ser mezclado con otros reactivos

(tal como un alcohol o agua), o con un solvente si es necesario. Sí se desea, el sustrato puede ser refinado antes de entrar a la columna. Refinación se refiere a la eliminación de impurezas tales como residuos y derivados, y
5 puede contribuir a prolongar la vida de las enzimas inmovilizadas.

Como se indicó anteriormente, el proceso de la presente invención puede ser usado para realizar cualquier tipo de reacción enzimática. No obstante, ahora serán
10 descritas un número de posibles modalidades en detalle adicional por medio de ilustración. Esto no debe ser interpretado en cualquier modo, como limitante del alcance de la invención. Mejor dicho, sus enseñanzas deben ser entendidas, cuando sea apropiado, para aplicar la invención
15 como un todo.

Interesterificación

Esta modalidad se refiere a la interesterificación de aceites. La interesterificación cambia el perfil acil-
20 glicerol del aceite o mezclas de aceite, de esta forma modifica sus propiedades físicas.

De conformidad con esta modalidad, la enzima de elección es una lipasa. Será idealmente inmovilizada en un soporte de sílice. Una enzima inmovilizada adecuada es la
25 lipasa Lypozyme TL IM de Novozymes.

El sustrato, el cual puede ser usado en un estado refinado o no refinado, dependiendo de los requerimientos, típicamente comprenderá un acilglicérido tal como un triacilglicérido, un diacilglicérido, un monoacilglicérido o una mezcla de dos o más de los mismos. Puede ser derivado de una fuente de planta, animal, pez, alga o de célula única. Preferiblemente, será derivado de una fuente de planta. Sustratos adecuados por lo tanto, incluirán pero no se limitarán a, aceite de palma kernel, aceite de palma, aceite de coco, aceite de oliva, aceite de semilla de colza, aceite de canola, aceite de semilla de lino, aceite de cacahuate, aceite de soya, aceite de semilla de algodón, aceite de semilla de girasol, aceite de semilla de calabaza, aceite de maíz, aceite de ricino, aceite de nuez y mezclas de dos o más de los mismos. El sustrato puede incluir aceites completos y/o fracciones de aceites tal como estearinas u oleínas.

De conformidad con una modalidad, el sustrato comprenderá estearina de palma, aceite de coco, aceite de palma kernel o una mezcla de dos o más de los mismos. Preferiblemente, el sustrato comprenderá una mezcla de estearina de palma más aceite de coco o una mezcla de estearina de palma más aceite de palma kernel. Aún más preferiblemente, la mezcla incluirá 50-80% en peso de estearina de palma y 50-20% en peso de aceite de coco y/o aceite de palma kernel, en base al peso total del sustrato.

Idealmente, la mezcla incluirá 65-75% en peso de estearina de palma y 35-25% en peso de aceite de coco o 60-70% en peso de estearina de palma y 40-30% en peso de aceite de palma kernel. De conformidad a una modalidad más preferida, el sustrato de la presente invención consistirá de una mezcla.

El sustrato puede ser bombeado a través de una o más columnas presentadas en paralelo con la enzima inmovilizada. El sustrato y material enzimático inmovilizado pueden fluir en direcciones opuestas con la entrada de aceite en o cerca del fondo de la columna y el material enzimático fresco se agregará en o cerca de la parte superior. Periódicamente, una parte de la enzima inmovilizada será intercambiada con material enzimático fresco. Por lo tanto, la enzima en la columna puede tener un perfil de calidad casi constante. La enzima inmovilizada en la parte superior de la columna será más fresca con actividad casi completa y la enzima inmovilizada cercana al fondo de la columna será casi agotada de actividad. Por lo tanto, existirá una gradación de actividad a lo largo de la longitud de la columna.

La reacción de interesterificación debe ser realizada a una temperatura definida. Esta temperatura será elegida con un número de factores en mente:

- como la mayoría de los aceites y grasas interesterificados producidos de conformidad con este proceso se planean para productos nutricionales, el proceso

preferiblemente estará libre de solventes orgánicos. Como tal, el proceso debe ser realizado a una temperatura la cual garantiza que los aceites/grasas permanezcan en un estado líquido;

5 - la temperatura debe ser suficientemente alta para permitir una velocidad de reacción elevada, a su vez, permitirá tiempos de residencia cortos;

 - la temperatura debe ser suficientemente baja para no afectar la estabilidad y actividad de la enzima. Lipasas
10 microbianas son muy termoestables y se encontró la inmovilización por mejorar adicionalmente la estabilidad de estas enzimas, así que las temperaturas óptimas para muchas lipasas varían de 30°C a 62°C (W.M. Willis and A.G. Marangoni, in Enzymatic Interesterification, Food Lipids -
15 Chemistry, Nutrition, and Biotechnology, editado por C.C. Akoh and D.B. Min). Algunas lipasas aún permiten temperaturas de 60°C a 75°C (WO 97/01632). El desarrollo enzimático futuro puede incluir aún variedades más resistentes a temperaturas, por ejemplo, resistente a 80°C, 90°C, 100°C o superior. A
20 estas temperaturas, muchos triacilglicéridos, aún los mismos completamente saturados, estarán en un estado líquido.

Tomando en cuenta estos factores, la persona experta será capaz de determinar la temperatura de reacción ideal.

25 Por medio de ejemplo, la interesterificación de

estearina de palma con aceite de coco por Lypozyme TL IM (Novozymes) preferiblemente será realizada a aproximadamente 70°C.

Después de calentar los aceites a la temperatura y
5 mezclado deseado, si es necesario, son dirigidos a la válvula de entrada en o cera del extremo de entrada del reactor. El reactor preferiblemente, será diseñado para operar bajo vacío y hasta una presión adecuada.

La enzima fresca inmovilizada es suministrada en la
10 parte superior de la columna a intervalos regulares. Una cantidad definida de aceite de producto interesterificado puede ser bombeada en el recipiente de suministro y mezclada con la enzima inmovilizada para producir una suspensión de flujo libre. Para mejorar el mezclado y mejorar la humedad de
15 los poros de la enzima inmovilizada, se puede aplicar un vacío al recipiente. Ventajosamente, el recipiente será chorreado con nitrógeno para prevenir la oxidación del aceite.

Cuando una carga fresca de la enzima es agregada a
20 la columna, el recipiente de suministro es des-aireado, si es aplicable, y la suspensión o una porción de la misma vaciada en el reactor. Si se requiere, el recipiente y tuberías pueden ser chorreados con un cierto volumen de aceite de producto para asegurar el vaciado completo del recipiente.
25 Preferiblemente, el recipiente de suministro será situado

arriba del reactor para facilitar la carga de enzima.

El proceso de esta modalidad será preferiblemente esencialmente continuo. El tiempo de reposo solamente será algunos momentos que toma para intercambiar una porción de la enzima a partir de la columna. Este tiempo de reposo es solamente una fracción muy pequeña del tiempo de corrida total del proceso.

Alcoholólisis

A menos que se declare de otro modo, los detalles de esta modalidad son los mismos como para la reacción de interesterificación descrita anteriormente.

El sustrato será una mezcla adecuadamente agitada de uno o más aceites y uno o más alcoholes. Los alcoholes adecuados incluyen pero no se limitan a, metanol, etanol, glicerol, monopropilenglicol, etilenglicol, butandiol, eritritol, pentaeritritol, sorbitol, esteroides, estanoles, tocoferoles, polifenoles, ésteres de tales alcoholes y mezclas de dos o más de los mismos. La enzima inmovilizada será preferiblemente lipasa.

La reacción preferiblemente se llevará a cabo a 50-70°C. Después de ser colectados en la salida del reactor, los productos (ésteres de ácido graso y glicerol), pueden ser aislados. El aislamiento puede ser realizado por enfriamiento a una temperatura en donde ocurre la separación de fase,

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seguido por aislamiento de la fase de éster de ácido graso y una etapa de secado. El alcohol puede ser recuperado por evaporación directamente de la mezcla o después de aislar los ésteres de ácido graso.

5

Glicerólisis

La glicerólisis es un tipo específico de alcoholólisis, como se describe anteriormente. El sustrato es una mezcla de uno o más aceites y glicerol. La enzima es una
10 enzima inmovilizada en un portador el cual es inerte contra glicerol.

El sustrato es preparado mezclando o disolviendo glicerol en el(los) aceite(s) a temperatura de reacción o ligeramente arriba. La cantidad de glicerol mezclado con el
15 aceite puede ser incrementada agregando un emulsificador. Dependiendo de la relación de glicerol a aceite, la enzima más cercana al extremo de salida del reactor, actuará tanto como un purificador de sustrato como para ajustar la concentración de equilibrio de glicerol en el sustrato.

20 La reacción preferiblemente se llevará a cabo a 50-70°C. El producto recuperado después de la modificación, dependerá de la relación de glicerol a aceite del sustrato.

Hidrólisis

En esta modalidad -la cual sigue a la modalidad de interesterificación descrita anteriormente a menos que se indique lo contrario- el sustrato es una solución de lecitina o lecitina bombeable. Una solución de lecitina se puede obtener mezclando lecitina con un solvente tal como hexano y agua.

La enzima será una lipasa o fosfolipasa. Dependiendo de la enzima, se obtendrán productos diferentes. De este modo, si se usa una lipasa, el producto será una mezcla de fosfolípidos y mono y/o diglicéridos. Usando fosfolipasa A o B, a una temperatura de reacción preferida de 20-70°C, conducirá a la producción de lisofosfolípidos. Usando fosfolipasa C, a una temperatura de reacción preferida de 20-50°C, conducirá a la producción de di y/o triglicéridos. Usando fosfolipasa D, a una temperatura de reacción preferida de 20-70°C, conducirá a la producción de ácido fosfatídico. Si es necesario, puede ser usada una mezcla de enzimas diferentes para obtener el producto deseado.

Después de la conversión, las mezclas de producto pueden ser usadas como tales o después de etapas de purificación adicionales (por ejemplo, lavado, secado, evaporación, fraccionación, etc.).

Isomerización

Otra posible reacción la cual podría ser realizada de conformidad con el proceso de la presente invención, es isomerización. Un ejemplo típico de isomerización es la conversión de glucosa a fructuosa. De este modo, de conformidad con una modalidad, el sustrato preferiblemente será glucosa (o un jarabe de glucosa) y la enzima inmovilizada será preferiblemente una glucosa isomerasa (EC 5.3.1.5). La reacción debe ser idealmente llevada a cabo entre 55-75°C. El producto será un jarabe que contiene tanto glucosa como fructuosa. Si se desea, el contenido de fructuosa puede ser además incrementado por purificación o por división de la glucosa y fructuosa en productos separados.

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Se hace constar que con relación a esta fecha, el mejor método conocido por la solicitante para llevar a la práctica la citada invención, es el que resulta claro de la presente descripción de la invención.

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REIVINDICACIONES

Habiéndose descrito la invención como antecede, se reclama como propiedad lo contenido en las siguientes reivindicaciones:

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1. Proceso para la modificación de un sustrato, que comprende pasar el sustrato a través de una columna de lecho empacado de un volumen específico de enzima inmovilizada, caracterizado porque:

10

a) el sustrato entra a la columna en o cerca de un extremo de la columna (el "extremo de entrada") y el sustrato modificado sale en o cerca del extremo opuesto de la columna (el "extremo de salida"),

15

b) una porción del volumen de la enzima modificada es periódicamente removida en o cerca del extremo de entrada de la columna, y

a) una porción de enzima inmovilizada es periódicamente agregada en o cerca del extremo de salida de la columna.

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2. Proceso de conformidad con la reivindicación 1, caracterizado porque la columna es sustancialmente vertical con su extremo de entrada en o cerca del fondo de la columna y su extremo de salida en o cerca de la parte superior de la columna.

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3. Proceso de conformidad con la reivindicación 1

o reivindicación 2, caracterizado porque es esencialmente un proceso continuo.

4. Proceso de conformidad con cualquiera de las reivindicaciones precedentes, caracterizado porque la porción
5 de la enzima inmovilizada la cual es removida de la columna, es menos de 35% del volumen total.

5. Proceso de conformidad con cualquiera de las reivindicaciones precedentes, caracterizado porque la porción de enzima inmovilizada la cual es removida de la columna, es
10 sustancialmente equivalente en volumen a la porción de la enzima inmovilizada la cual es agregada.

6. Proceso de conformidad con cualquiera de las reivindicaciones precedentes, caracterizado porque las etapas de remover la enzima inmovilizada de y de agregar enzima
15 inmovilizada a la columna, ocurren sustancialmente de manera concurrente.

7. Proceso de conformidad con cualquiera de las reivindicaciones precedentes, caracterizado porque la porción removida de enzima inmovilizada es usada para pre-tratar el
20 sustrato antes de que entre a la columna.

8. Proceso de conformidad con la reivindicación 7, caracterizado porque la porción removida de la enzima inmovilizada es regenerada o reactivada antes de ser usada para pre-tratar el sustrato.

25 9. Proceso de conformidad con cualquiera de las

reivindicaciones precedentes, caracterizado porque la modificación es una condensación o incluye etapas de condensación.

10. Proceso de conformidad con cualquiera de las
5 reivindicaciones 1 a 8, caracterizado porque la modificación es una interesterificación.

11. Proceso de conformidad con cualquiera de las reivindicaciones 1 a 8, caracterizado porque la modificación es una isomerización.

10 12. Proceso de conformidad con cualquiera de las reivindicaciones 1 a 10, caracterizado porque la enzima es una hidrolasa.

13. Proceso de conformidad con la reivindicación 12, caracterizado porque la enzima es una lipasa o una enzima
15 que modifica la lipasa y el sustrato comprende uno o más aceites crudos, uno o más aceites procesados o una mezcla de los mismos.

14. Proceso de conformidad con la reivindicación 12, caracterizado porque el sustrato comprende uno o más
20 triacilglicéridos, diacilglicéridos, monoacilglicéridos, fosfolípidos, ácidos grasos libres, ésteres de ácidos grasos o mezclas de dos o más de los mismos.

15. Proceso de conformidad con la reivindicación 14, caracterizado porque el sustrato además comprende un
25 alcohol.

16. Proceso de conformidad con la reivindicación 15, caracterizado porque el alcohol se selecciona de: metanol, etanol, glicerol, monopropilenglicol, etilenglicol, butandiol, eritritol, pentaeritritol, sorbitol, esteroides, estanoles, tocoferoles, polifenoles, ésteres de tales alcoholes y mezclas de dos o más de los mismos.

17. Proceso de conformidad con cualquiera de las reivindicaciones 14-16, caracterizado porque el sustrato además comprende agua.

18. Proceso de conformidad con la reivindicación 13, caracterizado porque el sustrato comprende uno o más aceites a base de planta o fracciones de aceite a base de planta.

19. Proceso de conformidad con la reivindicación 18, caracterizado porque el sustrato comprende estearina de palma, aceite de coco, aceite de palma kernel o una mezcla de dos o más de los mismos.

20. Proceso de conformidad con la reivindicación 19, caracterizado porque el sustrato comprende 50% hasta 80% en peso de estearina de palma y 50% hasta 20% en peso de aceite de coco.

21. Proceso de conformidad con la reivindicación 20, caracterizado porque el sustrato comprende 65% hasta 75% en peso de estearina de palma y 35% hasta 25% en peso de aceite de coco.

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22. Proceso de conformidad con la reivindicación 19, caracterizado porque el sustrato comprende 50% hasta 80% en peso de estearina de palma y 50% hasta 20% en peso de aceite de palma kernel.

5 23. Proceso de conformidad con la reivindicación 22, caracterizado porque el sustrato comprende 60% hasta 70% en peso de estearina de palma y 40% hasta 30% en peso de aceite de palma kernel.

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RESUMEN DE LA INVENCION

La presente invención se refiere a un proceso para
5 la modificación de un sustrato que comprende, pasar el
sustrato a través de una columna de lecho empacado de un
volumen específico de enzima inmovilizada, en donde el
sustrato entra a la columna en o cerca de un extremo de la
columna (el "extremo de entrada") y el sustrato modificado
10 sale en o cerca del extremo opuesto de la columna (el
"extremo de salida"), una porción del volumen de la enzima
modificada es periódicamente removida en o cerca del extremo
de entrada de la columna, y una porción equivalente de enzima
inmovilizada es periódicamente agregada en o cerca del
15 extremo de salida de la columna.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44bis)

Applicant's or agent's file reference 30A-100 850	FOR FURTHER ACTION		See item 4 below
International application No. PCT/EP2007/002596	International filing date (<i>day/month/year</i>) 23 March 2007 (23.03.2007)	Priority date (<i>day/month/year</i>) 31 March 2006 (31.03.2006)	
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237			
Applicant CARGILL, INCORPORATED			

1.	This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 <i>bis</i> .1(a).																								
2.	This REPORT consists of a total of 6 sheets, including this cover sheet. In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.																								
3.	This report contains indications relating to the following items: <table style="width: 100%; border: none;"> <tr> <td style="width: 10%; text-align: center;">☒</td> <td style="width: 30%;">Box No. I</td> <td style="width: 60%;">Basis of the report</td> </tr> <tr> <td style="text-align: center;">☐</td> <td>Box No. II</td> <td>Priority</td> </tr> <tr> <td style="text-align: center;">☐</td> <td>Box No. III</td> <td>Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</td> </tr> <tr> <td style="text-align: center;">☐</td> <td>Box No. IV</td> <td>Lack of unity of invention</td> </tr> <tr> <td style="text-align: center;">☒</td> <td>Box No. V</td> <td>Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</td> </tr> <tr> <td style="text-align: center;">☐</td> <td>Box No. VI</td> <td>Certain documents cited</td> </tr> <tr> <td style="text-align: center;">☐</td> <td>Box No. VII</td> <td>Certain defects in the international application</td> </tr> <tr> <td style="text-align: center;">☐</td> <td>Box No. VIII</td> <td>Certain observations on the international application</td> </tr> </table>	☒	Box No. I	Basis of the report	☐	Box No. II	Priority	☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability	☐	Box No. IV	Lack of unity of invention	☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement	☐	Box No. VI	Certain documents cited	☐	Box No. VII	Certain defects in the international application	☐	Box No. VIII	Certain observations on the international application
☒	Box No. I	Basis of the report																							
☐	Box No. II	Priority																							
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability																							
☐	Box No. IV	Lack of unity of invention																							
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement																							
☐	Box No. VI	Certain documents cited																							
☐	Box No. VII	Certain defects in the international application																							
☐	Box No. VIII	Certain observations on the international application																							
4.	The International Bureau will communicate this report to designated Offices in accordance with Rules 44bis.3(c) and 93bis.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44bis .2).																								

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Date of issuance of this report 30 September 2008 (30.09.2008) Authorized officer Agnes Wittmann-Regis e-mail: pt06.pct@wipo.int
--	---

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PCT

see form PCT/ISA/220

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/EP2007/002596

International filing date (day/month/year)
23.03.2007

Priority date (day/month/year)
31.03.2006

International Patent Classification (IPC) or both national classification and IPC
INV. C12M1/40 C12P7/64

Applicant
CARGILL, INCORPORATED

1. This opinion contains indications relating to the following items:

- | | |
|---|--|
| ☒ Box No. I | Basis of the opinion |
| ☐ Box No. II | Priority |
| ☐ Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| ☐ Box No. IV | Lack of unity of invention |
| ☒ Box No. V | Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| ☐ Box No. VI | Certain documents cited |
| ☐ Box No. VII | Certain defects in the international application |
| ☐ Box No. VIII | Certain observations on the international application |

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:



European Patent Office - Gitschiner Str. 103
D-10958 Berlin
Tel. +49 30 25901 - 0
Fax: +49 30 25901 - 840

Date of completion of this opinion

see form
PCTISA/210

Authorized Officer _____

Cubas Alcaraz, Jose

Telephone No. +49 30 25901-324



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2007/002596

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☐ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☐ on paper
 - ☐ in electronic form
 - c. time of filing/furnishing:
 - ☐ contained in the international application as filed.
 - ☐ filed together with the international application in electronic form.
 - ☐ furnished subsequently to this Authority for the purposes of search.
3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2007/002596

Box No. V Reasoned statement under Rule 43b/s.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>1-23</u>
	No: Claims	
Inventive step (IS)	Yes: Claims	
	No: Claims	<u>1-23</u>
Industrial applicability (IA)	Yes: Claims	<u>1-23</u>
	No: Claims	

2. Citations and explanations

see separate sheet

Re Item V

**Reasoned statement with regard to novelty, inventive step or industrial applicability;
citations and explanations supporting such statement**

1. Reference is made to the following documents:

D1: US-A-4629742

D2: US-A-4209591

D3: DE-U-9320595

D4: Eur. J. Lipid Sci. Technol. 105 (2003) pages 289-304.

2. The present application does not meet the requirements of Article 33 PCT because the subject-matter of claim 1 does not involve an inventive step in the sense of Article 33(3) PCT.

The document D1 is considered as the closest prior art to the subject-matter of claim 1. This document describes (col. 7, line 54-col. 8, line 25; figure 1) a process for the enzymatic modification of a substrate to produce a modified substrate comprising the introduction of the substrate at the bottom of a column containing a packed bed of immobilized enzyme and drawing off the modified substrate via a conduit on the top of the column. The document D4 (page 296, figure 14) also describes a process as referred in claim 1, comprising a packed bed of immobilized enzyme, addition of substrate at the bottom of the column and outlet of the modified substrate at the top of the column.

The difference between the subject-matter of claim 1 and the process disclosed in D1 is limited to the steps of removing periodically a portion of the immobilized enzyme at or near the inlet end of the column and adding periodically a portion of immobilized enzyme at or near the outlet of of the column.

The problem to be solved is to provide a process for the enzymatic modification of a substrate having a constant and increased reaction yield.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/EP2007/002596

However, the distinguishing feature is described in the documents D2 (col. 3, lines 10-21; claim 1; figure 1), related to a process for the enzymatic modification of a substrate using a fluidized bed, and D3 (claim 1; page 3, paragraphs 5 and 6), related to a biological process using a stirred bed, as providing the same advantages as in the present application. The loss of activity of the enzyme during the process (see col. 3, lines 48-55 in D1) as well as the importance of maintaining a constant activity of the enzyme during the process (see page 303 in D4) are known problems. The skilled person would therefore regard it as a normal option to include the distinguishing feature as described in D2 or D3 in the process described in the documents D1 or D4 in order to solve the problem posed.

3. The dependent claims 2-23 are related to features already disclosed in the prior art or are directed to alternatives not giving rise to an unexpected technical effect. Accordingly, the subject-matter of claims 2-23 does not involve an inventive step (Article 33(3) PCT).

PATENT COOPERATION TREATY

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From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To: WUESTHOFF & WUESTHOFF Allen. Wibelmann, Leiter Schweigerstrasse D-81541 München ALLEMAGNE	
WUESTHOFF & WUESTHOFF PATENT-UND RECHTSANWÄLTE Eingeg. 14. Juni 2007 68	Recd. 14/06/2007

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 30A-100 850	Date of mailing (day/month/year) 14/06/2007
International application No. PCT/EP2007/002596	International filing date (day/month/year) 23/03/2007
Applicant CARGILL, INCORPORATED	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland. Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Diana Dreschers
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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Volume I/A, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume I/A, paragraph 296).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 30A-100 850	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/EP2007/002596	International filing date (day/month/year) 23/03/2007	(Earliest) Priority Date (day/month/year) 31/03/2006
Applicant CARGILL, INCORPORATED		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☐ Certain claims were found unsearchable (See Box No. II)

3. ☐ Unity of invention is lacking (see Box No. III)

4. With regard to the title,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. _____
☐ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
 b. ☐ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

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International application No
PCT/EP2007/002596

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12M1/40 C12P7/64

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)
C12M C12P B01J

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 629 742 A (BRADY ET AL) 16 December 1986 (1986-12-16) column 3, line 48 - line 55 column 7, line 54 - column 8, line 25 figure 1	1-23
Y	US 4 209 591 A (HENDRIKS, PETRUS F A M) 24 June 1980 (1980-06-24) column 3, line 10 - line 21 claim 1 figure 1	1-23
A	DE 93 20 595 U1 (KUNZ, PETER, PROF. DR., 76185 KARLSRUHE, DE) 20 October 1994 (1994-10-20) page 3 claim 1	1-23

☒ 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
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *I* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

6 June 2007

Date of mailing of the international search report

14/06/2007

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Cubas Alcaraz, Jose

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/002596

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	XUEBING XU: "Engineering of enzymatic reactions and reactors for lipid modification and synthesis" EUR. J. LIPID SCI. TECHNOL., vol. 105, 2003, pages 289-304, XP002396995 page 296 page 303	1-23
A	----- US 4 029 546 A (BROUILLARD ET AL) 14 June 1977 (1977-06-14) figure 1; example 24 -----	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2007/002596

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
US 4629742	A	16-12-1986	CA	1297822 C	24-03-1992
			DE	3778176 D1	21-05-1992
			EP	0232933 A1	19-08-1987
			ES	2032431 T3	16-02-1993
			GR	3005178 T3	24-05-1993
			GR	3015235 T3	30-06-1995
			JP	1798471 C	12-11-1993
			JP	5003274 B	14-01-1993
			JP	62179388 A	06-08-1987

US 4209591	A	24-06-1980	BE	869496 A2	05-02-1979
			CA	1117043 A1	26-01-1982
			CH	646195 A5	15-11-1984
			DE	2835979 A1	01-03-1979
			DK	344378 A	20-02-1979
			FR	2400386 A1	16-03-1979
			GB	2003048 A	07-03-1979
			IT	1107204 B	25-11-1985
			JP	1445077 C	30-06-1988
			JP	54044088 A	07-04-1979
			JP	62050113 B	22-10-1987
			NL	7709179 A	21-02-1979

DE 9320595	U1	20-10-1994	NONE		

US 4029546	A	14-06-1977	AU	2368077 A	05-10-1978
			DE	2713858 A1	06-10-1977
			FR	2346042 A1	28-10-1977
			JP	52136990 A	16-11-1977
			NL	7703407 A	03-10-1977

PATENT COOPERATION TREATY

63

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/EP2007/002596

International filing date (day/month/year)
23.03.2007

Priority date (day/month/year)
31.03.2006

International Patent Classification (IPC) or both national classification and IPC
INV. C12M1/40 C12P7/64

Applicant
CARGILL, INCORPORATED

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:



European Patent Office - Gitschiner Str. 103
D-10958 Berlin
Tel. +49 30 25901 - 0
Fax: +49 30 25901 - 840

Date of completion of
this opinion

see form
PCT/ISA/210

Authorized Officer

Cubas Alcaraz, Jose

Telephone No. +49 30 25901-324



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2007/002596

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☐ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☐ on paper
 - ☐ in electronic form
 - c. time of filing/furnishing:
 - ☐ contained in the international application as filed.
 - ☐ filed together with the international application in electronic form.
 - ☐ furnished subsequently to this Authority for the purposes of search.
3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/EP2007/002596

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>1-23</u>
	No: Claims	
Inventive step (IS)	Yes: Claims	
	No: Claims	<u>1-23</u>
Industrial applicability (IA)	Yes: Claims	<u>1-23</u>
	No: Claims	

2. Citations and explanations

see separate sheet

Re Item V

**Reasoned statement with regard to novelty, inventive step or industrial applicability;
citations and explanations supporting such statement**

1. Reference is made to the following documents:

D1: US-A-4629742

D2: US-A-4209591

D3: DE-U-9320595

D4: Eur. J. Lipid Sci. Technol. 105 (2003) pages 289-304.

2. The present application does not meet the requirements of Article 33 PCT because the subject-matter of claim 1 does not involve an inventive step in the sense of Article 33(3) PCT.

The document D1 is considered as the closest prior art to the subject-matter of claim 1. This document describes (col. 7, line 54-col. 8, line 25; figure 1) a process for the enzymatic modification of a substrate to produce a modified substrate comprising the introduction of the substrate at the bottom of a column containing a packed bed of immobilized enzyme and drawing off the modified substrate via a conduit on the top of the column. The document D4 (page 296, figure 14) also describes a process as referred in claim 1, comprising a packed bed of immobilized enzyme, addition of substrate at the bottom of the column and outlet of the modified substrate at the top of the column.

The difference between the subject-matter of claim 1 and the process disclosed in D1 is limited to the steps of removing periodically a portion of the immobilized enzyme at or near the inlet end of the column and adding periodically a portion of immobilized enzyme at or near the outlet of of the column.

The problem to be solved is to provide a process for the enzymatic modification of a substrate having a constant and increased reaction yield.

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**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/EP2007/002596

However, the distinguishing feature is described in the documents D2 (col. 3, lines 10-21; claim 1; figure 1), related to a process for the enzymatic modification of a substrate using a fluidized bed, and D3 (claim 1; page 3, paragraphs 5 and 6), related to a biological process using a stirred bed, as providing the same advantages as in the present application. The loss of activity of the enzyme during the process (see col. 3, lines 48-55 in D1) as well as the importance of maintaining a constant activity of the enzyme during the process (see page 303 in D4) are known problems. The skilled person would therefore regard it as a normal option to include the distinguishing feature as described in D2 or D3 in the process described in the documents D1 or D4 in order to solve the problem posed.

3. The dependent claims 2-23 are related to features already disclosed in the prior art or are directed to alternatives not giving rise to an unexpected technical effect. Accordingly, the subject-matter of claims 2-23 does not involve an inventive step (Article 33(3) PCT).

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REPORTE DE BÚSQUEDA INTERNACIONAL

No. de Solicitud Internacional
PCT/EP2007/002596

A. CLASIFICACION DEL ASUNTO RELACIONADO		
INV. C12M1/40 C12P/64		
De acuerdo con la Clasificación Internacional de Patentes (IPC) o tanto la clasificación nacional como la IPC.		
B. CAMPOS BUSCADOS		
Documentación mínima buscada (sistema de clasificación seguido por los símbolos de clasificación) C12M C12P B01J		
Documentación buscada diferente de la documentación mínima, al grado que estos documentos se incluyen en los campos buscados.		
Base de datos, electrónica, consultada durante la búsqueda internacional (nombre de la base de datos, donde se puede practicar, términos de búsqueda usados). EPO-Interno, Datos WPI		
C. DOCUMENTOS QUE SE CONSIDERAN PERTINENTES		
Categoría*	Citación del documento, con indicación, donde es apropiado, de los pasajes pertinentes	Pertinente para la reivindicación No.
Y	US 4 629 742 A (BRADY ET AL) 16 Diciembre 1986 (1986-12-16) columna 3, línea 48- línea 55 columna 7, línea 54 - columna 8, línea 25 figura 1	1-23
Y	US 4 209 591 A (HENDRIKS, PETRUS F A M) 24 Junio 1980 (1980-06-24) columna 3, línea 10 - línea 21 reivindicación 1 figura 1	1-23
A	DE 93 20 595 UI (KUNZ, PETER, PROF. DR., 76185 KARLSRUHE, DE) 20 Octubre 1994 (1994-10-20) página 1 reivindicación 1	1-23
☒ Documentos adicionales se listan en la continuación del cuadro C. ☒ Los miembros de la familia de patentes se listan en el anexo		
* Categoría especial de los documentos citados: "A" Documento que define el estado general de la técnica, que no se considera que es de pertinencia particular "E" Documento anterior, pero publicado en o después de la fecha de presentación internacional "L" Documento que puede crear dudas de la reivindicación(es) de prioridad o que se cita para establecer la fecha de publicación de otra situación u otra razón especial (como se especifique) "O" Documento que se refiere a una descripción oral, uso, exhibición u otro medio "P" Documento publicado antes de la fecha de presentación internacional, pero después de la fecha de prioridad reivindicada "T" Documento posterior publicado después de la fecha de presentación internacional o la fecha de prioridad, y que no provoca conflicto con la solicitud, pero se cita para entender el principio o teoría que trata la invención "X" Documento de pertinencia particular; la invención reivindicada no se puede considerar nueva o no se puede considerar que comprende un paso inventivo cuando el documento se toma solo "Y" Documento de pertinencia particular; la invención reivindicada no se puede considerar que comprende un paso inventivo cuando el documento se combina con uno o más de estos documentos, estas combinaciones son obvias para una persona experta en la técnica "&" Documento miembro de la familia de patentes		
Fecha de la actual terminación de la búsqueda internacional 06 Junio 2007		Fecha de envío del reporte de búsqueda internacional 14/06/2007
Nombre y dirección de envío I SA/ Oficina Europea de Patentes, P.B. 5818 Patentlaan 2 NL- 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax. (+31-70) 340-3016		Funcionario autorizado Cubas Alcaráz, José

REPORTE DE BUSQUEDA INTERNACIONAL

No. Solicitud Internacional

PCT/EP2007/002596

DOCUMENTOS QUE SE CONSIDERAN PERTINENTES (continuación)

Categoría*	Citación del documento, con indicación, donde es apropiado, de los pasajes pertinentes	Reivindicaciones Relevantes No.
A	XUEBING XU: "Engineering of enzymatic Reactions and reactors for lipid Modification and synthesis" EUR. J. LIPID SCI. TECHNOL., Vol. 105, 2003, páginas 298-304, XP002396995 Página 296 Página 303	1-23
A	US 4 029 546 A (BROUILLARD ET AL) 14 Junio 1977 (1977-06-14) Figura 1; ejemplo 24	1-23

REPORTE DE BUSQUEDA INTERNACIONAL

Información sobre los miembros de familia de patentes

Solicitud Internacional No.:

PCT/EP2007/002596

Documento de la patente citado en el reporte de búsqueda	Fecha de publicación	Miembros de la familia de patentes	Fecha de publicación
US 4629742	A	16-12-1986	CA 1297822 C 24-03-1992
			DE 3778176 D1 21-05-1992
			EP 0232933 A1 19-08-1987
			ES 2032431 T3 16-02-1993
			GR 3005178 T3 24-05-1993
			GR 3015235 T3 30-06-1995
			JP 1798471 C 12-11-1993
			JP 5003274 B 14-01-1993
			JP 62179388 A 06-08-1987
US 4209591	A	24-06-1980	BE 869496 A2 05-02-1979
			CA 1117043 A1 26-01-1982
			CH 646195 A5 15-11-1984
			DE 2835979 A1 01-03-1979
			DK 344378 A 20-02-1979
			FR 2400386 A1 16-03-1979
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			JP 62050113 B 22-10-1987
			NL 7709179 A 21-02-1979
DE 9320595	U1	20-10-1994	NINGUNO
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			DE 2713858 A1 06-10-1977
			FR 2346042 A1 28-10-1977
			JP 52136990 A 16-11-1977
			NL 7703407 A 03-10-1977

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/002596

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12M1/40 C12P7/64

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)
C12M C12P B01J

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 629 742 A (BRADY ET AL) 16 December 1986 (1986-12-16) column 3, line 48 - line 55 column 7, line 54 - column 8, line 25 figure 1	1-23
Y	US 4 209 591 A (HENDRIKS, PETRUS F A M) 24 June 1980 (1980-06-24) column 3, line 10 - line 21 claim 1 figure 1	1-23
A	DE 93 20 595 U1 (KUNZ, PETER, PROF. DR., 76185 KARLSRUHE, DE) 20 October 1994 (1994-10-20) page 3 claim 1	1-23

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

6 June 2007

Date of mailing of the international search report

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Cubas Alcaraz, Jose

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/002596

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	XUEBING XU: "Engineering of enzymatic reactions and reactors for lipid modification and synthesis" EUR. J. LIPID SCI. TECHNOL., vol. 105, 2003, pages 289-304, XP002396995 page 296 page 303	1-23
A	US 4 029 546 A (BROUILLARD ET AL) 14 June 1977 (1977-06-14) figure 1; example 24	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2007/002596

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4629742	A	16-12-1986	CA 1297822 C	24-03-1992
			DE 3778176 D1	21-05-1992
			EP 0232933 A1	19-08-1987
			ES 2032431 T3	16-02-1993
			GR 3005178 T3	24-05-1993
			GR 3015235 T3	30-06-1995
			JP 1798471 C	12-11-1993
			JP 5003274 B	14-01-1993
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US 4209591	A	24-06-1980	BE 869496 A2	05-02-1979
			CA 1117043 A1	26-01-1982
			CH 646195 A5	15-11-1984
			DE 2835979 A1	01-03-1979
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			JP 1445077 C	30-06-1988
			JP 54044088 A	07-04-1979
			JP 62050113 B	22-10-1987
			NL 7709179 A	21-02-1979
DE 9320595	U1	20-10-1994	NONE	
US 4029546	A	14-06-1977	AU 2368077 A	05-10-1978
			DE 2713858 A1	06-10-1977
			FR 2346042 A1	28-10-1977
			JP 52136990 A	16-11-1977
			NL 7703407 A	03-10-1977

United States Patent [19]

Brady et al.

[11] Patent Number: 4,629,742

[45] Date of Patent: Dec. 16, 1986

[54] HYDROLYSIS OF FATS

[75] Inventors: Catherine D. Brady, Downers Grove; Lincoln D. Metcalfe, Lagrange; Dale R. Slaboszewski, Joliet; Dieter Frank, Naperville, all of Ill.

[73] Assignee: Akzo America Inc., New York, N.Y.

[21] Appl. No.: 823,114

[22] Filed: Jan. 27, 1986

[51] Int. Cl.⁴ C08J 9/36

[52] U.S. Cl. 521/55; 210/500.42; 210/502.1; 521/53; 521/84.1

[58] Field of Search 521/53, 55; 210/500.2, 210/502.1

[56] References Cited

PUBLICATIONS

"Continuous Hydrolysis of Olive Oil by Lipase in Microporous Hydrophobic Membrane Bioreactor", by HOQ et al, JAOCS, vol. 62, No. 6, (Jun. 1985).

"Continuous Hydrolysis of Tallow with Immobilized Lipase in a Microporous Membrane", Taylor et al, Jun. 12, 1985, OS-693, FT: SRM (5/1/85), Agricultural Research Service, U.S. Dept. of Agriculture, Eastern Center.

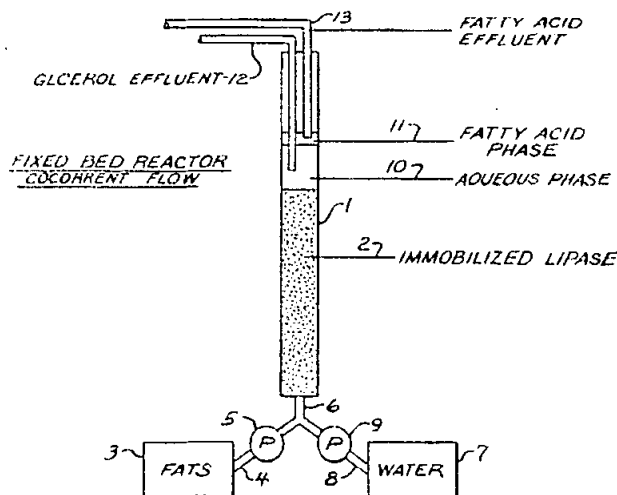
Primary Examiner—Morton Foelak

Attorney, Agent, or Firm—Francis W. Young; Louis A. Morris

[57] ABSTRACT

A process for the hydrolysis of liquid fats comprising contacting the fats, in the presence of water at hydrolyzing conditions, with lipase immobilized by adsorption from aqueous solution without pretreatment or pretreatment with a polar solvent on a microporous structure comprising a synthetic hydrophobic thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof. Various embodiments of the invention include immobilized lipase itself and an embodiment that employs a vertical packed column of particles of the immobilized lipase through which the liquid fat and water feed streams may be passed cocurrent or countercurrent, one that employs a horizontally disposed diaphragm that includes a layer of fibers of the immobilized lipase and an embodiment that employs a stirred reactor wherein a suspension of particles of the immobilized lipase is maintained in the reaction medium. The process of the invention has exhibited surprisingly high activity in the hydrolysis of fats and the immobilized lipase possesses significant longevity.

38 Claims, 11 Drawing Figures



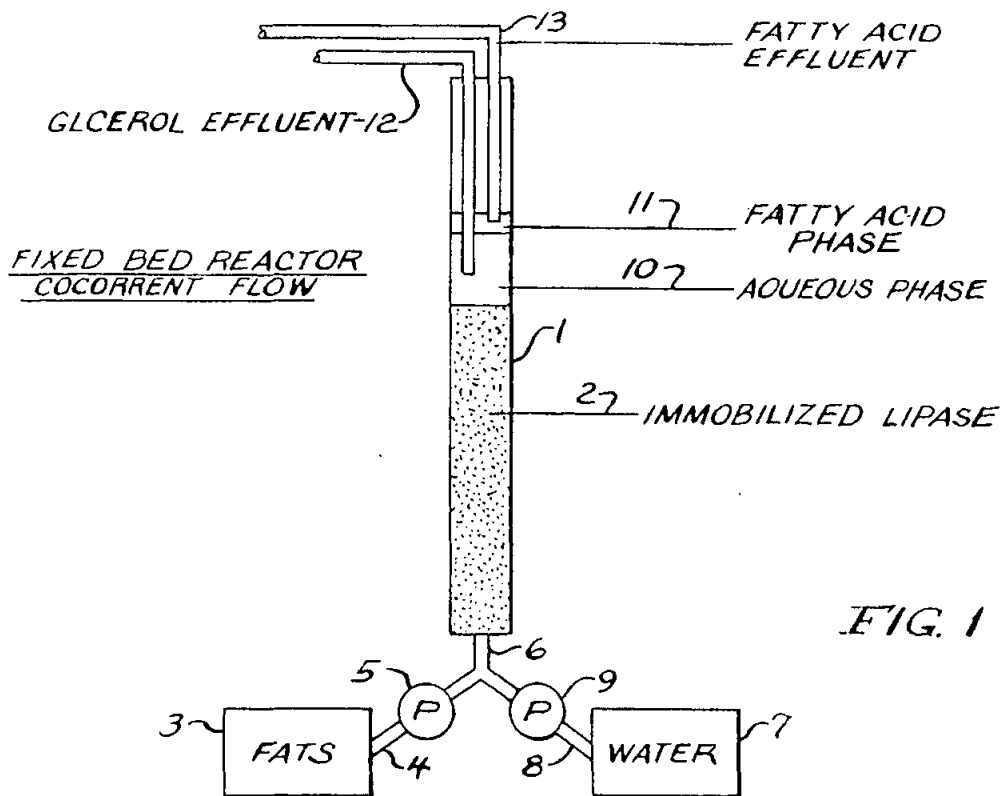


FIG. 1

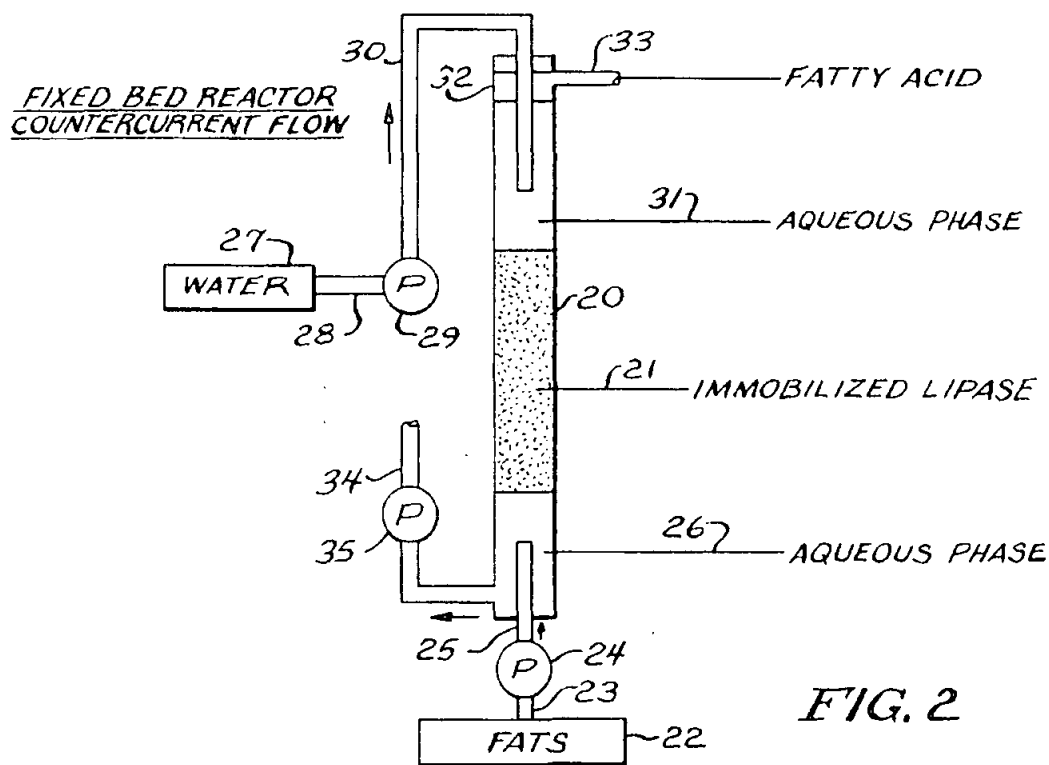
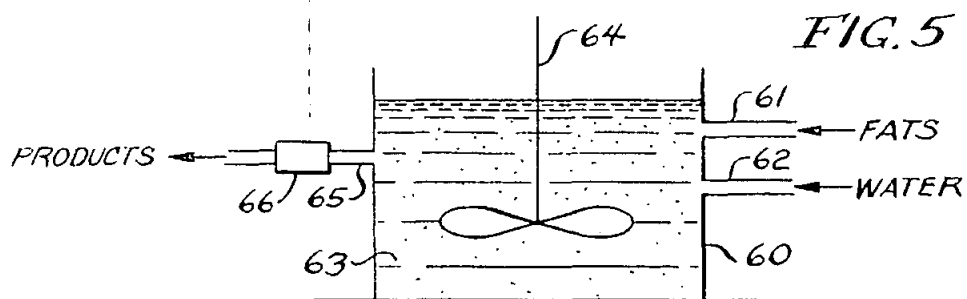
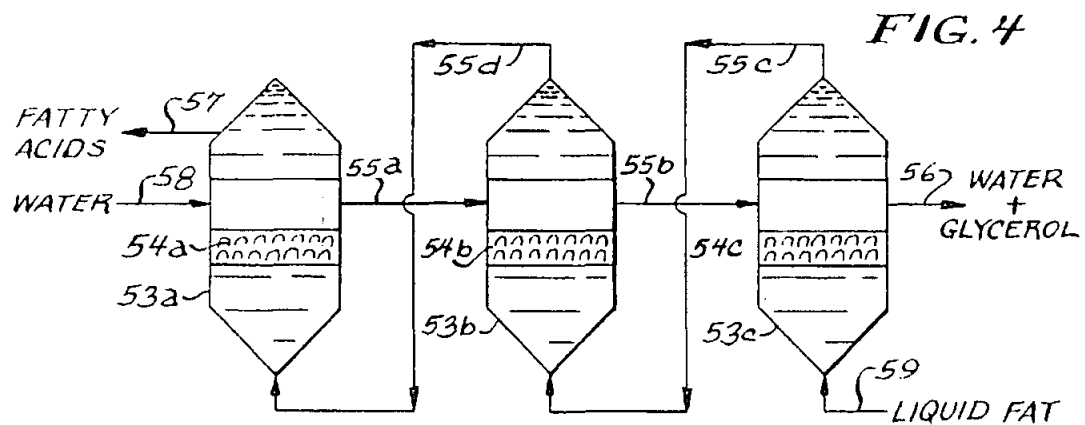
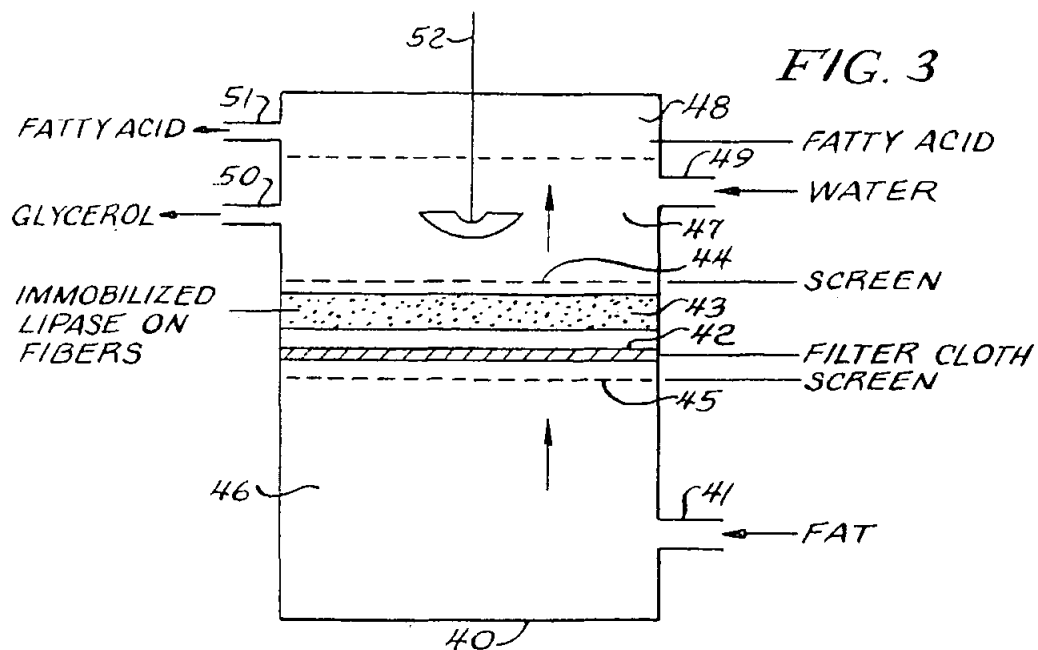


FIG. 2



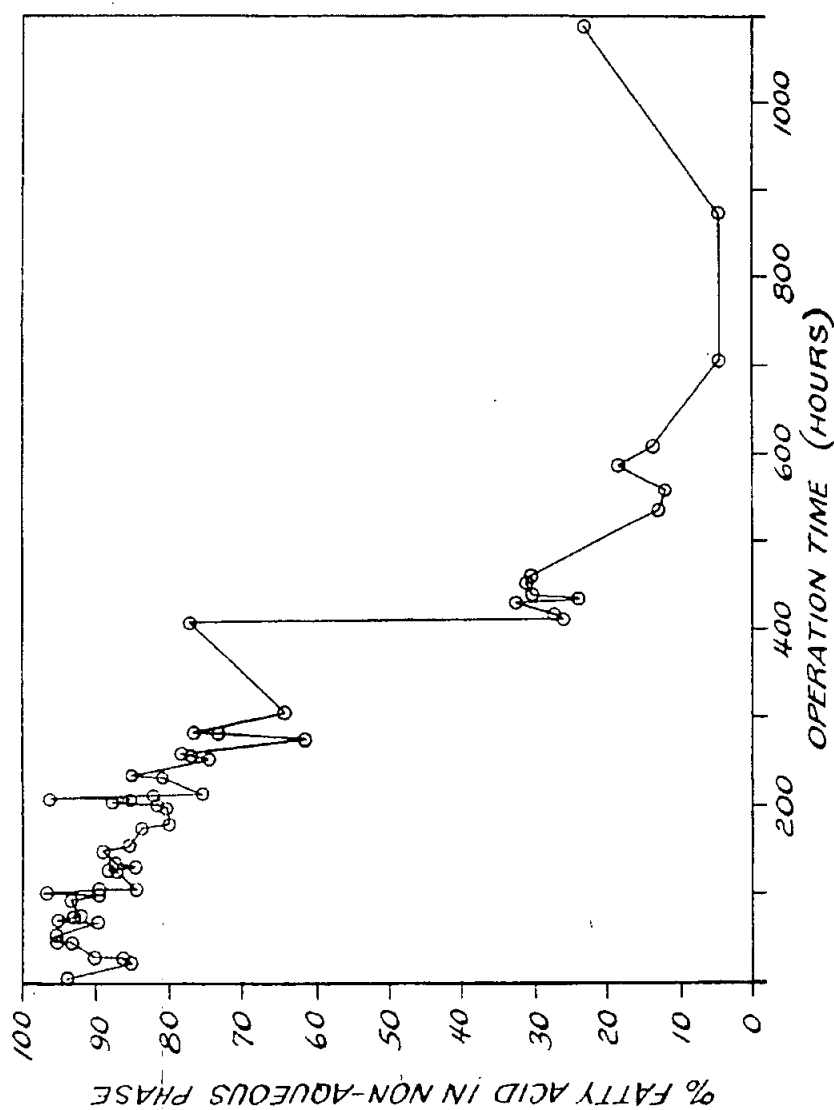


FIG. 6

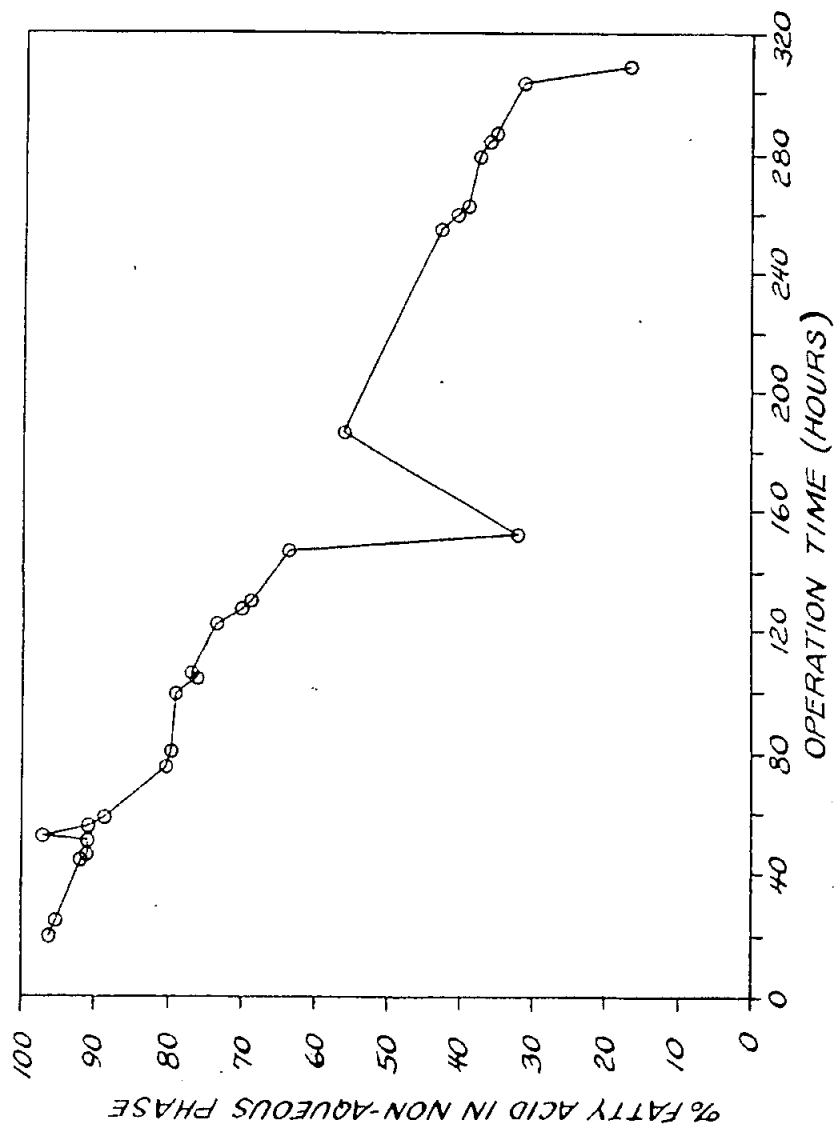


FIG. 7

IMMOBILIZED LIPASE FIXED BED COLUMN REACTOR
EFFECT OF GLYCEROL CONCENTRATION
OLIVE OIL SUBSTRATE

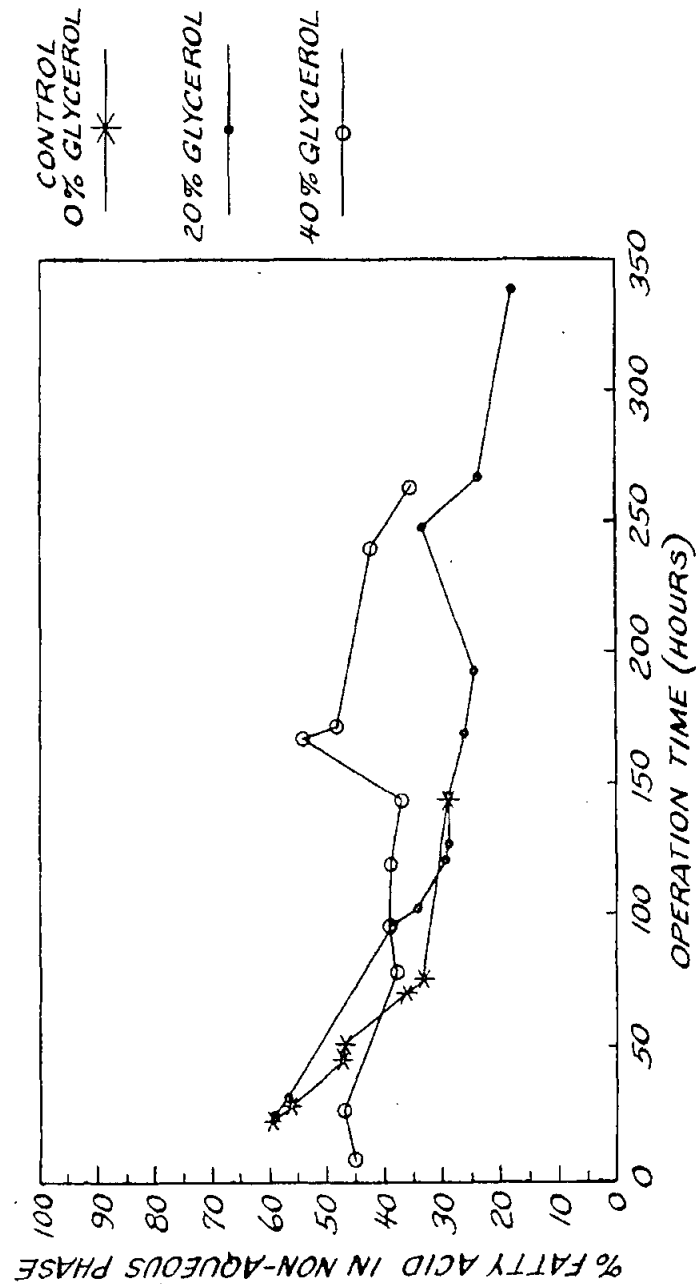


FIG. 8

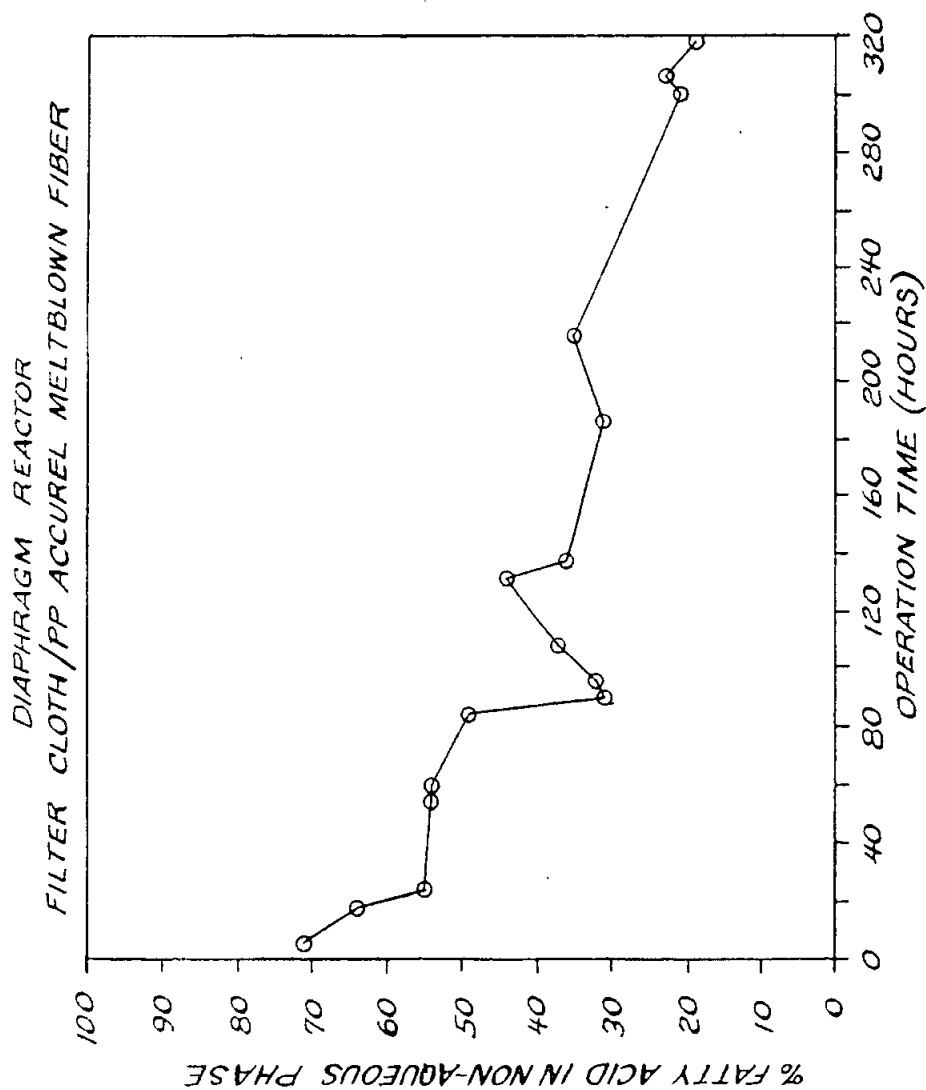


FIG. 9

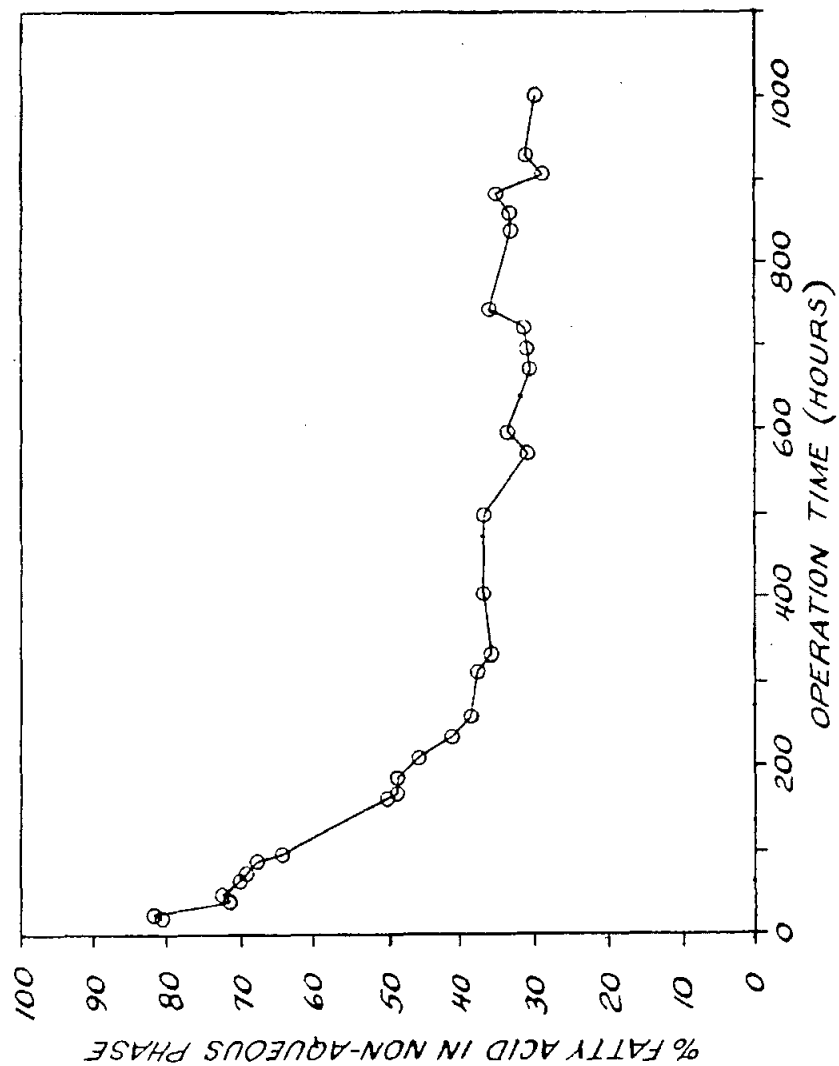


FIG. 10

23

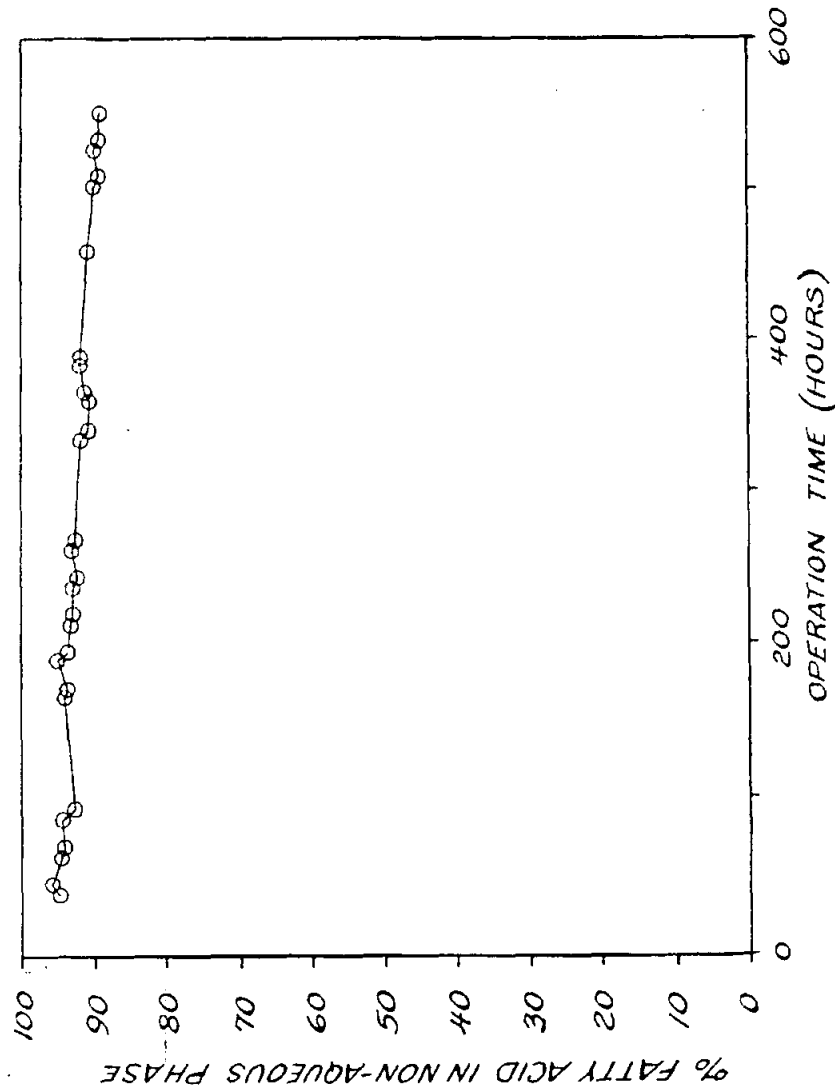


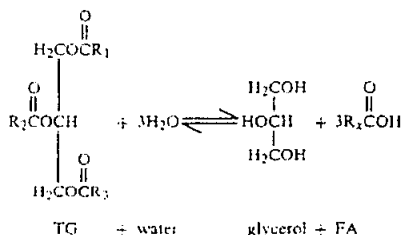
FIG. 11

HYDROLYSIS OF FATS

BACKGROUND OF THE INVENTION

The hydrolysis of fats, also known as fat splitting, has long been accomplished by the use of high pressure steam. Steam splitter reaction conditions are typically about 250° C. and 750 psig. To maintain these conditions a boiler is required to supply the high pressure steam as well as sophisticated pumps capable of pumping feedstock and water into the steam splitting column at high pressure. The costs involved for this type of operation, as required for capital investment as well as process costs such as for energy in the forms of steam, natural gas and electricity, are of course, very high.

There is thus a significant economic incentive to develop new more efficient processes for the hydrolysis of fats, and, as has already been demonstrated in Japan, enzymatic fat splitting is clearly the process of choice. The enzyme that catalyzes the hydrolysis of fats is called lipase, or more formally E.C. 3.1.1.3 glycerol-ester hydrolase. The overall chemistry of the reaction is shown below for a typical triglyceride:



It may be noted that the above reaction actually proceeds via stepwise hydrolysis of the acyl groups on the glyceride, so that any given time, the reaction mixture contains not only triglyceride, water, glycerol, and fatty acid, but also diglycerides and monoglycerides. Furthermore the reaction is reversible. The reverse reaction between an alcohol and a fatty acid to form an ester is called esterification. To force the forward reaction to completion, it is necessary to remove one of the products from the reaction mixture. This task is made easy by the fact that the reaction actually takes place in a biphasic medium. The triglyceride and fatty acid form an oil layer, while the water and glycerol form an aqueous layer. Thus the hydrolysis is easily forced to completion in the splitter by removing the glycerol in the sweetwater.

Lipase can be isolated from several sources soil, plants, animals, or microorganisms. However, there are important differences in the substrate specificity of the lipases harvested from different sources. For example, porcine pancreatic lipase is position specific for the terminal (sn-1 and sn-3) ester bonds on the triglyceride. Lipase from several microbes, such as *Rhizopus arrhizus* and *Mucor miehei* also show the same positional preference for the end acyl groups. Another type of specificity is exhibited by the lipase secreted by *Geotrichum candida*. This lipase preferentially liberates unsaturated fatty acid groups containing a cis double bond in the 9-position of the acyl group, such as oleic and linoleic acids.

A third type of substrate specificity shown by some lipases is that of chain length. Pregastric esterases of lamb, goat, and kid selectively split shorter carbon

chain length acyl groups (C₄-C₈). These lipases, are used in the manufacture of Italian cheeses. The distinctive flavor of these cheeses is caused by the release of short chain fatty acids. Lipase from *Aspergillus niger* has also shown a similar specificity for shorter chain lengths.

Lipases that show no substrate specificity and are thus random in their attack on the glyceride molecule also exist. This is the type of enzyme catalyst that is needed for the fat splitting reaction. The most prevalent nonspecific lipase is isolated from the yeast *Candida cylindracea*, which has been reclassified recently to *Candida rugosa*. Pseudomonas type bacteria have also been found to excrete a nonspecific lipase.

There are many references concerned with enzymatic fat hydrolysis. In *Household & Personal Products Industry*, August 1981, Page 31, there was disclosed the use of lipase in an enzymatic process in which oil and fat are separated into fatty acid and glycerine. Likewise, the following references discuss various methods of effecting enzymatic fat hydrolysis:

A. Mitsutani, *Research & Development Review Report No. 27, Application of Microbiological Technology to Chemical Process*, Nippon Chemtec Consulting, Inc., March 1984.

W. Linfield, R. Barauskas, L. Sivieri, S. Serota, R. Stevenson, "Enzymatic Fat Hydrolysis and Synthesis"; *JAOCs*, 61 (2), Feb. 1984, pp. 191-195.

W. Linfield, D. O'Brien, S. Serota, R. Barauskas, "Lipid-lipase Interactions I. Fat Splitting with Lipase from *Candida rugosa*"; *JAOCs*, 61 (6), June 1984, pp. 1067-1071.

G. Benzonana, S. Esposito, "On the Positional and Chain Specificities of *Candida Cylindracea Lipase*"; *Biochim. Biophys. Acta*, 231 (1971) pp. 15-22.

None of the above references discusses the use of immobilized enzymes. The immobilization of enzymes on solid supports has advantages that have long been recognized. A particular advantage is that the immobilized enzyme remains bonded to the support rather than passing through with the substrate upon which it is acting so that there is no need to recover the enzyme from the substrate and so that the enzyme remains in the support where it may be reused.

Japanese Patent Publication JP No. 84091883 (Abstract No. 84-168208) of May 5, 1984 discloses that an immobilized enzyme may be produced by bringing an aqueous solution of enzyme into contact with a porous synthetic hydrophobic adsorbent. Examples given of adsorbent materials are styrene and methacrylic acid ester. The reference, however, gives no hint to the hydrolysis of fats, nor to lipase as the enzyme.

Russian Patent Publication No. SU 804647 (Abstract No. 83249D) of Feb. 15, 1981 discloses crosslinked porous styrene polymers used as activity enhancing carriers for immobilized enzymes, but also does not hint to the composition, process, methods or apparatus of the present invention.

There is also art that teaches the hydrolysis of fats by use of immobilized lipase. In *Chemical Week*, Vol. 133, No. 22, Nov. 30, 1983, on page 33, it is generally mentioned that a number of useful enzymes may be immobilized by locking them to a carrier by adsorption, cross-linking or covalent bonding, and on page 34 there is mention that lipase may be used to hydrolyze fat, but there is no teaching in this reference of polymeric carriers, and there is a warning on page 33 that an enzyme

free in solution and the same enzyme locked to a carrier do not behave the same.

In J. Lavayre, J. Baratti, "Preparation and Properties of Immobilized Lipases", *Biotech & Bioengr.*, 24 (1982), pp. 1007-1013, hereinafter referred to as "Lavayre et al", there is discussed the use of lipase immobilized by adsorption onto a hydrophobic support for the hydrolysis of olive oil. The Lavayre et al article, however, states that when purified pancreatic lipase was used, the specific activity of the immobilized enzyme was 17 to 25% that of the soluble enzyme. Furthermore, the only support used in the hydrolysis tests was the iodopropyl derivative of porous glass (Spherosil).

The use of lipase immobilized onto polyacrylamide beads for the hydrolysis of triglyceride is discussed in "Bell, Todd, Blain, Paterson and Shaw", Hydrolysis of Triglyceride by Solid Phase Lipolytic Enzymes of *Rhizopus arrhizus* in "Continuous Reactor Systems", *Biotech & Bioengr.*, 23 (1981), pp. 1703-1719, and in Lieberman and Ollis, "Hydrolysis of Particulate Tributyrin in a Fluidized Lipase Reactor", *Biotech & Bioengr.*, 17 (1975), pp. 1401-1419. In those references, however, the immobilization is effected by covalent bonding (e.g. diazonium intermediate), not adsorption. The results were a significant decrease in the activity of the immobilized as compared to the free enzyme.

The hydrolysis of fats with lipase is a reversible reaction and there are teachings in the art of methods of producing fats by reacting a fatty acid with water and glycerol in the presence of lipase. One such reference is M. M. Hoq, T. Yamane, S. Shimizu, T. Funada, S. Ishida, "Continuous Synthesis of Glycerides by Lipase in Microporous Membrane Bioreactor", *JAOCs*, 61 (4), April 1984, pp 776-781, hereinafter referred to as "Hoq et al". Hoq et al advises against the use of immobilized lipase for the stated reason that its activity is commonly only several percent of the original activity of the free lipase. Hoq employs a device, it refers to as a bioreactor, which comprises supported hydrophobic microporous membrane, in particular one made from polypropylene, that is placed at the interface of an upper phase of fatty acid and lower phase of a solution of glycerol, water and lipase. The reactants and lipase come into contact at the interface of the two phases thereby causing the reaction, the glycerides diffusing back into the bulk flow of the fatty acid phase.

The present invention is based on the surprising discovery that lipase immobilized on certain porous polymeric supports in a certain manner loses very little of its fat hydrolysis activity as compared to soluble lipase, notwithstanding teachings of prior art such as Lavayre et al and Hoq et al that immobilization of lipase causes such activity to diminish to a small fraction of the free lipase.

SUMMARY OF THE INVENTION

It is a primary objective of the present invention to obtain a composition comprising an immobilized lipase that has a unique suitability for use in a process for the hydrolysis of fats. Another objective is to obtain a process for the hydrolysis of fats using such immobilized lipase without significant sacrifice of lipase activity as compared to free soluble lipase. Other objectives are to provide a method for the immobilization of the lipase as well as means and devices which use the immobilized lipase of the invention in a practical and efficient manner.

Accordingly, the present invention, in a first embodiment is a composition comprising lipase immobilized by adsorption from aqueous solution on a microporous structure comprising a synthetic hydrophobic thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof. The structure is not pretreated prior to the adsorption or is pretreated by wetting with a polar water miscible organic solvent in which the polymer is insoluble and which does not deactivate the lipase.

In a second embodiment, the present invention comprises a method for the immobilization of lipase on a microporous structure comprising a synthetic hydrophobic thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof. The immobilization is effected either without pretreatment of the structure or is pretreated by first wetting the polymer with a polar water miscible organic solvent in which such polymer is insoluble and which does not deactivate the lipase. The support is then soaked in a dilute aqueous solution of the lipase.

In a third embodiment, the present invention comprises a process for the hydrolysis of liquid fats. The process comprises contacting liquid fats, in the presence of water at hydrolyzing conditions, with lipase, immobilized by adsorption from aqueous solution on a microporous structure. The structure comprises a synthetic hydrophobic thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof. The structure may be pretreated prior to adsorption only by wetting with a polar water miscible organic solvent in which the polymer is insoluble and which does not deactivate the lipase. Pretreatment, however, is not essential.

In a fourth embodiment, the present invention comprises a process for the hydrolysis of liquid fats comprising contacting the fats in the presence of water at hydrolyzing conditions with lipase immobilized by adsorption from aqueous solution on a microporous structure comprising a synthetic hydrophobic thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof. The structure is either not pretreated or is pretreated prior to adsorption only by wetting with a polar water miscible organic solvent in which the polymer is insoluble and which does not deactivate the lipase. The contacting is effected by means of a column packed with a powder of the structure on which the lipase is immobilized. The powder in the cocurrent embodiment is preferably from about 150 to about 450 micron particle size.

In a fifth embodiment, the present invention is a process for the hydrolysis of fats. The process comprises maintaining a lower liquid phase of fats and an upper liquid phase which comprises water. The phases are separated at their interface with a horizontally disposed diaphragm which comprises three layers. The bottom most layer is a hydrophobic filter cloth. The middle layer is fibers of a support comprising a synthetic microporous thermoplastic polymer having lipase immobilized thereon. The top most layer of the diaphragm is a retaining means capable of maintaining the fibers of the middle layer in place. The fats flow upward through the bottom layer and into contact with the supported lipase of the middle layer, where in the presence of water from

the upper phase, and, at hydrolyzing conditions, the hydrolysis of the fats occurs. The fatty acids product of the hydrolysis rises to the surface of the upper phase to form a separate uppermost phase. The glycerol product of the hydrolysis dissolves in the upper phase and the fatty acids are removed as the uppermost phase and glycerol products are recovered from the upper phase. Additional fats and water are added as required to maintain the desired inventory of each.

In a sixth embodiment, the present invention is a process for the hydrolysis of fats which comprises maintaining a suspension comprising lipase immobilized by adsorption from aqueous solution on particles of a microporous structure. The process employs a synthetic hydrophobic thermoplastic polymer which is selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof, in a liquid reaction mixture. The structure may be pretreated prior to the adsorption by wetting with a polar water miscible organic solvent in which the polymer is insoluble and which does not deactivate the lipase. Pretreatment, however, is not essential. The reaction mixture comprises fats and water and is maintained by the continuous addition thereto of a stream of liquid fats and water. A portion of the reaction mixture containing reaction products which comprise fatty acids and a glycerol solution is continuously withdrawn.

A seventh embodiment of the present invention is an apparatus related to the above fifth embodiment comprising a diaphragm suitable for use in the hydrolysis of fats comprising:

- a. a first layer consisting of a hydrophobic filter cloth having openings from about 3 to about 5 microns in size;
- b. a second layer adjacent to said first layer comprising fibers of a hydrophobic microporous thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof, having lipase immobilized on the fibers by adsorption from an aqueous solution either without pretreatment or following pretreatment of the fibers only by wetting with a polar water miscible organic solvent in which the polymer is insoluble and which does not deactivate the lipase; and
- c. a third layer adjacent to the side of said second layer opposite said first layer comprising a retaining means capable of maintaining the fibers of the second layer in place.

Other embodiments of the present invention encompass details about process flow schemes, reaction conditions and materials compositions, and mechanical details all of which are hereinafter disclosed in the following discussion of each of the facets of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1 and 2 are, respectively, elevation views of the embodiment of the invention comprising cocurrent and countercurrent flow packed columns.

FIG. 3 is illustrative of the embodiment of the invention employing a multi-layered diaphragm.

FIG. 4 is illustrative of a multi-diaphragm embodiment of the invention.

FIG. 5 is illustrative of the stirred reaction embodiment of the invention.

FIGS. 6 through 11 comprise graphic presentations of data obtained as described in the examples.

DESCRIPTION OF THE INVENTION

There has long been an interest and unfulfilled need to immobilize lipase for use in fat splitting, particularly Candida lipases in view of their high activity for that purpose. A primary reason for such need is the high cost of such lipase which dictates that it be used in an immobilized form so that it can be reused many times. Otherwise, the enzymatic fat splitting process, at least in view of current costs of enzymes and energy, could never be competitive with non-catalytic high pressure steam fat splitting.

As mentioned above, however, the prior art teaches that the immobilization of lipase causes its activity to diminish to a small fraction of free lipase. The present inventors, therefore, were greatly surprised to find that little if any of such activity was lost when the lipase was immobilized on an appropriate porous polymeric support.

At about the time of the discovery of the surprising utility of lipase immobilized on porous polymeric support for fat splitting was the discovery that when immobilization was carried out in a certain way, an even superior product was obtained. The method then thought to be best for immobilization involved pretreating the polymer with a metal salt solution (e.g. stannous chloride) and/or a long chain cationic solution (e.g. a salt of N-coco-1,3-diaminopropane or trimethyltallowammonium chloride). The enzyme was then immobilized onto the pretreated polymer. When immobilization experiments started with lipase and polymeric supports for the splitting of fats, however, it was noted that not only did the pretreatment have no positive effect on the activity of the immobilized lipase, a lack of pretreatment (other than wetting the support with a polar solvent) was found to be beneficial.

The method of immobilization found to be most effective is to simply soak the support in a dilute aqueous solution of the lipase, or, optionally, first wet the polymer structure with a polar water miscible organic solvent in which the polymer is insoluble and which does not deactivate the lipase. The concentration of the lipase solution may vary. A dilute solution is about 35 lipase units per ml., while concentrated would be about 500 units per ml. A unit is defined as the amount of lipase required to produce one micro-mole of fatty acid per minute from an olive oil substrate under the conditions of the assay, typically at a pH of 7° and 35° C. The pH of the lipase solution is not important, and may be buffered to any value, with an optimum between about 4 to about 7.

The term "polar" as used herein shall mean the property of having a dipole moment of at least 0.1 debye. The term "water miscible" shall mean capable of mixing in any ratio with water without separation of phases. The term "insoluble" shall mean a solubility in the solvent in question of not greater than 0.1 g/l. The term "deactivate" shall mean the loss of the ability to catalyze the hydrolysis reaction.

The hydrophobic microporous cellular polymer selected must be a microporous (about 0.1-500 micron average pore diameter) synthetic hydrophobic thermoplastic polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof. Polypropylene and polyethylene are examples of nonionic polymers. The binding of lipase to the nonionic polymers is by hydrophobic adsorption. A minimum hydrophobicity is essential for

the nonionic polymers. Nonionic polymers effective for the present invention, and having a sufficient degree of hydrophobicity, are considered to be those having a surface tension less than 41 dynes/cm which includes polyethylene and polypropylene. For the ionic polymers, e.g. Surlyn®, the binding of lipase to polymer may no longer be simply hydrophobic bonding, but rather complicated by ionic interactions. Thus, surface tension would no longer be a relevant parameter. For these polymers for which surface tension is not a relevant parameter, the term "hydrophobic" may have its commonly understood meaning as defined in *Hackh's Chemical Dictionary*, 4th Edition, i.e. a substance that does not adsorb or absorb water.

The ideal microporous structure for the polymeric supports and method of obtaining such structure are as disclosed in U.S. Pat. Nos. 4,247,498 and 4,519,909 issued to Castro, both incorporated by reference herein in their entirety. Those patents disclose microporous cellular polymer structures known by the trademark Accurel® which are marketed by Enka America Incorporated, 1827 Walden Office Sq., Suite 480, Schaumburg, Ill. 60195. Accurel® structures may be characterized in one of three ways:

1. a cellular microporous structure which comprises a plurality of substantially spherical cells having an average pore diameter from about 0.5 to about 100 microns, distributed substantially uniformly throughout the structure, adjacent cells being interconnected by pores smaller in diameter than the microcells, the ratio of the average cell diameter to the average pore diameter being from about 2:1 to about 200:1, the pores and the cells being void.

2. A cellular microporous structure which is cellular and is characterized by a C/P ratio of from about 2 to about 200, an S value of from about 1 to about 30, and an average cell size from about 0.5 to about 100 microns.

3. An isotropic microporous structure that is characterized by an average pore diameter of from about 0.1 to about 5 microns and an S value of from about 1 to about 10.

In numbers 2 and 3 above "C" means average diameter of cells, "P" the average diameter of the pores, and "S" is the sharpness factor, determined by use of a Micromeritics Mercury Penetration Porosimeter, and defined as the ratio of the pressure at which 85 percent of the mercury penetrates the structure to the pressure at which 15 percent of the mercury penetrates.

MEANS TO ACHIEVE THE CONTACTING OF THE REACTANTS WITH THE IMMOBILIZED LIPASE

One means to achieve the contacting of the liquid fats with the immobilized lipase is a column packed with discrete particles of the immobilized lipase. The fixed bed column reactor is a very common design for immobilized enzyme reactors. In this type of reactor fats and water may either be passed cocurrently through the column in a direction parallel to the longitudinal axis of the column, or countercurrently through the column in directions parallel to the longitudinal axis of the column. In a packed column, the activity of the lipase may be restored by first flushing the contents of the column with a solvent suitable for the removal of spent lipase and residual fat from the porous polymeric support (e.g. alcohol), then flushing the contents of the column with water to remove the solvent, then flushing with a broth

of fresh lipase and finally flushing the contents of the column with water to wash away excess enzyme.

With the cocurrent type flow scheme fats and water are passed into the column at one end and reaction products comprising glycerol and fatty acids are removed at the opposite end. FIG. 1 illustrates the cocurrent fixed bed immobilized lipase reactor including vertical column 1 packed with bed 2 of particles of immobilized lipase. The particles are preferably in the form of powder of from about 150 to about 450 microns in particle size. Liquid fats are passed from tank 3 via conduit 4 and pump 5 into mixing conduit 6, and water is passed from tank 7 via conduit 8 and pump 9 into mixing conduit 6. In mixing conduit 6 vigorous mixing is effected so as to obtain a fat/water emulsion which passes into column 1 and one end of bed 2. The reaction mixture is passed through bed 2 over a period of time from about 2 to about 10 hours (or longer, depending on the degree of conversion desired). The reaction mixture exits the top of bed 2 and separates into an aqueous phase 10, also referred to as "sweetwater" since that is where dissolved glycerol product accumulates, and fatty acid phase 11. Aqueous phase product may periodically or continuously be drawn off via conduit 12 and fatty acid phase product may be drawn off via conduit 13.

FIG. 2 illustrates the countercurrent fixed bed immobilized lipase reactor, including vertical column 20 packed with bed 21 of particles of immobilized lipase. Liquid fats are passed from tank 22 via conduit 23, pump 24 and conduit 25 into the bottom of column 20 and first aqueous phase 26. Water is passed from tank 27 via conduit 28, pump 29 and conduit 30 into the top of column 20 and second aqueous phase 31. The water and fats will flow countercurrently through bed 21 due to the effect of gravity and the difference in specific gravity between the phases, the fats being the lighter of the two phases. The temperature and residence time of the reactants in bed 21 will be about the same as for the above cocurrent reactor. Fatty acid will accumulate in fatty acid phase 32 and may periodically or continuously be drawn off via conduit 33, while at the same time sweetwater is drawn from aqueous phase 26 via conduit 34 and pump 35.

Another type of continuous fat hydrolysis reactor is the diaphragm reactor as illustrated in FIG. 3. Fat in a liquid form enters a bottom portion of vessel 40 via conduit 41. Vessel 40 is separated into a bottom and top portion by a horizontal diaphragm comprising three layers. The bottom most layer 42 is a hydrophobic filter cloth. Suitable materials for filter cloth 42 are PTFE (Teflon) and polypropylene. The middle layer 43 comprises fibers of the porous polymeric support on which the lipase is immobilized. The fibers that have heretofore been used averaged about 3-7 microns in diameter. The thickness of the fiber layer heretofore observed, following compression through use was about 2-4 mm. The top most layer 44 is a retaining means capable of keeping the fibers in place and may comprise any type of screen having appropriately sized openings. There may be an additional screen 45 supporting the entire diaphragm if filter cloth 42 is considered inadequate for that purpose.

Choice of the hydrophobic filter cloth comprising bottom most layer 42 is particularly important. The openings in the cloth must be large enough to permit an acceptable flow rate of fat through the cloth, but not so large that water would pour through from above. Filter cloths found to be effective were Gore-Tex® Ex-

panded PTFE Membranes and Laminates with 3-5 μ openings from W. L. Gore and Associates, Inc. and 0.5-1.0 CFM rated (air flow measured at $\frac{1}{2}$ " H₂O pressure on a Frazier Permeometer) PP cloth from Crosble, Inc.

In operation, the diaphragm will serve to separate at their interface a lower liquid fat phase 46 and an upper water phase 47. The fats will flow upward through bottom layer 42 and come into contact with the supported lipase of middle layer 43 where in the presence of the water from the upper phase 47, and with a residence time in the diaphragm itself of from about 20 minutes to about 60 minutes, the hydrolysis of the fats will occur. The fatty acids that are formed, rather than remaining in the fat phase, will advantageously due to their inherent buoyancy, rise to the surface of the upper phase 47 to form a separate uppermost phase 48. The glycerol product of the hydrolysis will dissolve in the upper aqueous phase 47.

Water may enter the top portion of tank 40 via conduit 49. Fatty acid may be withdrawn as a product stream via conduit 51 and glycerol via conduit 50. Stirrer 52 will serve to maintain a reasonably homogeneous solution in phase 47. Additional fat and water may be added as required to maintain the desired inventory of each.

In the process as shown in FIG. 3, the activity of the lipase with respect to the hydrolysis of the fats may be restored by flushing the diaphragm sequentially with three flushing liquids which enter the diaphragm at top layer 44 and exit through a means provided to bypass the filter cloth, such as a conduit and valve. The first flushing liquid comprises a solution of water and a solvent suitable for the removal of spent lipase from the support. Suitable solvents are the same as those that may be used to pretreat the polymer in effecting the initial immobilization. The second flushing liquid comprises a broth of fresh lipase. The third flushing liquid comprises water.

A multi-stage diaphragm reactor is as illustrated in FIG. 4. Each stage in this illustration comprises a separate vessel in which a diaphragm is contained. Three vessels, 53a, 53b and 53c, are shown, but any number (two or more) may be used. Diaphragms 53a, 53b and 53c, as described above, of horizontal orientation are placed in sealed contact with the interior surface of the walls of vessels 54a, 54b and 54c, respectively. Conduits 55a and 55b connect vessels 53a to 53b and vessels 53b to 53c, respectively, at points immediately above the diaphragms in each vessel. Conduits 55c and 55d connect vessels 53c to 53b and vessels 53b to 53a, respectively, at points above the diaphragms of vessels 53c and 53b and at or near the tops of those vessels to below the diaphragms of vessels 53b and 53a. There is a water-glycerol product withdrawal conduit 56 connected to vessel 53c immediately above diaphragm 54c. There is a fatty acid product withdrawal conduit 57 connected to vessel 53a above diaphragm 54a and at or near the top of the vessel. There is a water inlet conduit 58 connected to vessel 53a above diaphragm 54a. There is a liquid fat (oil) inlet conduit 59 connected to vessel 53c below diaphragm 54c.

The apparatus of FIG. 4 may be used for that embodiment of the process of the present invention, employing a multiplicity of the diaphragms discussed above with reference to FIG. 3. Since the conversion per pass of the diaphragm appears to not be extremely high, multiple stages are considered advantageous. In using the

multi-diaphragm scheme of the invention, as shown in FIG. 4, each diaphragm is associated with a stage which also includes the lower, upper and uppermost phases as discussed above. Each stage is contained in a separate vessel. For reason of simplicity, various pumps and control valves that one skilled in the art would understand to be required are not shown in FIG. 4.

In the operation of the apparatus of FIG. 4, the direction of flow of the non-aqueous streams from vessels 53c to 53b to 53a via lines 55c and 55d is considered "downstream" flow. Conversely, the direction of flow of the aqueous streams from vessels 53a to 53b to 53c via lines 55a and 55b is considered "upstream" flow and counter-current to the non-aqueous stream. The liquid fat to the process is introduced to vessel 53c via line 59 below diaphragm 54c into the lower phase of the first upstream stage. The water to the process is introduced to vessel 53a via line 58 into the upper phase of the last downstream stage above diaphragm 54a. Although line 58 is shown as passing the water directly into the upper phase in vessel 53a, it may be more convenient to introduce the water at the top of the vessel into the uppermost phase whereupon it would simply flow through the uppermost non-aqueous phase, with which it is immiscible, and thereby, in effect, be introduced into the upper phase. The aqueous stream from the upper phase of each stage in vessels 53a and 53b is passed via lines 55a and 55b, respectively, to the upper phases of vessels 53b and 53c, respectively, while the aqueous stream from the upper phase of the first upstream stage in vessel 53c is withdrawn as the glycerol product stream via line 56. The non-aqueous stream from the uppermost phases of the stages in vessels 53c and 53b are passed via lines 55c and 55d, respectively, to the lower phases of the stages in vessels 53b and 53a, respectively, while the non-aqueous stream from the uppermost phase of the stage in vessel 53a is withdrawn as the fatty acid product stream via line 57.

The embodiment of the present invention illustrated in FIG. 4 is only one possible way of achieving a multi-stage configuration. There are other possibilities including a multiplicity of diaphragms in a single vessel. The FIG. 4 configuration, however, is considered advantageous from the standpoint of simplicity of construction of the equipment and its operation.

Yet another type of continuous fat hydrolysis reactor is one as illustrated in FIG. 5 which employs a suspension in the liquid reaction mixture of particles of immobilized lipase. This embodiment of the invention may be referred to as the "stirred reactor" or "CSTR".

Looking to FIG. 5, fats and water streams are continuously introduced into vessel 60 via lines 61 and 62, respectively. Particles or a powder of the polymer support on which the lipase is adsorbed are kept in suspension in reaction medium 63 by means of stirrer 64. A portion of the reaction mixture containing the fatty acids and glycerol solution products is continuously withdrawn via line 65. The residence time in reaction medium 63 may comprise from about 2 to about 60 hours.

There is preferably a filter 66 associated with line 65 to enable recovery of the products along with the retention of the particles in the reaction mixture.

As far as reaction conditions for the fat splitting process of the present invention are concerned, the most important consideration is that the temperature be high enough to enable the fat to be in liquid phase but not so high as to cause deactivation of the lipase. Deactivation

of *Candida cylindracea* becomes significant at about 50° C. The pH of the reaction mixture is not critical, but it may be maintained at about 7.0 by use of water reactant buffered with sodium phosphate.

EXAMPLES

The following non-limiting examples will serve to illustrate the preferred and advantageous method for achieving immobilization of lipase on a porous polymeric support, the superiority of lipase immobilized on such support in a process for the hydrolysis of fats, and various embodiments of the present invention employing certain devices and flow schemes.

EXAMPLE 1

In this example it is shown that pretreatment of Accurel® prior to lipase immobilization thereon with anything other than a polar solvent is disadvantageous. Four samples were prepared of *Candida cylindracea* lipase immobilized on polypropylene Accurel® powder. The *Candida cylindracea* lipase for this example was obtained from Sigma Chemical Co. as its Type VII Lipase, catalog no. L-1754, with a stated activity of 470 U/mg solid. On three of the four samples Accurel® was pretreated with various materials. On one sample the immobilization was effected by first wetting the polymer with ethanol and then, without further pretreatment, soaking the support in a dilute aqueous solution of the lipase. The results obtained are as shown in Table 1.

TABLE 1

Pretreatment	Lipase Activity* (IU/g)
1. Ethanol	103
2. SnCl ₄ + ethanol	91
3. N-coco-1,3-diaminopropane + ethanol	83
4. SnCl ₄ + N-coco-1,3-diaminopropane + ethanol	76

*Lipase activity units (IU's) are a measure of the micro moles of fatty acid per minute liberated from an olive oil substrate at a pH of 8 and room temperature.

The results from Table 1 illustrate that not only is pretreatment (other than only with a polar solvent) unnecessary, significantly improved activity is realized when pretreatment, other than with a polar solvent, is avoided.

EXAMPLE 2

In this example there was an investigation of the use of various solvents to prewet Accurel® polypropylene powder (150-450u) before the immobilization on such powders of lipase (*Candida cylindracea*) from solution. The lipase obtained for this example was from Enzyme Development Corporation, and is known as Enzeco® lipase with a stated activity of 30,000 U/g. Also investigated was the same powder that was not pretreated prior to immobilization. The procedure in each case to obtain the immobilized lipase was as follows:

1. 1 g of powder was prewet with solvent (except where prewetting was not to occur);
2. 0.2 g. of the lipase was dissolved in 100 ml. of water buffered to pH 7.0 with 0.1M sodium phosphate;
3. the powder was soaked in the lipase solution for thirty minutes; and
4. the resulting immobilized lipase was filtered from the solution and flushed three times with additional quantities of water buffered as above.

A series of tests was then run wherein each of the immobilized lipase samples prepared by the above pro-

cedure was used to hydrolyze fat. In each test 50 ml. of Bleachable Fancy Tallow was stirred in a beaker with the immobilized lipase and 100 ml of buffered water at 42° C. over a 24 hr. period. The results obtained, in terms of % fatty acids in the non-aqueous product phase, at the end of the 24 hour period, are as given in the following Table 2:

TABLE 2

Pretreatment	% FA
ethanol	58.0
isopropanol	58.2
methanol	55.9
acetone	51.0
tetrahydrofuran	52.6
none	66.0

The above results show that high levels of fatty acid may be obtained either when pretreatment is effected with a polar solvent, or where there has been no pretreatment. In the latter case the highest degree of fat hydrolysis is achieved, but as will be discussed in the following example, prewetting with a polar solvent greatly facilitates the rate at which the lipase may be immobilized.

EXAMPLE 3

To illustrate a further advantage of the method of lipase immobilization of the present invention, a determination was made of the time required to obtain the immobilization of lipase on a polypropylene Accurel® in a powdered form (150-450u) and pretreated with ethanol. Immobilization could be effected in about two minutes. Without any pretreatment immobilization took about five times as long. Thus, although pretreatment with the appropriate polar solvent is not necessary to obtain high activity immobilized lipase, it will enable rapid loading of the lipase onto the support which is particularly advantageous with in situ loading and regeneration.

EXAMPLE 4

For this example *Candida cylindracea* lipase (the same as the aforementioned Enzeco® lipase) was immobilized on untreated Accurel® products (treated only with a polar organic solvent) by simple adsorption from a buffered enzyme solution. The procedure for a typical immobilization was to dissolve 0.20 grams of lipase in 100 milliliters of 0.1M sodium phosphate, pH 7.0 buffer. Next, 1.0 gram of Accurel® powder was prewet with as little 3A ethanol as possible and added to the enzyme solution. After stirring for 5-60 minutes, the immobilized catalyst was filtered and then rinsed with several bed volumes of buffer. The immobilized lipase was assayed for activity using a triglyceride substrate, such as olive oil or Bleachable Fancy Tallow.

Other materials, besides Accurel® were screened as supports for lipase. Table 3 lists all the enzyme support materials that were screened and lists the fatty acid levels generated during the assay at 6 and 24 hours on a tallow substrate relative to the soluble lipase as a control. The surprising result was that the porous polymer powders of the present invention performed as vastly superior supports for lipase. Specifically, polypropylene (PP), high density polyethylene (HDPE), and Surlyn® Accurel® powders, the more hydrophobic of the polymers used, were superior supports.

On the other hand, inferior results were obtained from polymers of low hydrophobicity such as cellulose and nylon (nylon is also a condensation polymer which is not a genus of polymer included in the invention), olefinic polymers which are not aliphatic (styrene) and non-polymeric material as well as non-porous material.

Furthermore, comparison of the immobilized enzyme results with those of the soluble lipase control in Table 3 indicates that very little of the lipase activity was lost upon immobilization on these supports. This result is diametrically opposite claims in the literature, as previously discussed, that lipase loses 75-99% of its activity upon immobilization.

TABLE 3

Support	Lipase Immobilization Supports	
	% FA by GPC* Relative to Soluble Lipase	
	6 HR	24 HR
<u>Johns-Manville</u>		
Celite 545	8%	12%
Celkate	12	
R-600	15	
R-640	15	19
CS-30K	15	17
<u>Misc</u>		
<u>Microcrystalline Cellulose:</u>		
Avicel	16	21
Avicel, 25μ	19	23
Avicel, 50μ	16	29
Ethyl Cellulose	31	52
Silica Gel	22	23
Kieselguhr	19	30
Bentonite Clay	12	13
Alumina, neutral	8	9
CPG-100	42	
Glycophase-G	15	
Styrene dvb x 4% (non-porous)	11	
Celgard 2500 (Celanese Corporation)	75	85
Amberlite XAD-2 (porous styrene)	8	9
Hercules Profax-PP (non-porous)	15	14
USI Microthene-HDPE (non-porous)	13	12
Tenax (porous oxidation polymer of 2,6 di-phenylparaphenylene oxide)	75	82
Versapor 200 (porous acrylic copolymer cast on nonwoven nylon)	9	9
AP-200 (porous acrylic copolymer cast on nonwoven polyester)	9	11
<u>Accurel Powders</u>		
Nylon	16	18
Surllyn	82	88
HDPE 150μ	93	98
HDPE 150-450μ	62	76
PP - std. grind	73	88
PP - Enka	78	88
PP - Friable	86	99
PP - Friable	73	91
PP - Friable	72	88
PP - Friable	81	88
<u>Control</u>		
Soluble Lipase	100	100

*1.0 gm support, 3750 IU's Lipase, 1 hour immobilization, 50 mls BFT, 100 mls buffer, 40° C.

EXAMPLE 5

This example describes the operation of fixed bed column reactors as illustrated in FIGS. 1 and 2.

The fixed bed column reactor is a very common design for immobilized enzyme reactors. The columns studied for splitting fat contained lipase immobilized on some form of Accurel®. Accurel® products used as support materials in the columns were Enka America Incorporated melt blown PP Accurel® fibers, Enka's HDPE Accurel® granules (2-3 mm), and HDPE Ac-

curel® powder (150-450 microns). The lipase was immobilized on the Accurel® by simple adsorption. In all cases, lipase from Candida (Enzeco®), as specified above) was dissolved in 0.1M, pH 7.0, sodium phosphate buffer, the support was prewet with 3A ethanol, and then the support was contacted with the lipase solution. These steps were performed either in a batch-wise process in a beaker or in the column reactor itself.

To get an idea of how much of the lipase actually adsorbed on the support, samples of the lipase solution before and after contact with the support and/or samples of the support material before and after contact with the lipase were analyzed for lipase content using the ANTEK model 707 Chemiluminescent Nitrogen Analyzer. For example, 8.0 g of HDPE Accurel® powder were placed in a glass column, wet with 100 mls of 3A ethanol, rinsed with 200 mls of buffered water, contacted with 100 mls of lipase solution containing 1.0 g of lipase dissolved in 100 mls of buffered water, and then rinsed with 150 mls of buffered water. Analysis of the lipase solution before and after contact with the support indicated that 64.5% of the lipase was removed from solution. Assuming all the removed lipase was immobilized on the powder, the support contained $(0.645 \text{ g} / (8.0 + 0.645) \text{ g}) \times 100\% = 7.5\%$ lipase w/w.

The fixed bed reactors were glass columns jacketed with plexiglass sleeves. The columns were maintained at 40° C., by circulating water baths. The actual column dimensions varied. Continuing the example involving 8.0 g of HDPE powder, the dimensions of the fixed bed were 1.7 cm diameter by 21 cm in length (48 cm³).

Substrate was pumped into the columns (5-20 ml/hr), with a piston pump to maintain close control over the rate. The substrate most used was 25/75 olive oil/0.1M, pH 7.0 phosphate buffer. Two column flow patterns were investigated: (1) countercurrent flow of the buffered water (enters column at top) and olive oil (enters at bottom), and (2) cocurrent flow of the oil and buffered water (both enter at bottom).

FIG. 1 illustrates the cocurrent fixed bed immobilized lipase reactor. Triglycerides (TG) and buffered water were pumped in the bottom of the column. As mentioned earlier, a variety of Accurel® materials were tried as support for the lipase. Table 4 below compares with % FA obtained from three fixed bed cocurrent reactors operated under conditions which were similar except for support material:

TABLE 4

Support	Weight (gm)	% FA
PP, melt blown fibers	4	34
HDPE granules	10	42
HDPE powder	8	94

Since the HDPE powder, 150-450 micron particle size, was clearly a superior support to a very surprising and unexpected extent as compared to the fibers, this powder was used in all the subsequent reactor columns. Conditions for two of these columns are given below:

	Column I	Column II
Substrate:	25/75 o.oil/buffered water <u>Emulsion Feed</u>	25/75 o.oil/buffered water <u>Separate Feed</u>
Total Flowrate:	10-15 ml/hr	20 ml/hr
Temperature:	40° C.	40° C.
Support wt:	8.0 gm	10.0 gm
Bed size:	1.7 cm diameter x	2.1 cm diameter x

-continued

	Column I	Column II
	21 cm length	18 cm length
Lipase adsorbed:	650 mg.	975 mg.

Column I was still in operation after 1100 hours. In this column the olive oil and buffered water were mixed together in a beaker prior to entering the column. Continuous agitation was supplied to the emulsion by a magnetic stirrer. A single pump drew off emulsion to feed the column. The volumes of buffer and oil that flowed out of the column were recorded daily. Considerable variation was observed in the ratios of oil:water that were collected from the 25:75 ratio which was supplied to the emulsion reservoir. Thus, throughout the run of this column, the substrate composition was not fixed. The half-life (time for the activity of the immobilized lipase to decrease by 50%) of the immobilized lipase in this column was 234 hrs. The results for this column in terms of % fatty acid in the nonaqueous product over time are graphically shown in FIG. 6.

Column II was operated for 310 hours before being terminated. This column had a higher support load and a higher support:lipase ratio in hopes of increasing the half-life of the lipase. In addition, separate pumps were used to pump the olive oil at 5 ml/hr and the buffered water at 15 ml/hr in order to maintain a constant substrate composition to the column. Also, in the bottom of the column was a glass frit which was used to break the oil into smaller droplets before contact with the lipase. This column in spite of the efforts made, achieved a half-life of only 157 hrs. The results for this column are graphically shown in FIG. 7.

FIG. 2 illustrates the countercurrent fixed bed immobilized lipase reactor. The column bed contained 10 grams of HDPE Accurel® granules. Lipase was immobilized on the granules prior to packing of the column. The catalyst bed measured 2.5 cm diameter by 26 cm in length.

Buffered water was pumped into the top of the column and out the bottom at 5 ml/hr by using two pump heads on the same pump drive. Olive oil was pumped in the bottom of the column at approximately 10 ml/hr. Problems were encountered in balancing the flows of the oil and water to maintain a steady state in the column. Yields of only 10-20% FA were obtained on the olive oil substrate, but it is believed that far better results will be obtained from this embodiment of the invention once it is optimized.

EXAMPLE 6

A study was made using the fixed bed co-current flow column reactor of the effect of maintaining a high glycerol content in the water phase. It was observed that such content had a profound effect in maintaining the activity of the lipase although there is a reduction in the degree of fat splitting due to reaction equilibrium effects. FIG. 8 comprises a plot of data of the fat split obtained over an extended period of time with the reaction medium having various levels of glycerine concentration, and shows the surprising improvement in lipase longevity that can be achieved by maintaining a concentration of about 40 wt. % glycerol. Concentrations in excess of 40 wt. % would probably not be practical because of the low conversion of fats that would be obtained.

EXAMPLE 7

The vertical diaphragm reactor embodiment of the invention is shown in FIG. 3. The reactor was constructed of 6" plexiglass pipe, contained a lower olive oil reservoir and an upper buffered water reservoir. Olive oil was pumped into the lower reservoir and was forced up through the diaphragm that separated the upper and lower reservoirs. The diaphragm was supported on top and bottom by plastic mesh screens. The diaphragm itself consisted of three layers including the top screen. Olive oil first passed through the bottom layer which was a teflon filter cloth which broke the oil into fine droplets. Next the oil passed up through a middle layer comprising a pad of lipase immobilized on melt blown polypropylene Accurel® fibers made by Enka America Incorporated. The fat was split as it passed through the pad. The fatty acid then rose to the top of the upper reservoir which was initially filled with 1.8 L of 0.05M EDTA buffered water to pH 7.0, to form an uppermost phase. Overhead agitation was supplied to the upper reservoir.

To evaluate the operational stability of the immobilized lipase, this diaphragm reactor was run in a batch mode for approximately two months. The reactor contained 5.0 g of melt blown Accurel® polypropylene fibers that contained approximately 0.3 g. of lipase from Candida (Enzeco®) as stated above. Each day, 450 mls of olive oil were fed into the reactor by gravity feed over a 6 hour period. At the end of 6 hours, the fatty acid layer on top of the upper reservoir was analyzed by GPC. The top reservoir was then emptied and filled with fresh buffer the following day.

The plot of % FA versus time in hours, as shown in FIG. 9, gives an indication of the half-life of the immobilized lipase. Linear regression of % FA versus time of actual operation indicates a half-life of 223 hours.

One can use data from the first day to calculate an estimate of the productivity of the reactor. On that day 450 mls of 71% FA were collected to arrive at a production rate of 301 mg FA/hr/cm².

EXAMPLE 8

This example illustrates the stirred reactor embodiment of the present invention as shown in FIG. 5.

In a first test run immobilized *Candida cylindracea* lipase (Maxazyme LP from Gist-Brocades N.V.) was prepared in the following manner:

1. 10.0 gm. of HDPE Accurel® powder (150-450 μ m diameter) was wetted with 20.0 ml of 3A ethanol.
2. 1.0 gm of Maxazyme LP lipase was dissolved in 100 ml of 0.1M Na₂HPO₄ in aqueous solution at pH 7 by stirring for 10 min.
3. The lipase solution was centrifuged to remove any insoluble material.
4. The lipase solution was added to the wetted Accurel® powder and stirred for 30 min.
5. The immobilized Accurel® powder was filtered using a Buchner funnel and washed with 300 ml of buffered water before it went into the reactor.

A bench scale reactor was constructed comprising a 500 ml round bottom flask with four necks. Two of the necks were for the inlet of liquid fats (oil) and buffered water, the third was for the product stream outlet, and the fourth was for an overhead stirrer. A glass wool plug was placed in the product stream outlet neck.

The 500 ml flask was filled with deionized water. Then the above immobilized enzyme was added. The

reactor was assembled, an overhead stirrer started, and two feed pumps were turned on, one for each of the feed streams with a combined flow rate of 12 ml/hr. and a volume ratio of oil to water of 4.5/7.5. The reaction was at room temperature.

The results of the first test run are shown in FIG. 10 in terms of fatty acid concentration in the non-aqueous product phase vs. time. The figure shows a rapid initial fall-off in conversion of fats to a rather low constant level.

The first test run was repeated except that 2.0 g. of lipase immobilized on 20.0 g. of support were added to the reactor. All other conditions and details of the procedure and apparatus remained the same. The results are shown in FIG. 11.

The results of FIG. 11 are startling. Unlike in the first test run where less amount of lipase was present, in the second run there was no initial fall-off of conversion and almost no fall-off of conversion even after 600 hours of operation were approached. There thus appears to be an amazing and unexpected criticality in a minimum amount of lipase that must be present in the reactor mixture for optimum effect.

Since it was determined that only about 65% of lipase initially introduced into the CSTR reactor is retained on the support within a short time after initiation of the test runs, the critical lipase concentration is calculated to be about 2.5 g per liter of reactor volume for the oil charge rate of 4.5 ml/hr, or 556 grams per liter of reactor volume per liters per hour of liquid fat charge.

EXAMPLE 9

This example provides a further comparison of the performance of the previously discussed embodiments of the present invention comprising the diaphragm, cocurrent fixed bed and stirred tank reactors. In each case the embodiment used was the one known to be the optimum, including the diaphragm as described in Example 7, the cocurrent fixed bed as in Example 5 with the HDPE Accurel® Powder and the stirred tank and immobilized lipase on Accurel® as described in Example 5.

The results of the comparison are shown in Table 5 where the half-life (in hours) and productivity (pounds fatty acid produced per pound of immobilized lipase) are given for each embodiment. Two runs were made for each of the fixed bed and stirred reactor embodiments.

The results show that the diaphragm clearly comprises the best mode of the process of the invention at least as far as productivity is concerned. In the course of the half-life of the immobilized lipase in the diaphragm, the productivity of the diaphragm was over five times that of the best run of the stirred reactor and over ten times that of the fixed bed reactor.

It was also observed that the production rate of the diaphragm in terms of mg/hr. per cm² of fatty acid produced was, depending on the choice of hydrophobic filter cloth, as high as 301.

TABLE 5

IMMOBILIZED LIPASE REACTOR SUMMARY		
Olive Oil Substrate		
Type	Half-Life (hours)	Productivity $\frac{\text{lbs FA}}{\text{lbs IME}}$
Diaphragm	223	1695
Fixed Bed	378	119
(Powder columns)	207	88
Stirred Reactor	348	342

TABLE 5-continued

IMMOBILIZED LIPASE REACTOR SUMMARY		
Olive Oil Substrate		
Type	Half-Life (hours)	Productivity $\frac{\text{lbs FA}}{\text{lbs IME}}$
	468	274

As can be seen from the above examples, and corresponding FIGS. 6 through 11, regardless of the embodiment of the invention employed, whether it be a packed column reactor with cocurrent or countercurrent flow, a diaphragm reactor or a stirred reactor, not only is the initial activity of the immobilized lipase comparable to that of free lipase, the activity remains usefully high for a significant time. Enzymatic fat splitting has thus become an economic reality.

What is claimed is:

1. A composition comprising lipase immobilized by adsorption from aqueous solution on a microporous structure comprising a synthetic hydrophobic thermoplastic aliphatic olefin polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof, said structure not being pretreated prior to said adsorption or being pretreated only by wetting with a polar water miscible organic solvent in which said polymer is insoluble and which does not deactivate said lipase.
2. The composition of claim 1 wherein said microporous structure is cellular and comprises a plurality of substantially spherical cells having an average diameter from about 0.5 to about 100 microns, distributed substantially uniformly throughout the structure, adjacent cells being interconnected by pores smaller in diameter than said microcells, the ratio of the average cell diameter to the average pore diameter being from about 2:1 to about 200:1, said pores and said cells being void.
3. The composition of claim 1 wherein said microporous structure is cellular and is characterized by a C/P ratio of from about 2 to about 200, an S value of from about 1 to about 30, and an average cell size from about 0.5 to about 100 microns.
4. The composition of claim 1 wherein said microporous polymer structure is isotropic and is characterized by an average pore diameter of from about 0.1 to about 5 microns and an S value of from about 1 to about 10.
5. The composition of claim 1 wherein said organic solvent comprises an alcohol having from 1 to 4 carbon atoms per molecule.
6. A method for the immobilization of lipase on a microporous structure comprising a synthetic hydrophobic thermoplastic aliphatic olefin polymer selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers and blends thereof, said immobilization being effected without pretreatment of said structure or pretreatment by first wetting said polymer with a polar water miscible organic solvent in which such polymer is insoluble and which does not deactivate said lipase, and then soaking said support in a dilute aqueous solution of said lipase.
7. The method of claim 6 wherein said organic solvent comprises an alcohol having from 1 to 4 carbon atoms per molecule, said solution is pH adjusted from about 4.0 to about 7.0 and the concentration of said lipase in said solution is such as to provide at least about 35 lipase units per ml, but less than 500 lipase units per ml.

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18. The process of claim 8 wherein said microporous structure is cellular and is characterized by a C/P ratio of from about 2 to about 200, an S value of from about 1 to about 30, and an average cell size from about 0.5 to about 100 microns.

19. The process of claim 8 wherein said microporous polymer structure is isotropic and is characterized by an average pore diameter of from about 0.1 to about 5 microns and an S value of from about 1 to about 10.

10 20. The process of claim 8 wherein a concentration of glycerol is maintained in said water of not greater than about 40 wt. %.

21. A process for the hydrolysis of liquid fats comprising contacting said fats in the presence of water at 15 hydrolyzing conditions with lipase immobilized by adsorption from aqueous solution on a microporous structure comprising a synthetic hydrophobic thermoplastic aliphatic olefin polymer selected from the group consisting of aliphatic olefinic polymers, oxidation poly-

mers, ionic polymers and blends thereof, said structure not having been pretreated or having been pretreated prior to said adsorption only by wetting with a polar water miscible organic solvent in which said polymer is insoluble and which does not deactivate said lipase, said contacting being effected by means of a column packed with a powder of said structure on which said lipase is immobilized, the flow of said liquid fats and said water in said column being cocurrent, said powder being from about 150 to about 450 micron particle size.

30 22. The process of claim 21 wherein said microporous structure is cellular and comprises a plurality of substantially spherical cells having an average diameter from about 0.5 to about 100 microns, distributed substantially uniformly throughout the structure, adjacent cells being
35 interconnected by pores smaller in diameter than said microcells, the ratio of the average pore diameter being from about 2:1 to about 200:1, said pores and said cells being void.

23. The process of claim 21 wherein said microporous
40 structure is cellular and is characterized by a C/P ratio
of from about 2 to about 200, an S value of from about
1 to about 30, and an average cell size from about 0.5 to
about 100 microns.

24. The process of claim 21 wherein said microporous
45 polymer structure is isotropic and is characterized by an
average pore diameter of from about 0.1 to about 5
microns and an S value of from about 1 to about 10.

25. A process for the hydrolysis of fats comprising maintaining a lower liquid phase of said fats and an upper liquid phase comprising water, said phases being separated at their interface with a horizontally disposed diaphragm comprising three layers, the bottom most of said layers being a hydrophobic filter cloth, the middle layer being fibers of a support comprising a synthetic

55 hydrophobic microporous thermoplastic aliphatic olefin polymer, said fibers of said support in said middle layer having lipase immobilized thereon, the top most layer of said diaphragm being a retaining means capable of maintaining the fibers of said middle layer in place, 60 said fats flowing upward through said bottom layer and into contact with said supported lipase of said middle layer where in the presence of water from said upper phase, and at hydrolyzing conditions, the hydrolysis of said fats occurs, the fatty acids product of said hydrolysis rising to the surface of said upper phase to form a 65 separate uppermost phase and the glycerol product of said hydrolysis dissolving in said upper phase, said fatty acids being removed as said uppermost phase and glycerol being removed as said lowermost phase.

erol products in aqueous solution being recovered from said upper phase and additional fats and water being added as required to maintain the desired inventory of each.

26. The process of claim 25 wherein the activity of said lipase with respect to the hydrolysis of said fats is restored by sequentially flushing said diaphragm with three flushing liquids which enter said diaphragm at the top layer and exit through means provided to bypass the bottom layer, the first said flushing liquid comprising a solution of water and a solvent suitable for the removal of spent lipase from said support, the second flushing liquid comprising a broth of fresh lipase and the last liquid comprising water.

27. The process of claim 25 wherein said hydrolyzing conditions comprise a temperature high enough to enable said fat to be in liquid phase and less than the deactivating temperature of the lipase and a residence time of about 20 minutes to about 60 minutes.

28. The process of claim 25 wherein the average diameter of said fibers is about 3 to about 7 microns.

29. The process of claim 25 wherein the openings in said hydrophobic filter cloth are from about 3 to about 5 microns in size.

30. The process of claim 25 wherein said lipase being immobilized on said support by adsorption from aqueous solution either without pretreatment or following pretreatment of said structure only by wetting with a polar water miscible organic solvent in which said polymer is insoluble and which does not deactivate said lipase.

31. The process of claim 30 wherein said microporous structure is cellular and comprises a plurality of substantially spherical cells having an average diameter from about 0.5 to about 100 microns, distributed substantially uniformly throughout the structure, adjacent cells being interconnected by pores smaller in diameter than said microcells, the ratio of the average cell diameter to the average pore diameter being from about 2:1 to about 200:1, said pores and said cells being void.

32. The process of claim 30 wherein said microporous structure is cellular and characterized by a C/P ratio of from about 2 to about 200, an S value of from about 1 to about 30, and an average cell size from about 0.5 to about 100 microns.

33. The process of claim 30 wherein said microporous polymer structure is isotropic and is characterized by an average pore diameter of from about 0.1 to about 5 microns and an S value of from about 1 to about 10.

34. A process for the hydrolysis of fats comprising a series of at least two stages in series, each stage comprising the phases and diaphragm as defined in claim 25, the

liquid flow in said series being considered downstream with respect to the direction of flow of the non-aqueous phase from one stage to another in said series, the direction of flow of the aqueous phase from one stage to another being considered upstream and countercurrent to said non-aqueous phase, the liquid fat to said process being introduced into the lower phase of the first upstream stage in said series and the water to said process being introduced into the upper phase of the last downstream stage in said series, an aqueous stream from the upper phase of each stage being passed to the upper phase of the next upstream stage, except that the aqueous stream from the upper phase of said first upstream stage is withdrawn as the glycerol product stream, the non-aqueous stream from the uppermost phase of each stage being passed to the lower phase of the next downstream stage, except that the non-aqueous stream from the uppermost phase of said last downstream stage is withdrawn as the fatty acid product stream.

35. A process for the hydrolysis of fats comprising maintaining a suspension comprising lipase immobilized by adsorption from aqueous solution on particles of a microporous structure comprising a synthetic hydrophobic thermoplastic aliphatic olefin polymer and blends thereof, said structure having not being pretreated, or having been pretreated prior to said adsorption only by wetting with a polar water miscible organic solvent in which such polymer is insoluble and which does not deactivate said lipase, said suspension being in a liquid reaction mixture comprising fats and water by the continuous addition thereto of a stream of liquid fats and a stream of water, and by the continuous withdrawal therefrom of a portion of said mixture containing reaction products comprising fatty acids and a glycerol solution, the concentration of lipase in said liquid reaction mixture being at least about 556 grams per liter of reactor volume per liter per hour of liquid fats added.

36. The process of claim 35 wherein said hydrolyzing conditions comprise a residence time of from about 2 to about 60 hours and a temperature sufficient to maintain liquid phase and less than the deactivating temperature of the lipase.

37. The process of claim 35 wherein a stirring means is operated in said reaction mixture to maintain said suspension.

38. The process of claim 35 wherein said portion of said reaction mixture withdrawn is passed through a filter means to enable recovery of said products and the retention of said particles in said reaction mixture.

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 4,629,742

DATED : December 16, 1986

INVENTOR(S) : Catherine D. Brady, Lincoln D. Metcalfe,
Dale Slaboszewski, Dieter Frank

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In Claim 1, Column 18, lines 22-24 of the Patent, delete "selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers".

In Claim 6, Column 18, lines 53-55 of the Patent, delete "selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers".

In Claim 8, Column 19, lines 6-8 of the Patent, delete "selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers".

In Claim 21, Column 20, lines 18-20 of the Patent, delete "selected from the group consisting of aliphatic olefinic polymers, oxidation polymers, ionic polymers".

Signed and Sealed this
Fifth Day of December, 1989

Attest:

JEFFREY M. SAMUELS

Attesting Officer

Acting Commissioner of Patents and Trademarks

96

100 850

United States Patent [19]

Hendriks

[11] **4,209,591**[45] **Jun. 24, 1980**[54] **ENZYMATIC CONVERSION PROCESS**

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[73] Assignee: **Stamicarbon, B.V., Geleen, Netherlands**[21] Appl. No.: **930,860**[22] Filed: **Aug. 3, 1978**[30] **Foreign Application Priority Data**

Aug. 19, 1977 [NL] Netherlands 7709179

[51] Int. Cl.² **C12M 1/40**[52] U.S. Cl. **435/288; 435/813**[58] Field of Search 195/2, 108, 127, 115,
195/116, 139, DIG. 11; 422/140, 142, 191, 195;
210/17, 150, 151; 435/288, 813[56] **References Cited****U.S. PATENT DOCUMENTS**

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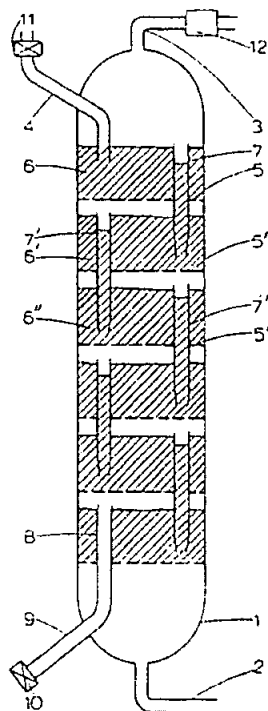
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Primary Examiner—R. B. Penland*Attorney, Agent, or Firm*—Cushman, Darby & Cushman[57] **ABSTRACT**

Chemical enzymatic conversions are conducted by contacting an aqueous solution of the substrate with a granular immobilized enzyme, the substrate solution being passed through several series-connected, separate fluidized beds of the granular enzyme, while the enzyme particles are passed from one fluidized bed to the next countercurrently and against the direction of flow of the substrate solution. Multi-compartment reactor columns are also disclosed.

5 Claims, 2 Drawing Figures

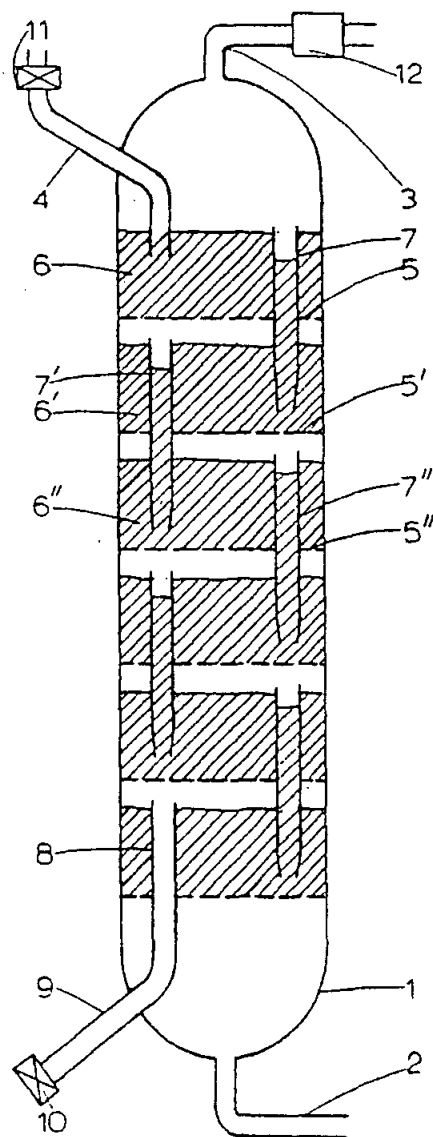


FIG.1

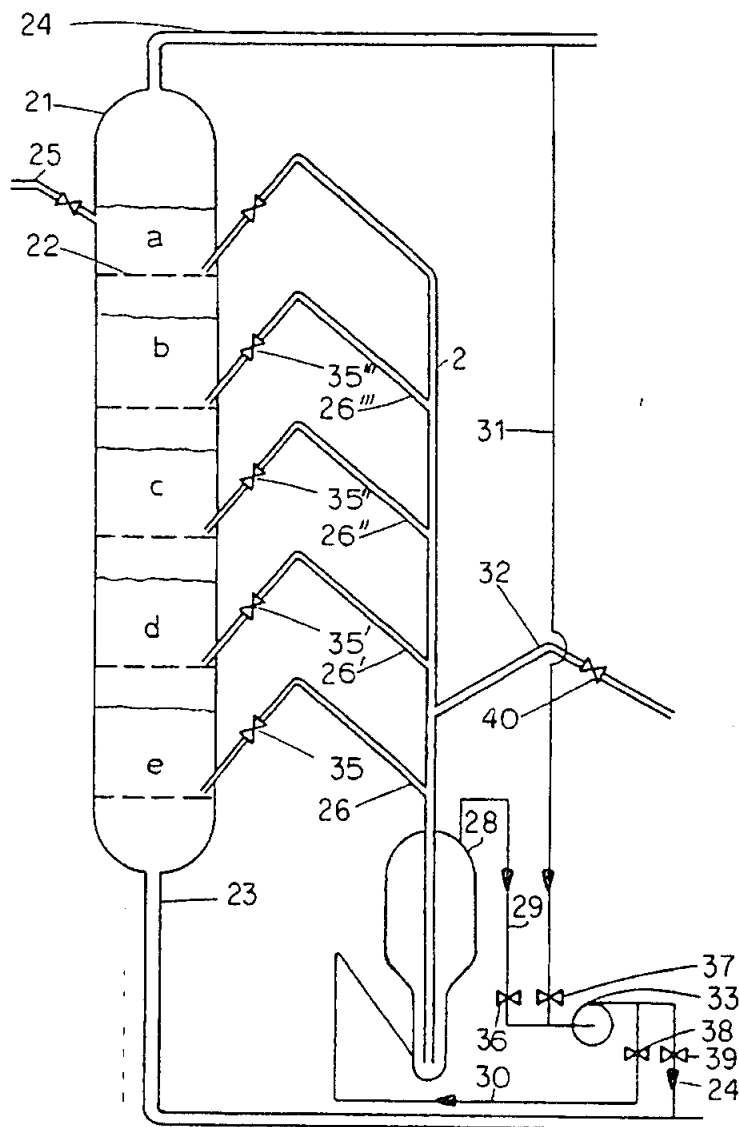


FIG.2

ENZYMATIC CONVERSION PROCESS

BACKGROUND OF THE INVENTION

The invention relates to a process for effecting a chemical conversion by contacting a substrate in the liquid phase with an immobilized enzyme.

It is known to chemically convert an organic or inorganic compound into one or more other compounds by putting a solution of the substrate into contact with an immobilized enzyme or mixture of enzymes. Chemical conversion here comprises any change of the molecular structure of an organic or inorganic compound, such as those caused by hydrolysis, oxidation, reduction, isomerization, or racemization. Enzymes can be immobilized by physical or chemical binding to an organic or inorganic material or by crosslinking the enzyme or cell material with enzymatic activity, optionally in the presence of a filler.

On a technical scale, glucose is thus converted into a mixture of glucose and fructose by means of immobilized glucose isomerase, and N-acetyl L-amino acids are deacylated by means of immobilized amino acylase. In this process it is customary to pass the solution of the substrate through a fixed or fluidized bed of immobilized enzyme particles. Owing to the loss of enzymatic activity, the content of the reactor must be renewed at intervals. For reasons of process economy, it is customary in nearly all cases to replace the immobilized enzyme preparation as soon as its activity has dropped to a given value, usually 20 to 25% of the original activity, both in a discontinuous process in a single reactor and in a semicontinuous process in a single series-connected reactors. A drawback of this process is that the residual activity of the enzyme preparations is not utilized.

The use of a fixed bed of enzyme particles has the additional drawback that the pressure build-up of such a bed is great owing to coagulation or swelling of the enzyme particles. It is also possible that channels are formed in the fixed enzyme bed. It is an object of the present invention to provide a process in which the enzymatic activity of the enzyme preparation can be utilized fully or almost fully. Another object is to make the conversion of the substrate as complete as possible.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, the chemical conversion of a compound is effected by putting an aqueous solution of the substrate into contact with a granular immobilized enzyme so that the substrate solution is passed through a number of series-connected, separate fluidized beds of granular immobilized enzyme, while the enzyme particles are passed discontinuously from a fluidized bed to an adjacent fluidized bed countercurrently against the direction of flow of the substrate solution.

Some advantages of the process according to the present invention are that it can be effected continuously in a direct manner that the enzyme preparation can be used until the activity has dropped to only a small percentage of the original activity, that the pressure differences over the column are small, and that the degree of conversion of the substrate can be very high, especially when dilute substrate solutions are used. A further advantage is that the costs of construction and operation of the column used according to the present invention are lower than for known processes even

when the process is conducted until 20 or 25% of the activity remains, as in the known processes mentioned above.

The process of the present invention is conveniently conducted in a column reactor filled with liquid, the column being divided by horizontal partitions, which are pervious to the substrate solution, into several superposed compartments, each such compartment containing a fluidized bed of granular immobilized enzyme. The reactor is provided with means for the transport of enzyme from one compartment to the next against the direction of flow of the substrate solution. A column reactor of this type is compact and can be constructed and operated in a simple manner.

According to one process aspect of the present invention, a granular immobilized enzyme is used with a specific gravity lower than that of the substrate solution. In this case the substrate solution is fed to the top of the column and discharged at the bottom, while the fresh immobilized enzyme is fed to the lowest compartment of the column. An immobilized enzyme for use in this embodiment can be obtained by immobilizing a free enzyme by cross-linking in the absence of fillers, or by using a filler or support of low specific gravity in the immobilization. In this case the fluidization is brought about by the tendency of the enzyme particles to rise in the contacted liquid and the opposite downward force of the flow of substrate solution.

It is preferred to use a granular immobilized enzyme with a specific gravity greater than that of the substrate solution. In this case the substrate solution is passed upwards through the column and fresh enzyme is fed to the top compartment of the reactor. From the top compartment of the reactor it is passed successively through the lower series of compartments, in each of which the enzyme loses part of its activity. A more detailed discussion of this embodiment follows. This embodiment is to be preferred as it is compatible with the technological know-how of fluid-beds and allows greater variation in the types of granular immobilized enzyme to be used.

The process according to the present invention has some marked advantages in addition to the utilization of the enzyme and the almost complete conversion of the substrate as mentioned above. Thus, the column can be operated at a constant feed rate of the substrate solution and, unlike the reaction in a fixed bed, it is not necessary to make up for the loss in activity of the enzyme preparation by decreasing the amount of substrate supplied to the column. In conversions in which a gaseous reactant is used, such as air in oxidative conversions, the air may be fed to the bottom of the column. A number of sieve trays or grids are used causing the gas to be redistributed over and over again, and no channels or large gas bubbles are formed.

The reaction conditions can be accurately and conveniently controlled by adding some reactants or auxiliary substances to one or more of the compartments. For instance, when a base or acid is released in the conversion, the best pH can still be maintained by adding an acid, base or buffer to the column as required in one or several places.

Substrate characteristics can be matched with enzyme activity requirements most conveniently according to this process. Thus, if the substrate itself has an activating effect, this effect is utilized fully, as the substrate concentration is highest in those parts of the reactor where the enzymatic activity of the enzyme prepara-

ration has fallen most. Conversely, inhibition, if any, by the conversion products or byproducts causes comparatively less interference, as the concentration of such inhibitors is highest in those places where the activity of the enzyme preparations is also highest. The process furthermore has the advantages inherent in the use of a fluidized bed, such as better temperature control, proper transfer of matter, and reduced susceptibility to contamination, such as infections and bacterial growth.

The process of the present invention is particularly suitable for effecting reactions that take place fully or partly in the Michaelis range. In this range, the conversion rate depends not only on the enzymatic activity, but also on the substrate concentration. The substrate concentration is low in the upper part of the column, but the activity of the immobilized enzyme preparation is high, so that a satisfactory conversion rate can still be reached. As a result, the process of this invention can be used for conversions with an enzyme with a low Michaelis constant and for conversions that have to proceed as completely as possible. Some examples are the treatment of waste water from which materials such as phenol are removed by means of phenol oxidase, or urea is removed by means of urease; the treatment of optically active substrates, such as the preferential hydrolysis of L-phenyl-glycine amide in the presence of D-phenyl-glycine amide by means of amino-acid amide deamidase; and the conversion of penicillin G into 6-amino-penicillanic acid by means of penicillin amidase. The advantage of the invention clearly appears from the fact that the urea content of a waste-water flow can be reduced to below 10 p.p.m. by the present process.

The process according to the present invention furthermore has the advantage that the apparatus required is simpler to construct and to operate. In the known processes at most three or five reactors could be arranged in series. In such system the reactor that has operated longest may be shut down at regular intervals, emptied and filled with fresh enzyme and started again as the last of the series. Each time the substrate solution is connected to another reactor. A larger number of reactors is hardly feasible because of the cost and the pressure drop across the system. In the invention ten or more fluidized beds can be used in series without any problem, so that the enzyme can be more efficiently used and a more complete conversion of the substrate is possible.

The transport of enzyme particles from one compartment to the next lower compartment may be effected in various ways, illustrative embodiments of which will now be described. As a first embodiment, overflow pipes may be installed that pass through the partition between two compartments and connect the two compartments. The overflow pipe ends at the top side at some distance over the partition. The distance between the end of the overflow pipe and the partition is the maximum height of the fluidized bed. When fresh enzyme particles are fed to the top compartment, the height of the fluidized bed rises and the surplus flows through the overflow pipe to the next lower compartment, where it causes the transport of enzyme particles to the next lower compartment. Enzyme particles that have completely lost their activity or nearly all of their activity are discharged from the lowest compartment.

In this first embodiment, the partition between the compartments permits passage of the substrate solution only, under normal operating conditions. The partition used may be a sieve plate or gauze. The overflow pipes

may also be arranged to extend to the outside of the column with outlet opening in the column wall. The lower side of the overflow pipe ends in the fluidized bed and preferably tapers towards the end. The pipes are staggered with respect to each other. A column reactor of this type is simple to construct and to operate. A minor drawback is that the flow rate of the substrate solution must be controlled properly, otherwise the fluidized bed might expand too strongly at peak values of the flow rate, so that enzyme products would flow prematurely to the lower compartment.

Enzyme particle movement and transfer may also be controlled by providing each compartment with a pipe through which enzyme particles can be discharged to the next lower compartment by a pump, optionally by way of a buffer vessel. The procedure may be such that the enzyme is first discharged from the lowest compartment. Next, the enzyme is pumped from the last compartment in the form of a slurry which is transferred to the lowest compartment, optionally by way of a buffer vessel. In this embodiment the flow rate of the substrate solution is much less critical. The larger investment in equipment pipes and a pump is usually offset by greater reliability in operation.

The transport of immobilized enzyme particles can also be controlled by partitioning the compartments using sieve trays or other functionally equivalent members that do not allow enzyme product to pass at a liquid flow rate of over a given predetermined threshold value, but do allow enzyme product to pass to the next lower zone at a flow rate between the minimum fluidization rate and that threshold value. Such a partition may consist of a plate with a number of adjacent truncated conical recesses that are open at the lower end. In this case the liquid feed is reduced periodically, so that the enzyme particles sink to the lower zone and exhausted enzyme particles are discharged from the bottom of the column while an equal amount of fresh enzyme particles is supplied to the top of the column.

It will be understood that further embodiments for the transport of the enzyme particles are possible. Use might also be made of trays provided with valves, either located flush with the surface or mounted in short overflow tubes. If the above-described methods, the first two are preferred because of their simplicity and reliability.

The reaction conditions are preferably selected such that the reactivity of the enzyme discharged from the reactor is at most 10 to 15% of the initial activity, and, preferably, less than 5% of the starting activity. Under certain conditions it may be advantageous, as described below hereinafter, to have one or more compartments even contain fully deactivated enzyme. Other reaction conditions will depend on the nature of the liquid phase substrate being treated and the particular enzymatic conversion or conversions to be accomplished. In some cases it may be economically sound to carry out the reaction under such conditions that the residual activity of the discarded enzyme is somewhat higher, for instance between 15% and 20% of the initial activity.

The typical reactor will include at least three separate compartments and, preferably, at least five. For practical reasons, one will preferably use a reactor from 5 to 20 compartments. The compartments, or the fluidized beds contained in them, need not all have the same volume.

The average retention time of the granular immobilized enzyme in the reactor to a large extent depends on

the type of enzyme, the substrate and the reaction conditions. The average retention time will generally range between about a week and several months. The average retention time of the substrate in the reactor depends on a number of factors, some of which include the type of substrate, the type of enzyme, the substrate concentration and the desired degree of conversion. The average retention time of the substrate generally ranges between 0.5 and 120 minutes, in most cases between 5 and 60 minutes.

The substrate to be converted is fed into the reactor in the form of a solution, preferably aqueous, that may optionally contain other reactants as may be required, such as pH regulators, activators, agents for binding interfering impurities, and inert compounds. Emulsions may also be used. If so desired, the substrate solution may be pre-treated, such as by degassing, sterilization, treatment with activated carbon, or similar customary procedures. The substrate solution first contacts the enzyme product that has fully or largely lost its enzymatic activity.

The substrate solution may contain impurities, for instance, heavy metals, that deactivate the enzyme by physical or chemical combination with the enzyme. In this case it is advantageous to use a first zone containing a sufficient number of enzyme particles such that the impurities are substantially or completely to the enzyme particles that are then discharged from the system.

The concentration of the substrate in the solution may vary within wide limits, the only requirement being that the solution can still cause proper fluidization of the enzyme particles as described above. If the conversion step is part of a synthesis system, the substrate concentration may be as much as 50% by weight or more, but in most cases it will be between about 5 and about 30% by weight, which also depends on the solubility of the substrate. However, if the object of the conversion is to remove impurities, the concentration is usually much lower, in the order of about 0.01 to about 1% by weight. The rate at which the substrate solution is passed through the column primarily depends on the fluidization behavior of the enzyme particles and the reactor design. The optimum flow rate can be determined from theoretical calculations or tests by a skilled operator. In most cases, a flow rate of between about 0.5 and about 20 cm/sec may be used.

The granular enzyme products used in the process of the present invention are prepared in various ways. Preference is generally given to products of a granular organic or inorganic support to which the enzyme, cells, or cell parts containing enzyme have been bound either directly or by means of a coupling agent. Products obtained by cross-linking enzymes or cell materials, optionally in the presence of a filler, followed by shaping or any other process yielding fluidizable particles are also used. Various other enzyme products may be used, for instance, products obtained by precipitation of enzymes or a cell material with a flocculant, followed by molding, or products obtained by incorporation of enzymes or cell material in an organic polymer matrix, or binding enzymes or cell material to a polymer, which may be cross-linked. Particularly suitable supports or fillers include glass, sand, silica, carbon, alumina, zirconium oxide, titanium oxide and metals such as nickel. If desired, the support or filler may have been pretreated to effect a better bond. Specific coupling agent may be selected from poly-functional compounds such a glu-

taric aldehyde, silanes, polyisocyanates, azides, polycarboxylic acids and anhydrides thereof.

The overall dimensions, shape and specific gravity of the particles of the enzyme product are chosen such that these particles are readily fluidized and do not only slightly separate into various fractions under operating conditions. As is generally known and reported, there exists a relationship between the diameter and shape of the particles, their specific gravity, and the flow rate to be used in the fluidized bed in the liquid phase. When possible, the dimensions and shape of the particles are selected such that the bed expansion is about 2 at a flow rate of about 4 to about 5 cm/sec. The specific gravity of the particles depends largely on the amount and the nature of the filler.

Many of the granular enzyme products useful in the present invention are themselves described in the art, including their manner of preparation, physical size and properties as well as their biological specificity for the substrate which is to be treated. The physical aspects of these granules such as specific weight and size are selected so as to be functional to best advantage in the process of the present invention by maintaining a substantially or completely fluidized condition in the substrate liquid under operational conditions.

The particular type of enzyme used in the process of the present invention depends upon the type of enzymatic conversion desired provided, of course, the enzyme has sufficient activity once immobilized to carry out the required conversion. The choice of enzyme type need not be limited and as representative classes or types of enzymes belonging to the classes of the hydrolases, the oxidases, the isomerases and the transferases may be used. Some specific examples include amino-acid acylase, amino-acid amidase, peptidase, urease, phenol oxidase, inulase, lactase, asparaginase, fumarase, glucose-oxidase, penicillin amidase, hydantoinase, trypsin, papain, chymotrypsin, aminopeptidase. It is also possible to use a mixture of enzymes instead of only one enzyme. One may also feed an additional amount of granular immobilized enzyme into the column at a position between the inlet and outlet of the enzyme. This additional enzyme may be an enzyme type other than the one fed to the top of the column.

The temperature at which the conversion reaction is conducted is selected within the range defined by the melting point of the substrate solution and the temperature at which the enzyme is deactivated, which range is generally between 0° and 80° C. and preferably between about 20° and about 60° C. It will be apparent that the temperature chosen depends primarily on the enzyme used.

The pressure at which the conversion is effected is of little importance in most cases the process is conveniently conducted at approximately atmospheric pressure. If gaseous reactants are used in the conversion, or gaseous products are formed, it may be advantageous to use a higher reaction pressure in order to maintain the gaseous compounds dissolved in the aqueous phase. As an example, in the decomposition of urea with immobilized urease, an absolute pressure of between 5 and 10 bar may be used in order to keep ammonia and carbon dioxide in solution. The reaction may also be effected at a lower pressure so that gaseous reactants or products are present. In this case the column must be provided with a gas-liquid separator connected to a gas outlet.

The pH at which the conversion is effected is selected such that the optimum enzymatic activity of the immo-

bilized enzyme is obtained which, of course, is related to the particular enzyme employed. In most cases this is a pH of between 6.0 and 8.0. However, there are certain enzymes which may be used at a much lower pH such as between 3.5 and 5, or on the other hand, at higher pH such as between 8.5 and 10.5. The skilled operator will easily determine the appropriate pH required for optimum activity of the enzyme or enzyme system used.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention, in both its process and apparatus aspects will now be elucidated with reference to the appended drawings, in which

FIG. 1 is a cross-section view of an interconnected compartment counterflow fluidized-type reactor; and

FIG. 2 is a part schematic, part-cross-sectional view of a multi-compartment reactor including a common transfer pipe system, and associated fluid transfer equipment according to the present invention.

DETAILED DESCRIPTION OF THE DRAWINGS

In FIG. 1 a column reactor 1 is provided at the bottom with a supply tube 2 for substrate solution and at the top with an outlet 3 for the discharge of the converted product solution. The column is also provided at the top with an enzyme supply tube 4 for immobilized granular enzyme in dry form or in the form of an aqueous slurry.

A number of horizontal sieve trays 5, 5', 5'' divide the column into a corresponding number of compartments containing fluidized beds 6, 6' and 6'' of enzyme particles. The compartments communicate through overflow pipes 7, 7' and 7''.

Overflow pipe 8 from the lowest compartment becomes a discharge pipe 9 for the exhausted enzyme, which is connected through valve 10 with a settling vessel or other means for separating and discharging the enzyme.

Under operating conditions the column is completely filled with liquid; the substrate solution is passed through the column from the bottom to the top. The level of the fluidized beds is preferably maintained somewhat below the top of the overflow pipes. Just before fresh particulate immobilized enzyme is fed to the column the level of the fluidized beds may be brought up to the top of the overflow pipes by increasing the liquid flow. Intermittently an amount of fresh enzyme in the form of a slurry is forced in the column through valve 11 and conduit 4. This causes a transport of enzyme downwards through the column and discharge of enzyme through overflow 8, pipe 9 and valve 10. The outlet may be connected to a gas-liquid separator 12. If the presence of enzyme particles in the product solution is not tolerated, outlet 3 may also be connected to a filter in order to remove any fine particles that may have been entrained by the liquid flow.

Another embodiment of the invention is shown in FIG. 2 in which the column reactor 21 is provided with a number of sieve trays 22 that divide the reactor into a corresponding number of compartments a, b, c, d and e. The reactor is also fitted with a supply tube 23 for the substrate solution, an outlet 24 for the process liquor containing the substrate, and a feed tube 25 for fresh granular enzyme. Each compartment is provided with a pipe 26 connected to a common pipe 27, which in turn, terminates in a storage tank 28. Storage tank 28 is provided, at the top, with a liquid discharge pipe 29 that is

connected to the suction side of a pump 33. Tank 28 is also provided, at the restricted side, with a liquid feed pipe 30 that is connected to the delivery side of pump 33. Pipe 31 connects pipe 24 to the suction side of pump 23 and pipe 24 connects the delivery side of pump 33 to pipe 23. Valves 35, 35' and 35'' are provided in pipes 26, 26' and 26''. Pipe 29 is provided with valve 36, pipe 30 with valve 38, pipe 31 with valve 37, and pipe 34 with valve 39. Pipe 27 is connected to a discharge pipe 32 with valve 40 for discharging the exhausted enzyme.

Under normal operating conditions the entire system is filled with liquid and all valves are closed. The substrate solution flows upwardly through the column and keep the enzyme in each of the compartments in a fluidized state. In the enzyme transfer mode the enzyme in compartment c is emptied first. While the supply of substrate solution is maintained unchanged, pipe 33 is started and valves 35, 36 and 39 are opened. As a result, the enzyme particles are passed in the fluidized state or as a slurry from compartment c through conduits 26 and 27 into tank 28.

The liquid flow thus transferred is so small that conditions in the other compartments hardly change. Common pipe 27 terminates at the bottom of tank 28 which is provided with a restricted portion which serves to promote discharge of the particles under conditions of permanent fluidization. As the liquid flow pumped through pipes 29 and 34 is small, the enzyme particles collect in tank 28 in the fluidized state while pipes 29 and 34 and pump 33 remain free of solids. When all the enzyme has been removed from compartment c, valves 35, 36 and 39 are closed and valves 37, 38 and 40 are opened. The enzyme particles are then pushed from tank 28 and discharged through pipes 27 and 32. In the next step, the contents of compartment d are transported to the tank in the manner described above and then transported to compartment c. Likewise, the contents of the other compartments are transferred to the next lower compartment. Finally a charge of fresh enzyme particles is fed to the top compartment. If so desired, the various pipes, valves and the intermediate storage tank may be provided with connections to a rinsing system for rinsing the equipment with clean water.

It will be noted that the pipes 26, 26', 26'' and 26''' extend into the fluid beds at an angle of for instance between 30° and 80° to the horizontal. The purpose of this positioning is so the interior of these pipes stays free of solid particles when valve 35 is closed. The restricted lower portion of the intermediate storage tank is important to ensure that all enzyme particles can be removed from the tank again with the aid of the delivery pipe. The transport medium used for emptying the various compartments is substrate solution, which is returned to the column. The medium used to fill the various compartments is the substrate-free effluent. In this manner, contamination is avoided. For convenience of illustration, a reactor having only five compartments is shown in the figure. It will be understood that a greater number of compartments, such as 10 or more, will be used in a practice with this type of reactor.

The partitions may consist of sieve trays provided with apertures having a diameter greater than the size of the particulate material. If the liquid flow decreases or is interrupted. The enzyme could in such case fall down the length of the column and gather at the bottom. In order to avoid this other partitions, e.g. bubble-cap trays, may be used or obstructions may be provided.

Very suitable are beams, of triangular cross-section mounted above each row of apertures, such that the base of the beam which is wider than the diameter of the apertures is at a small distance above the surface of the tray. The substrate solution can flow upwards through the apertures and the space between the tray and the base of the beam. If the liquid flow decreases, the particulate material gathers and settles in the through-like space between adjacent beams. When the liquid flow again reaches the fluidisation velocity the particles move upwards and are fluidised.

The invention will be further described by means of the following working Examples. Unless otherwise indicated all parts and percentages are by weight.

EXAMPLE 1

150 grams of sand (screening fraction 175-250 μm) are added with stirring and under nitrogen conditions 2150 ml of fermentation liquid obtained by culturing *Bacillus pasteurii* in a 5-liter vessel. Then over a period of 10 minutes, 350 ml of 0.1% solution of the flocculant Praestol 44 K were added, after which stirring was continued for another 15 minutes. The precipitate was filtered and washed three times with 300 ml of water containing 1 mmol/l of mercapto-ethanol. The solid mass consisting of sand and cellular material was dried at 55° C. and a pressure of 16 kPa for 2.5 hours. 151.2 grams of dry solid product were obtained.

In the next stage, 149 g of the solid mass were stirred in a mixture of 175 ml of acetone and 69 ml of an aqueous solution of mercapto-ethanol (1 mmol/l) while nitrogen was passed through. Over a period of 2 minutes 6.5 ml of a 20% aqueous solution of glutaric dialdehyde at a pH of 7.0 were added to this suspension at room temperature, after which stirring was continued for another 20 minutes. The solid mass was then filtered, washed four times with 150 ml of aqueous mercapto-ethanol solution (1 mmol/l), dried for 2 hours at 50° C. under reduced pressure (16 kPa) and ground into particles.

The urease activity of the resulting granular product was 28.5 units per gram of solids. The activity was determined according to known procedures by adding 2.0 g of solids to 50 ml of a 2% urea solution in a 0.1 molar Sorensen glycine buffer with a pH of 9.2 at a temperature of 40° C. The amount of CO_2 formed at 40° C. in 15 minutes was determined calorimetrically. The activity was expressed in units, being the amount of substrate, in micromoles, that is converted per weight unit of enzyme in one minute.

When the sand consists of the screening fraction in excess of 250 μm granular immobilized enzyme is obtained which has an activity of 26.6 units per gram of solids.

These granular enzyme preparations are used in the apparatus described in the following Example.

EXAMPLE 2

Flow Rate And Bed Expansion Study

A reactor for conducting the process according to the present invention consisted of a tube with an internal diameter of 114 mm and a length of 2200 mm, in which six trays were fitted 300 mm apart. Each tray was provided with 42 apertures 2 mm in diameter and was perforated by an overflow pipe 11 mm in internal diameter extending 100 mm above the tray and 280 mm below the tray. The lower end of each overflow pipe was tapered to a diameter of 5 mm. The lower end of

the tube communicated with a collecting tank fitted with stop-cock for exhausted enzyme and a liquid feed pipe. The tube was provided at the top with a liquid outlet. The apparatus can be used at flow rates of up to 0.05 m/s. At a flow rate of 0.01 m/s the bed expansion is about 2 if particles having an irregular shape with a diameter of 176-380 μm and a specific gravity weight of 2650 kg/m^3 are used. When particles of 516-840 μm in diameter are used a bed expansion of 2 is reached at a flow rate of 0.025 m/s.

EXAMPLE 3

Treatment of Urea-Containing Waste Liquid

Waste-water containing 1.0 wt. % of urea can be treated by the process according to the present invention in a reactor formed by a column 9.45 m length and 1.20 m in internal diameter that is divided, by means of horizontal sieve trays, into ten compartments each with a height of 0.67 m and containing 453 kg of immobilized urease granules in the fluidized state. The sieve trays are designed as described in Example 2 and are provided with overflow pipes reaching to 0.46 m above the trays. The top of the column is provided with a liquid inlet and the bottom is provided with a liquid outlet. A feed pipe for fresh immobilized enzyme empties itself in the top compartment and the overflow pipe of the lowest compartment is connected to a discharge line.

A suitable enzyme preparation is obtained by immobilising cell material containing urease, obtained from a culture of *Bacillus pasteurii*, with sands as a filler in a weight ratio of 1 to 5. A fluidisable particulate material is thus obtained with an average particle size of 300 to 700 μm , a specific gravity of 1780 kg/m^3 , an initial activity of 300 units/g and a half-life of 1000 hours. The fluidisation velocity is from 0.0035 to 0.025 m/s. The amount of waste-water is about 30.9 m^3/hr , at a pH of about 9, a temperature of 35° C. and a pressure of 300 kPa. Each compartment contains 453 kg of enzyme preparation and under normal conditions the bed height is 0.45 m. The amount of urea left in the treated waste-water and the residual activity of the enzyme which is discarded both depend on the amount of fresh enzyme. If 72 kg of the fresh particulate immobilised enzyme are introduced once every 48 hours, the treated solution will contain about 10 ppm of urea and the activity of the enzyme discarded will be about 9% of the initial activity. If 100 kg of enzyme are introduced once every 48 hours the treated solution will contain about 2 ppm of urea and the activity of the enzyme discarded will be about 16.5% of the initial activity.

What is claimed is:

1. A continuous process for the enzymatic chemical conversion of a substrate solution by contacting the substrate solution with granular immobilized enzyme in an expanded or fluidized state, said process comprising:

- passing a substrate solution continuously in an upward direction through a single column reactor which is divided by horizontal partitions pervious to the substrate solution into at least three compartments, each compartment containing a fluidized bed of granular immobilized enzyme having a specific gravity greater than that of the substrate solution, said partitions being substantially impervious to the granular enzyme
- periodically passing said granular immobilized enzyme in a downward direction from each compartment to the next lower compartment, feeding

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fresh enzyme to the upper compartment and discharging spent enzyme from the lowest compartment, and

(c) continuously removing the solution containing converted substrate from the top of the column.

2. The process according to claim 1 wherein the granular immobilized enzyme has a specific weight in excess of about 1.0 and is passed downwardly while the substrate solution passes through said enzyme in an upward direction.

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3. The process according to claim 1 wherein said discharged spent enzyme has less than about 15% of its original enzymatic activity.

4. The process according to claim 3 wherein said discharged spent enzyme has less than about 5% of that original enzymatic activity.

5. The process according to claim 1 wherein said column reactor contains from 5 to 20 of said compartments.

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Gebrauchsmuster

U 1

- (11) Rollennummer G 93 20 595.3
- (51) Hauptklasse C12M 1/18
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Vorrichtung zur Optimierung biologischer Festbett-Reaktoren

Beschreibung

Die Erfindung betrifft eine Vorrichtung zur Optimierung kontinuierlicher biologischer Prozesse für die mikrobielle Wirkstoffproduktion oder auch Behandlung von Emissionen in Bereichen, in denen trägerfixierte Mikroorganismen einen Vorteil darstellen.

Es ist bekannt, Abwasser und Abluft biologisch mit Hilfe von immobilisierten oder suspendierten Mikroorganismen in Form von Tropfkörper-Rieselselfilm-Systemen oder auch mit eingetauchten Trägermaterialien in Form von Scheiben, Waben, "Lockenwickler"-Paketen oder Pellets unterschiedlicher Ausprägung von Aktivkohle bis zu Schaumstoffwürfeln zu reinigen (s.d. ausführlich KUNZ, P.: Behandlung von Abwasser - Kapitel 5, Vogel-Verlag, Würzburg; 3. Auflage 1992).

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Weiterhin ist bekannt, daß auf Festbetten immobilisierte Mikroorganismen Vorteile bei der mikrobiellen Synthese von Wirkstoffen in der pharmazeutischen und chemischen Produktion bringen (vgl. SCHÜGERL, DELLWEG, EINSELE/FINN/SAMHABER: Grundlagen der Biotechnologie, Bioverfahrenstechnik u.s.w.).

Weniger bekannt sind die in der Praxis mit dem Einsatz der Festbetten verbundenen Probleme und Nachteile: Zum einen überziehen sich im Laufe der Zeit die eingesetzten Trägermaterialien mit unkontrollierbaren Biofilmschichten, so daß ein großer Teil der Biomasse abstirbt oder ineffektiv ist, weil der Stoffaustausch mit dem Fluid zu gering ist. Zum anderen wachsen die Zwischenräume zu. Als Vorteil von Submersreaktoren wird weiterhin beispielsweise im Vergleich zu Festbettreaktoren angeführt, daß über die Vermischung des Fluids eine gleichmäßigere Verteilung der Inhaltsstoffe und ein Zugriff auf die Biomasse besteht. Als Zwischending versuchte man deshalb schwebende Träger aus Schaumstoffwürfeln (LINDE) oder verschiedene Kohlesorten (Aktivkohle, Anthrazitstaub usw.) einzusetzen.

Der Erfindung liegt deshalb konkret die Aufgabe zugrunde, eine Vorrichtung zu schaffen, die die oben genannten Einsatzgrenzen der Verwendung von leistungsfähigen immobilisierten Mikroorganismen aufhebt.

Die Aufgabe wird erfindungsgemäß dadurch gelöst, daß in den biologisch wirksamen Reaktionsraum eine Art "Förderschnecke" eingebaut wird, die selbst auch einen Träger für die Besiedelung mit Mikroorganismen darstellt, wobei der Raum längs der Förderschnecke durch geschlitzte oder gelochte Trennbleche segmentiert ist und in die Zwischenräume zwischen Schneckenwendel/Trennblech/zylindrischer Außenbegrenzung zusätzliche Trägermaterialien in lockerer Form eingefüllt werden. Die dabei verwendeten Trägermaterialien werden auf die jeweilige Aufgabe angepaßt und können angefangen bei sich auflösenden Calciumcarbonat-haltigen Strukturen bis hin zu Tennisball-großen Kunststoffgerippen mit Nährboden-Überzug den gesamten bekannten Trägermaterialienbereich überspannen.

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Die Vorrichtung kann gemäß spezieller Aufgabenstellungen so gestaltet sein, daß die Schneckenwendel das zu behandelnde Fluid eher zum Ablauf transportiert, um eine zielgerichtete Rohrreaktor-Kinetik zu ermöglichen; sie kann aber auch entgegen dem Einlauf gerichtet sein, um dem Zulauf ständig aktive Biomasse entgegenzuführen und eine ideale Vermischung wie in einem idealen Mischungsreaktor innerhalb des jeweiligen Segmentes herbeizuführen.

Die Vorrichtung kann horizontal, schräg oder vertikal aufgestellt werden.

Die Vorrichtung kann in einem zylindrischen Behälter, aber auch in einem rechteckigen untergebracht werden, wenn im letzteren Fall ein gitternetzartiger Käfig die Schneckenwendel in geringem Abstand umspannt. In diesem Fall können über außenliegende Einrichtungen zusätzliche Versorgungsmaßnahmen der Biomasse eingeleitet werden (Belüftung, Nährlösungsdosierung usw.). Der umspannende Käfig kann auch in entgegengesetzter Richtung zur Schneckenwendel gedreht werden, um eine noch bessere Durchmischung herbeizuführen.

Die Vorrichtung ist weiterhin darauf ausgelegt, die Träger auch entnehmen zu können.

Die Vorrichtung kann auch ohne Segmentierung vorteilhaft sein, wenn die Trägermaterialien von Zeit zu Zeit entnommen und beispielsweise extern reaktiviert werden sollen.

Die Vorrichtung sieht in diesem Fall auch einen Umlauf der Trägermaterialien vom Kopf der Anlage zum Fuß, aber auch umgekehrt vor, wie auch bei horizontaler Aufstellung von Anfang zum Ende und auch hier in umgekehrter Richtung.

Die Vorrichtung kann auch in einen vorhandenen biologischen Reaktor (bspw. Abwasserreinigungsanlage aus Belebungsbecken) eingebaut und parallel oder sequentiell angeströmt werden.

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Schließlich kann die Vorrichtung auch ohne Schneckenwendel, dafür aber mit einem starren oder angetriebenen Krähwerk versehen werden, um die Trägermaterialien in eine relative Bewegung zueinander zu bringen. In dieser Vorrichtungsform lassen sich bestehende Scheibentauchkörperanlagen optimieren.

Die Vorteile des erfindungsgemäßen Verfahrens liegen auf der Hand: In einfacher Weise können die bekannten kinetischen Verfahrensprinzipien nun auch in einem Festbettreaktor-System realisiert werden.

Die Biomasse-Konzentration kann im Vergleich zu üblichen Festbettreaktor-Systemen gewaltig erhöht werden, weil die Stoffaustausch-Limitierungen positiv beeinflußt und Verstopfungen vermieden werden können.

Die erfindungsgemäße Vorrichtung überwindet somit die Leistungsgrenzen der bekannten Festbettverfahren. Das Prinzip ist äußerst einfach und robust. Die Energiekosten sind vergleichsweise gering wie bei einem Scheibentauchkörper-verfahren.

Die erfindungsgemäße Vorrichtung ermöglicht den Betrieb als total aerobes oder total anaerobes Reaktionssystem.

Anhand Figur 1 läßt sich die Vorrichtung schematisch veranschaulichen:

Das Fluid strömt über den Zulauf (zu) in einen Trommelreaktor (a), in dessen Inneren eine Schneckenwendel (d) eingesetzt ist. In auf den jeweiligen Anwendungsfall optimierten Abständen weist der Trommelreaktor Trennelemente (b,e) mit Schlitzten oder Löchern auf, die das Fluid passieren kann, jedoch die Trägermaterialien (c) zurückhalten. Der Trommelreaktor (a) kann dabei unterschiedliche Zonierungen mit (B) und ohne (A,C) Trägermaterialien enthalten. Die Trägermaterialien können über Schieber (nicht eingezeichnet) sowohl aus den Segmenten (hier B) über die Trommelwand oder über die Trennwand (hier e) entnommen bzw. weitertransportiert werden.

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Betreff: Patentanmeldung:

Vorrichtung zur Optimierung biologischer Festbett-Reaktoren

~~Patent~~ansprüche

1. Vorrichtung zur kontinuierlichen biologischen Wirkstoffproduktion, Reinigung von Abluft oder Abwasser mittels Festbetten, dadurch gekennzeichnet, daß der biologisch wirksame Reaktionsraum in mehrere Sektoren unterteilt ist und eine Förderschnecke aufweist, innerhalb deren Zügen Trägermaterialien für den Aufwuchs von Mikroorganismen eingebracht sind, die die einzelnen Sektoren nicht verlassen können.
2. Vorrichtung nach Anspruch 1, dadurch gekennzeichnet, daß der biologisch wirksame Reaktionsraum zylindrisch ummantelt ist oder die Förderschnecke von einem gitternetzartig begrenzten Käfig ummantelt wird.
3. Vorrichtung nach den Ansprüchen 1 und 2, dadurch gekennzeichnet, daß außerhalb des gitternetzartigen Käfigs Einrichtungen für den Eintrag von Gasen oder Flüssigkeiten angeordnet sind.
4. Vorrichtung nach den Ansprüchen 1 bis 3, dadurch gekennzeichnet, daß die Trägermaterialien kugelförmige oder quaderförmige, offene oder geschlossene, im Inneren hohle, mit Querrippen versehene Kunststoffkörper mit definierter Oberfläche sind.

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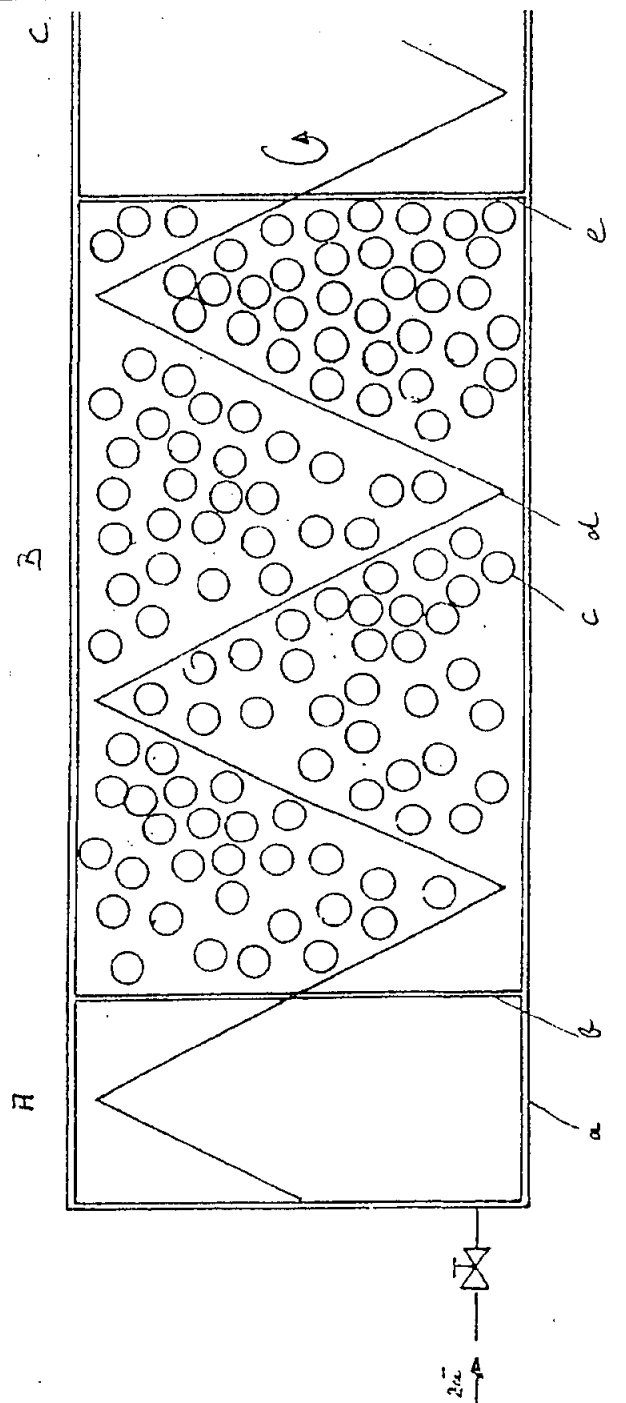
5. Vorrichtung nach den Ansprüchen 1 bis 3, dadurch gekennzeichnet, daß die Trägermaterialien organischer oder anorganischer Natur sind und sich im Verlauf der Zeit auflösen können.
6. Vorrichtung nach den Ansprüchen 1 bis 3 sowie 5, dadurch gekennzeichnet, daß die Wand des biologisch wirksamen Reaktionsraumes oder der gitternetzartige Käfig Öffnungen zur Aufgabe und/oder Entnahme der Trägermaterialien aufweist.
7. Vorrichtung nach den Ansprüchen 1 bis 6, dadurch gekennzeichnet, daß anstelle der die Sektoren begrenzenden geschlitzten oder gelochten Trennwände eine Entnahme der mit dem Fluid in Richtung des Ablaufes transportierten Trägermaterialien durch eine seitlich neben dem biologisch wirksamen Reaktionsraum angeordnete mechanische Rückführung der Trägermaterialien möglich ist.
8. Vorrichtung nach den Ansprüchen 1 bis 6, dadurch gekennzeichnet, daß anstelle der Förderschnecke ein Krählarms eingebaut ist, der die Trägermaterialien während der Rotation des biologisch wirksamen Reaktionsraumes in reaktiver Bewegung zueinander hält.
9. Vorrichtung nach den Ansprüchen 1 bis 8, dadurch gekennzeichnet, daß mehrere der genannten Vorrichtungen parallel oder sequentiell angeordnet werden.
10. Vorrichtung nach den Ansprüchen 1 bis 9, dadurch gekennzeichnet, daß die genannten Vorrichtungen in einen größeren Reaktionsraum eingebracht und sowohl an den Stirnseiten als auch quer angeströmt werden können.

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**Schnitt-
zeichnung**

**Schnecken-
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Figur 1



Prof. Dr. P. Kunz
BIO-LOGIK-CONTROL
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Engineering of enzymatic reactions and reactors for lipid modification and synthesis

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1 Introduction

Enzymatic lipid modification and synthesis have been dramatically developed in the last 20 years from a simple laboratory idea at the beginning to an industrial reality at present. This development has produced countless scientific publications worldwide through various channels. A simple literature search through useful databases such as ISI and Food Science and Technology Abstract can give more than 3000 of related publications in the area of lipids. This development has also generated a variety of potential products in every part of lipid processing and applications. This in turn has attracted wide attention from our related industry sectors. A strong push from this trend to the industrial strategies for future development has been quietly under way. More and more companies have organized their efforts to exploit the possibility using enzyme technology for their future commercial business. So far more than ten international companies have used enzyme technology for their product development and industrial production. A few products are also in commercial markets such as human milk fat substitutes (HMFS), cocoa butter equivalents (CBE), confectionary anti-bloom agents, diacylglycerol (DAG) cooking oil, polyunsaturated fatty acid concentrates, etc.

These interesting progresses are largely attributed to the development of micro-aqueous enzymology. Traditionally, enzymes of such kind (hydrolases) are supposed to work in water-abundant systems for hydrolysis, as demonstrated in nature. Under these conditions, hydrolysis is the only reaction that can be applied for limited applications in lipid areas. Although lipases for lipid hydrolysis have been known for decades before the discovery of possible uses of enzymes in micro-aqueous systems, none of the industrial applications had really been used in practice. In 1984, Zaks and Klibanov published a paper in *Science*, indicating that enzymes could work in a solvent micro-aqueous medium at 100 °C [1]. This is an important milestone in enzyme history. It means that enzymes can also be used for the catalysis of reverse reaction, i.e. esterifi-

cation. This opens up a variety of potential applications. In the lipid area, a reaction such as interesterification (acydolysis, alcoholysis, and ester-ester exchange) is also possible with the catalysis of lipases in a probable hydrolysis-esterification two-step mechanism. Thus chemical interesterification, which has been implemented in the current industry can be fully replaced.

However, the current situation is the joint result of many progresses. A simple summary of these progresses can be given in the following list:

- understanding of the enzyme 3D structure based on protein engineering,
- understanding of interfacial activation of lipases,
- use of genetic engineering in enzyme production,
- improvement of lipase immobilization technology,
- understanding of water thermodynamics in microaqueous enzymatic reactions,
- use of “solvent” engineering for certain reactions,
- development of new interesting products and the increase of their market demands,
- development of more efficient enzyme processes.

It is certain that more items can be added to this list by different people in different areas of research. In general, the joint effort of all these developments provides us with the possibility, flexibility, versatility, and economical potentiality to be applied in commercial sectors and industrial exploitation. The driving forces for the involvement of industrial sectors can be also listed as follows:

- very interesting products and potential market demands,
- improvement of product quality referring to mild conditions,
- reduction of enzyme price and balanced economy in general,
- simple processes and low investment,
- environmental considerations,
- customer perception and marketing strategy,
- know-how preparation and technology takeover/control.

This list can be prolonged: “image” strategy by using enzyme technology is important for many industrial companies; energy-efficiency of the technology is also often mentioned. A healthy industrialization of the technology can be reflected by the economical turnover and overall performance of the commercial sectors. The latter is often more complicated than the technology itself.

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* This paper is dedicated to Professor Carl-Erik Høy, Biochemistry and Nutrition, BioCentrum-DTU, who was one of my close colleagues and friends. He died on February 8, 2003.

There are many updated books, journal reviews, and book chapters that have been dedicated to the progresses in the area. A few selected references, purely from the author's preference, are provided for further reading [2–7]. In this paper, an overview of the enzymatic reactions and reactors for the modification and synthesis is provided. The major enzymatic reactions for the modification of lipids are often referring to hydrolysis, esterification, acidolysis, alcoholysis, and ester-ester exchange from the reaction mechanisms. Interesterification or transesterification is also commonly used in literature to describe some of the above reactions. A detail description is given below for the following two terms:

- **Interesterification:** a general term for the reactions between an ester and a fatty acid, an alcohol, or another ester, which includes acidolysis, alcoholysis, and ester-ester exchange. In literature, it sometimes describes only ester-ester exchange or alkaline-catalyzed randomization of oils and fats.
- **Transesterification:** a term often used for ester-ester exchange. It is also used in literature for the acidolysis reaction or as a general term for all the three reactions including alcoholysis.

Particular concern is given to some selected examples to illustrate the progresses in the area. The engineering aspects of reactions and reactors lie in the stable and practical reaction system, acceleration of the reaction equilibrium, shifting of reaction equilibrium towards products through process design, reaction and reactor optimization through modeling of related factors, and others. Some of these points will be discussed in this paper.

2 Enzymatic hydrolysis

There are three major routes currently used for the hydrolysis of fats and oils in the production of fatty acids: high pressure steam splitting, alkaline and enzymatic hydrolysis. The high temperatures and pressures (typically 250 °C, 7×10^6 Pa) needed for steam splitting make this process unsuitable for splitting sensitive triacylglycerols (TAGs). There are also difficulties associated with alkaline hydrolysis, namely high energy costs and the need to acidify the soaps formed. Thus enzymatic processes are imperative alternatives in certain circumstances.

Lipases are known in nature for hydrolysis. This reaction catalyzed by lipases has been well known for more than five decades. There had been some applications in early stages for the purpose of producing free fatty acids in certain industrial sectors. However, this application had not really attracted much interest, probably due to the high cost of the lipases, limited product potentialities, and complex reaction systems.

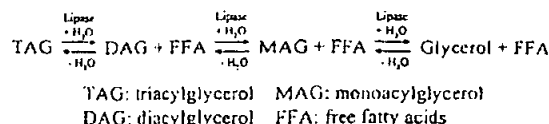


Fig. 1. Steps of enzymatic hydrolysis of oils and fats.

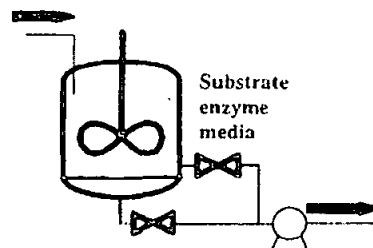


Fig. 2. A simplified illustration of a batch-stirred tank reactor.

This situation has been changed much in the last 10–20 years due to the overall progresses in the area. Enzymatic hydrolysis of oils and fats involves three steps from TAGs to glycerol and free fatty acids (FFA). The reaction is described in Fig. 1.

As shown in the figure the reaction is a multi-phase system consisting of at least the oil phase and the water phase. In most cases, a liquid enzyme is used which is dissolved in the water phase. A batch-stirred tank reactor (STR) is often used for the reaction (Fig. 2). Enzyme recovery for long-term reuse is not possible since the latter will be in the same phase with water and glycerol, even though circulation of the water phase can be used in certain applications. In some cases, a solvent system is used in order to obtain a practical system, especially with immobilized lipases. A few factors affecting the reaction equilibrium and efficiency are listed Tab. 1.

Packed bed reactors (PBR) are another type of reactors that can be used for the hydrolysis of oils and fats through lipase catalysis. Solvent media might help to maintain the stable system. In general this is not very practicable, especially in large scales. The large amount of water and the production of glycerol will easily clog the packed bed and make it impossible to operate. However, for low-degree hydrolysis, the operation might be still feasible with limited amounts of water in the system, as in the application described in the last paragraph of this section.

One of the interesting systems for the enzymatic hydrolysis of oils and fats is the application of a membrane reac-

Tab. 1. A description of factors that influence the reaction equilibrium and efficiency of enzymatic hydrolysis.

Factors	Remarks
Lipase	Lipase dispersion is the main concern to affect the reaction efficiency. Lipase water solution can be dispersed in a few different ways: – Mechanistic stirring: this is often difficult in large-scale operation for a strong stirring. In a small-scale operation, reaction rates were increased with the increase of stirring rates to 1000 rpm (unpublished results). Strong stirring enforces the contact between oils and water/lipase phase. – Electrostatic dispersion: negatively charged lipase solution is sprayed into the oil phase. This operation provides very small droplets into the oil phase and the contact surface is increased. The dispersion is much more stable due to the negative charges. – Emulsion system with surfactants: surfactant is added in the beginning to increase the contact between oil and water/lipase.
Water	The increase of water content can shift the reaction to hydrolysis as shown in Fig. 1. On the other hand, too much water also dilutes the lipase and makes the system more difficult. 5–50 wt-% water has been used in various publications. In case solvent is used, the supply of water is often conducted by the maintenance of water activity in the system.
FFA	FFA are formed during the reaction. They are inhibitive to the reaction. In certain cases, FFA can be continuously removed from the system by certain manipulation such as membrane separation, soap formation, etc.
Glycerol	Glycerol is also the direct product from the reaction. Glycerol exists in the water phase. This can be separated through water phase circulation or membrane separation.

tor [8–9]. In the typical example, the lipase is immobilized on the surface of the membrane (Fig. 3). The oil phase is circulated in the lipase section and the water phase is circulated on the other side. Reactions occur on the membrane surface and the formed glycerol will be transported through the membrane to the water phase. The FFA in the oil phase can be separated by a separate membrane system or by distillation and the acylglycerols are re-circulated for further hydrolysis. The glycerol in the water phase

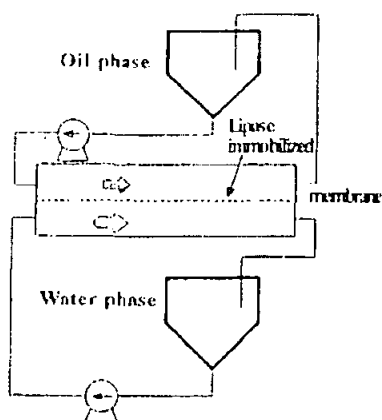


Fig. 3. A membrane system for the enzymatic hydrolysis of oils and fats for the production of free fatty acids.

can be also further treated before being circulated back by a third membrane system or another purification step. In theory this is a perfect set-up. However, much effort has to be made before the actual application is feasible. The membrane might be the hurdle to develop this process into potential production.

One of the interesting applications of hydrolysis is the enrichment of polyunsaturated fatty acids (PUFA) from fish oil [10–11]. Some lipases have less activity towards very long chain fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Partial hydrolysis of fish oil can largely release those shorter chain fatty acids such as 12, 14, 16, and 18 carbon fatty acids. Therefore the content of PUFA in the acylglycerols can be dramatically increased. A typical operation in a 1-kg scale could produce an acylglycerol product with more than 50% PUFA from a fish oil with 23% PUFA (D. Zhou, X. Xu, unpublished observations). A time course of the reaction is given in Fig. 4.

3 Enzymatic esterification

Esterification is the reverse reaction of hydrolysis. This reaction is only possible and useful in a microaqueous reaction system where hydrolysis can be minimized and controlled with limited amounts of water in the system. In water-abundant reaction systems, hydrolysis is the main reaction and it is difficult to make a useful reaction for esterification. A large number of applications has been

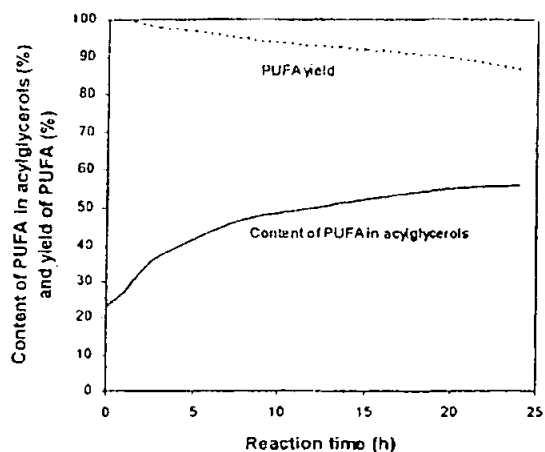


Fig. 4. Time course of the polyunsaturated fatty acids (PUFA, EPA+DHA) in acylglycerols and PUFA yields for the Lipzyme RM IM-catalyzed hydrolysis of fish oil in a 1-kg level batch stirred tank reactor. Reaction conditions: 6 wt-% lipase, 45 °C, water/oil 0.6/1 (wt/wt), stirring 500 rpm, and 100 ppm Toco 50.

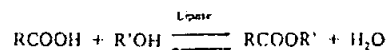


Fig. 5. A simple description of enzymatic esterification.

studied on the basis of extensive studies of microaqueous enzymology. This gives a variety of possibilities to exploit the enzyme technology for ester synthesis.

Esterification is the simple reaction between an organic acid and an alcohol. The reaction is depicted in Fig. 5. Water is one of the direct products from the reaction and it has dramatic effects on the shifting of reaction equilibrium. It has to be continuously removed from the reaction system in order to minimize the reverse hydrolysis reaction. The common methods to remove water from the reaction system in laboratory experiments or industrial applications are listed in Tab. 2.

Water in the reaction system is in one way the reaction by-product, which should be removed in order to force the reaction to the product side. On the other hand, a certain dynamic water environment should be maintained in or-

Tab. 2. Possible methods to remove water from the reaction system in laboratory experiments or industrial applications during the enzymatic esterification.

Vacuum evaporation	The system is simply applied with vacuum. The optimal vacuum should be used to maintain a certain water activity in the reaction system. A vacuum too high may lead to low enzyme activity due to the non-optimal water activity in the reaction system.
Membrane pervaporation	A membrane pervaporation system with vacuum is applied to remove the water from the reaction system. A suitable table water activity in the reaction system can be maintained by an automatic retro-control system with a humidity sensor.
Azeotropic solvent distillation (external drying agents)	The azeotrope property of alcohol or other solvent medium can be used to remove water from the reaction system. A distillation system is applied and the distilled solvent or alcohol can be dried in a separate connected chamber with a drying agent. After drying the solvent alcohol can be fed back to the reaction system.
Drying agent (internal or external)	Drying agents such as a molecular sieve or silica gel are added to the reaction system in certain stages of the reaction to remove water. It is very useful for large-scale plant operations. In a solvent system, non-contact drying can also be used by placing the drying agents in the vapor.
Air or N ₂ bubble	Bubbling by dry air or nitrogen can be also used to remove water, particularly in small-scale experiments.
Alcohol partition	Water will be largely kept in the alcohol phase in the partition between the hydrophilic alcohol phase and the hydrophobic fatty acids phase. By using a large amount of alcohol phase, the water in the system can be kept away from the reaction interface.
Solvent engineering	In certain laboratory operations, a hydrophilic solvent may be used to extract water and keep water away from the reaction.
Salt pair (internal)	In laboratory experiments, salt pairs with certain water activity can be used to maintain the water activity of the system.
Water activity control (external)	Vapor phases in the reaction system are circulated with the vapor phase of the saturated salt solution or salt pair. Water content or activity in the reaction system can be thus regulated.

der to have a high enzyme activity. This is especially important for the long-term production in an industrial plant. The evacuation rate of water from the reaction system should be carefully controlled in order not to dry out the whole system. In certain developments, automatic control of water humidity is a useful solution with a humidity sensor in the reaction system and a retro-control system to the vacuum pump.

A higher lipase activity usually requires a certain water activity (a_w) that often locates in the higher a_w range. The product yield, however, requires an a_w as low as possible in practical experiments. For this reason, the first stage of reaction can be controlled at a higher level of a_w to maintain a higher reaction rate. For the last stage of reaction, however, lower a_w can be used in order to improve the product yields. This strategy has been used in a large number of studies [12–13]. The reaction efficiency and yield have been indeed improved.

In Fig. 5, a number of different organic acids and a large range of alcohols can be used for the synthesis of a variety of products. Some selected examples are listed in Tab. 3. In most of these applications, the reactions involve three phases in the simple reaction system in a batch STR: a hydrophobic phase, a hydrophilic phase, and a solid enzyme phase (Fig. 6). Particular concern should be paid to ensure an efficient reaction system including water removal, homogeneity, and system sustainability. In many cases, solvents have to be used as the medium to increase the homogeneity depending on the particular application. Solid-phase biocatalysis has also been used for the reaction system in cases like sugar esters and other carbohydrate esters [14].

Packed bed reactors have been applied in previous work for the esterification to produce esters [15]. Due to the difficulty to remove water from the system, a circulation sys-

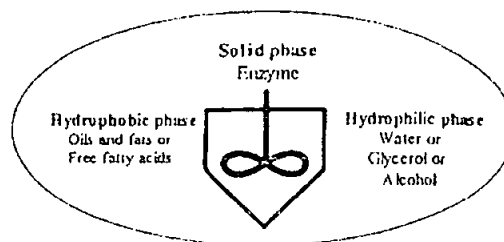


Fig. 6. An illustration of the three-phases reaction system.

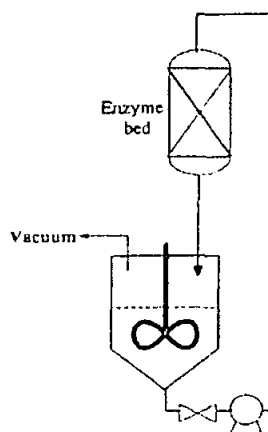


Fig. 7. A circulation system with a packed bed reactor unit and a storage tank with vacuum applied for esterification to produce esters.

tem is often used, in which a packed enzyme bed is the reaction unit (Fig. 7). Vacuum is applied to the circulation tank to remove water. In a practical process, stepwise operation instead of circulation can be implemented in which a packed bed together with a tank is a unit step. The num-

Tab. 3. Typical acids and alcohols for the enzymatic esterification together with a list of useful ester products that have been studied.

-COOH providers	-OH providers	Products
Fatty acids (C1–C26)	Straight or branched chain alcohols (C1–C26)	Biodiesels, fatty acid esters, wax
Other acids:	Glycerol (DAG, MAG)	MAG, DAG, TAG
Ferulic acid	Glycols	Glycol esters
Sinapic acid	Geraniol (citronellol)	Terpene esters and short chain esters
Caffeic acid	Sterols	Sterol esters
Anthranilic acid	Flavonoids	Flavonoid esters
Quinic acid	Lutein	Lutein esters
Glucuronic acid	Ascorbic acid	Ascorbyl esters
Kojic acid	Glucose	Sugar esters
Others	Lyso-phospholipids	Phospholipids
	Pyridoxine	Sunscreens
	Others	Others

ber of steps can be designed depending on the reaction efficiency in each step and the final product quality. An on-line water removal along the packed enzyme bed, in principle, can be also designed with an internal layer penetrative only for water vapor or with a tube in order to allow continuous vacuum. Other technical solutions to improve the feasibility of easy and practical operation, process efficiency and stability, etc. can be expected.

Membrane reactors have been widely used in the esterification system for different purposes [16]. As in the hydrolysis system for the production of FFA, the two-phases system can be also used for esterification applications (Fig. 3). Lipase can be immobilized on the membrane surface and products formed will be either in the hydrophobic phase or the hydrophilic phase depending on the product property. In most cases, products are likely to be in the hydrophobic phase such as acylglycerols formed from the reaction between glycerol and FFA.

Membrane has been also used for the removal of water through water pervaporation [17]. This is better implemented in large-scale or practical processes. Water can be more efficiently removed through the membrane matrix and oil phase will not be affected. A process diagram is provided to illustrate the process (Fig. 8).

4 Enzymatic acidolysis

Acidolysis has been more widely explored for the modification of lipids using enzyme technology. Chemical acidolysis has fewer applications in reality due to no positional specificity. The progress of enzyme technology makes acidolysis an important key word. The reaction takes place between an ester and an acid. The acid will

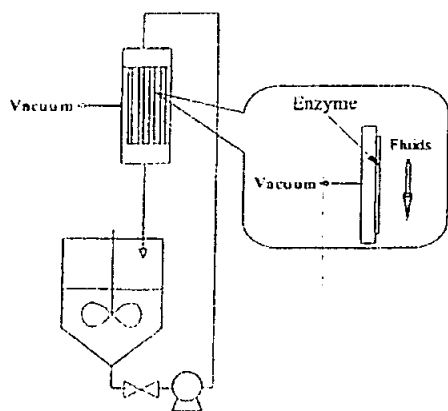


Fig. 8. A membrane reactor system together with pervaporation and circulation through a storage tank for esterification to produce esters.

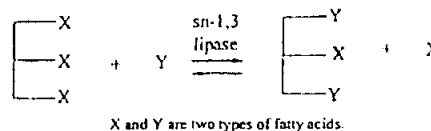


Fig. 9. A principle illustration of enzymatic acidolysis between a TAG (XXX) and a fatty acid (Y).

be exchanged with the acid in the ester. The reaction is depicted between a triacylglycerol and a FFA with a *sn*-1,3 specific lipase (Fig. 9).

In principle the reaction can be conducted between any organic esters and acids. In most applications in the lipid field, esters can be tri-(di-, or mono-)acylglycerols, glycerophospholipids, alkyl fatty acid esters, etc. and acids can be fatty acids or other acids as listed in Tab. 3 in the esterification section.

Enzymatic acidolysis reaction is a reversible reaction. The reaction is commonly considered as a two-step reaction: hydrolysis and esterification. For this consideration, diacylglycerols are considered as the reaction intermediates. Through steps of hydrolysis and esterification, the new fatty acids in the system are incorporated into triacylglycerols. The reaction will finally reach equilibrium. This procedure for the reaction between a TAG (LLL) and a fatty acid (M) is depicted in Fig. 10.

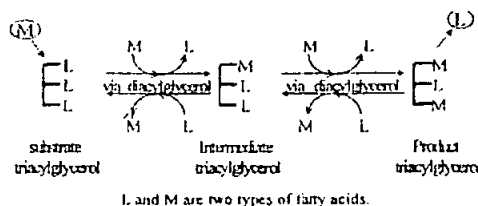


Fig. 10. Reaction illustration of enzymatic acidolysis between LLL and M with *sn*-1,3 specific lipases.

Diacylglycerols (DAGs) are also the precursor of side reactions since they are not thermodynamically stable [18]. The fatty acids will migrate to the neighbor position in the glycerol backbone. This leads to a positional non-specificity of the lipases and the formation of by-products. The whole reaction between LLL and M is depicted in Fig. 11. There are relationships between DAGs and product and byproduct yields. The relationship is illustrated in a reaction between a linoleic acid-based oil and capric acid for the production of C10:0/C18:2/C10:0 (Fig. 12).

system by simple vacuum. This leads to a yield of tri-PUFA of up to 95% compared to very small yields without vacuum application since butyric acid is strongly inhibitive for many lipases. This has been an alternatively good method for the synthesis of tri-PUFA instead of esterification method.

The reaction has been widely used for the production of structured TAGs such as cocoa butter substitutes (SOS; S = saturated fatty acids, O = oleic acid), anti-bloom agent (BOB; B = behenic acid), structured lipids (MLM; M = medium-chain fatty acids, L = long-chain polyunsaturated fatty acids), human milk fat substitutes (UPU; U = unsaturated fatty acids, P = palmitic acid), *etc.* The reactions are obviously catalyzed by *sn*-1,3 specific lipases since the positional specificity is essential for the final product.

The system for this reaction is considerably simple compared to esterification since both material oils and acyl donors are hydrophobic and in the same phase for most applications. This makes the process setup simple and easy. Both batch STRs and PBRs have been widely used for this reaction system from laboratory small scales to industrial plants. Since positional specificity is essential for this reaction in most applications, the reaction time should be as short as possible since acyl migration during the reaction process is proportional to the reaction time. For this consideration, PBRs have been considered the best reactors for this reaction system [22-23]. Reaction in a PBR has shown to generate very low acyl migration compared to the reaction in a STR (Fig. 13). A simple pilot plant setup of the PBR is given in Fig. 14.

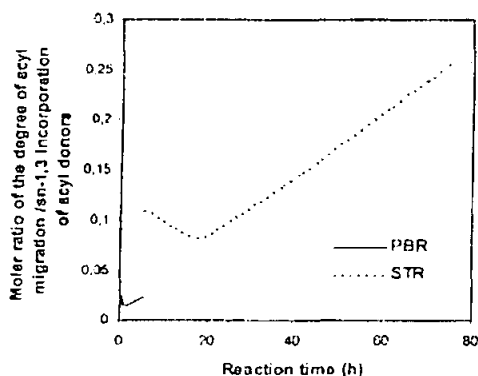


Fig. 13. A comparison between packed bed reactor (PBR) and stirred tank reactor (STR) with respect to the extent of acyl migration formed per unit of *sn*-1,3 incorporation during the time course of the acidolysis reaction between rapeseed oil and capric acid with the catalysis of Lipozyme RM IM. Very low acyl migration occurs in PBR because of the much shorter reaction time.

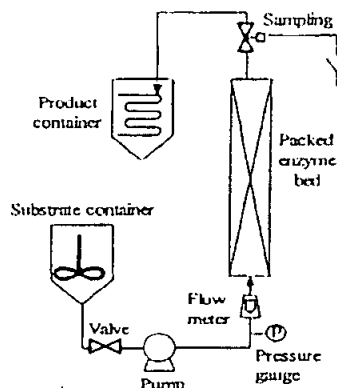


Fig. 14. A simple description of packed bed reactor.

5 Enzymatic alcoholysis

Alcoholysis is the reaction between an ester and an alcohol. Chemical alcoholysis has been widely used in industry for the production of partial acylglycerols, such as MAGs and DAGs, and biodiesels such as fatty acid methyl esters or ethyl esters. In the case of the former the reaction between oils and fats and glycerol (glycerolysis) is used, whereas the reaction between oils and fats and methanol or ethanol (methanolysis or ethanolysis, respectively) is exploited for the latter. Enzymatic alcoholysis has been widely studied for the production of similar products. The benefits of using enzyme processes vary from case to case but the general advantages of enzyme technology can be applied as described in the introduction section. Some particular applications can be highlighted as follows:

- *sn*-2 MAGs, the intermediate for the synthesis of structured TAGs, can be produced with *sn*-1,3 specific lipases.
- MAGs containing PUFA of higher quality can be produced, since milder conditions are applied than in chemical processes.
- Easy recovery of glycerol and possible uses of waste oils and fats are a partial argument for the enzymatic production of biodiesels.

Alcoholysis is also a reversible reaction as shown in Fig. 15. The starting ester (Ester¹) can be acylglycerols, TAGs or alkyl esters for example. The starting alcohol (Alcohol¹) can be glycerol, methanol, ethanol, or sterol for example. The product esters (Ester²) can be partial acylglycerols,

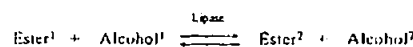


Fig. 15. An illustration enzymatic alcoholysis.

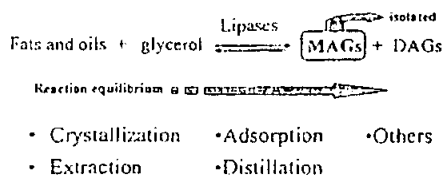


Fig. 16. Scheme and methods to drive the enzymatic glycerolysis of oils and fats towards the formation of monoacylglycerols (MAGs). DAGs – diacylglycerols.

alkyl fatty acid esters, or sterol esters and product alcohol (Alcohol²) can be glycerol or other alcohols.

To drive the reaction to the direction of the product side, we can use the different properties of the starting materials, which commonly exist for most applications [24–25]. For the reaction of glycerolysis, the product ester and alcohol are both MAGs and DAGs. In order to force the reaction towards more MAGs for example, we can isolate the formed MAGs from the reaction system by many different ways (Fig. 16). A brief description of the techniques is summarized in Tab. 5.

For the reaction of methanolysis or ethanolysis of oils and fats, the main products are the fatty acid methyl esters or ethyl esters and glycerol will be the by-product. Glycerol can be separated with the help of a membrane interface

through nano-filtration by applying pressure, or membrane extraction by applying methanol or ethanol as the extraction phase on the other side of the membrane. The formed glycerol will be transported to the alcohol side. The alcohol phase will also supply necessary alcohol for the proceeding of the reaction in the oil and fat phase.

Another way to drive the reaction to the product side is the use of special chemical agents, vinyl ester for example. For the production of MAGs, the reaction between vinyl fatty acid ester and glycerol can be conducted. The reaction should produce MAG and vinyl alcohol. The formed vinyl alcohol will quickly change to acetaldehyde. The latter is volatile even at low temperature and can be easily removed. This makes the alcoholysis reaction irreversible and directly leads to the formation of MAGs.

Glycerolysis of oils and fats has been widely used for the production of partial acylglycerols since 1 mol TAG and 2 mol glycerol can, in principle, form 3 mol MAG, with no unwanted byproducts. The content of partial acylglycerols is reached after reaction equilibrium and can be theoretically calculated from the ratio between glycerol and TAGs, provided the reaction is fully non-specific. The progress of the acylglycerols profile with the increase of the substrate molar ratio is described in Fig. 17. It shows that maximum 44% MAGs can be obtained at reaction equilibrium under the ratio of 2. With the increase of the ratio, the yield of MAGs is not increased if glycerol is included. Usually glycerol can be

Tab. 5. A description of techniques to isolate monoacylglycerols (MAGs) from the enzymatic glycerolysis of oils and fats system.

Methods	Brief description
Crystallization	MAGs have different melting points from TAGs and DAGs. The difference is shown in Tab. 6 for typical fatty acids. From this difference, the system can be controlled in a lower temperature after a certain period of reaction at higher temperatures. Under the lower temperature, MAGs will be solidified and this will drive the reaction to the formation of MAGs. Higher yield of MAGs up to 90% has been reported. Since the solidification, the system cannot be stirred at the lower temperature and the reaction is actually slow. Reaction time can be a few days. Every oil or fat has a suitable temperature to achieve this effect. This temperature is called critical temperature (T_c). Usually the lipase cannot be reused since it is difficult to separate it from the system.
Extraction	Particular solvents have different solubility to MAGs from oils and fats, DAGs, and glycerol. By this difference, the reaction can be driven to the formation of MAGs. In many cases, the system is actually no need to apply a separation stage during the reaction. The solubility difference makes MAGs away from the reaction interface.
Adsorption	In a two-phase operation system especially with a membrane between the oils and fats phase and glycerol phase (as shown in Fig. 3), the formed MAGs can be continuously removed by an external adsorption column implemented in the oil phase circulation line and the TAGs and DAGs left will be re-circulated back to the system for further reaction.
Distillation	Distillation is a separate operation to remove MAGs. The residues can be fed back for further reaction. This in general also drives the reaction toward MAG formation. Usually molecular or short path distillation can be used.

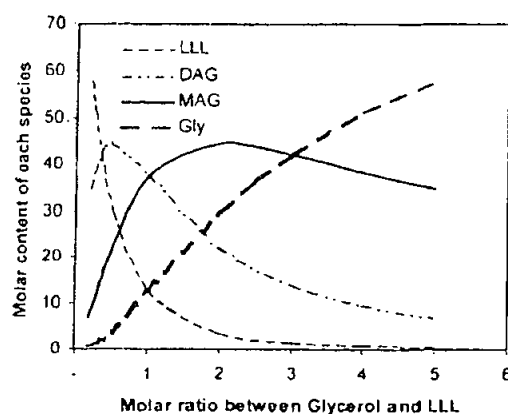


Fig. 17. The relationships between substrate molar ratio (glycerol/LLL) and the contents of acylglycerol species at equilibrium during reactions catalyzed by nonspecific lipases, which are assumed to have the same preference to the three positions. LLL, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; Gly, glycerol.

easily removed and is not present in the final products. The content of MAGs can thus be increased and the amount of leftover TAGs can be further reduced.

Besides the production of partial acylglycerols and alkyl esters, alcoholysis can be used as part of the reaction steps for the production of structured TAGs. Ethanolysis is often used in this case for the first step of reaction to produce *sn*-2 MAGs with *sn*-1,3 specific lipase. The formed *sn*-2 MAGs can thus be used for the esterification with a particular fatty acid to form ABA-type structured TAGs [27, 28]. With different process set-ups, the formed *sn*-2 MAGs can be separated through crystallization before further esterification is conducted, or the reaction can be driven to form high purity MAGs in the system using particular lipases and reaction systems in which no purification steps are necessary before the esterification is proceeded. This process can lead to structured TAGs of high purity compared to a single-acidolysis process.

Alcoholysis is usually a three-phase system as described in Fig. 6. STRs are usually used since it is more difficult to

make a homogeneous system, in particular when no solvents are used. For the glycerolysis of oils and fats, glycerol is the difficult part, limiting the reaction rate. Glycerol usually binds to the lipase surface and makes the reaction impossible since oils and fats are very difficult to diffuse through this hydrophilic medium. The reaction rate is thus very low in a simple mixing system. Many ideas have been explored to increase the reaction rate [24–25]. Some of these ideas can in one way improve the reaction efficiency by improving the distribution of glycerol in the system as listed below:

- absorption of glycerol in silica gel,
- micelle formation by adding dioctyl sulfosuccinate in a solvent system,
- micro-emulsion system,
- use of solvents such as methyl-tert-butyl ether,
- use of supercritical fluids to form the system,
- two-phase system with a membrane interface.

Unless a stable single-phase system can be built, PBRs are not practical for the alcoholysis reaction since the reaction needs two phases in the stable fluid. For methanolysis or ethanolysis of oils and fats, a stable system using a lower alcohol ratio can be easily created at higher temperatures. Since alcohol at a high ratio can lead to lipase inhibition, stepwise addition of alcohol is imperative [29]. Therefore it is not a big problem to apply the continuous operation with PBRs. However, pressure drop of the PBR will be high and the system will need special care to ensure a stable system. For the glycerolysis reaction of oils and fats, solvent(s) has/have to be used to obtain a stable system in order to use a continuous process with PBRs. With the use of particular solvents, this can be achieved without big problems.

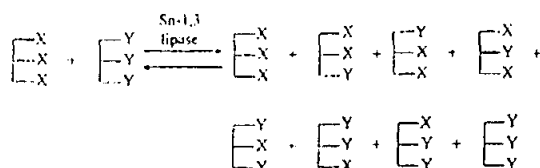
6 Enzymatic ester-ester exchange

Ester-ester exchange is a reaction between two esters. Acids will be exchanged between the hydroxyl moieties. Chemical ester-ester exchange, often referring to as chemical interesterification, is dominantly used for the randomization of oils and fats, which has been widely applied in industry for oil and fat modification. Enzymatic ester-ester exchange is an alternative to the chemical

Tab. 6. Difference of melting points of different acylglycerols with different fatty acids[†].

Fatty acids	FFA	<i>sn</i> -2 MAG	<i>sn</i> -1(3) MAG	<i>sn</i> -1,2 (2,3) DAG	<i>sn</i> -1,3 DAG	TAG
C16:0	62.9	69.0	77.0	62.8	73.2	65.5
C18:0	69.6	75.2	81.5	71.0	78.2	72.5
C18:1	13.0	23.5	35.2	–	25.8	4.9
C18:2	–5.2	8.9	12.3	–	–2.6	–13.1

[†] Figures are obtained from Lipid Bank for web (lipid.bio.m.u-tokyo.ac.jp).



X and Y are two types of fatty acids.

Fig. 18. Reaction scheme of enzymatic ester-ester exchange between two triacylglycerols (XXX and YYY) with *sn*-1,3 specific lipases.

method for the modification of oils and fats. The reaction is given in Fig. 18.

Enzymatic interesterification is different, in principle, from the chemical one in the case that the lipase used is *sn*-1,3 specific. It is not a random reaction in this case as shown in Fig. 18. The *sn*-1,3 positions will be exchanged and the *sn*-2 position will be maintained. In chemical interesterification, all positions will be randomized even though 8 triacylglycerols are still formed as shown in Fig. 18, but the contents of each TAG will be different, depending on how the fatty acids are located in *sn*-1,3 positions and *sn*-2 po-

sition. Since the DAGs are formed as intermediates, acyl migration is also taking place during the reaction. This will lead to randomization if the reaction time is not limited. Suppose that the reaction is randomized, the TAG profile of the interesterified product can be calculated from its fatty acid composition. The equations for the calculation of different types of TAG species are listed below:

$$[\text{AAA}] (\text{mol-}\%) = a^3/10^4$$

$$[\text{ABB}] (\text{mol-}\%) = 3ab^2/10^4$$

$$[\text{ABC}] (\text{mol-}\%) = 6abc/10^4$$

where a, b, c are the fatty acid content (mol-%) of A, B, C, respectively. Comparing to the blended mixture, the TAG profiles of randomized and enzymatically interesterified products differ a lot. The two interesterified products are slightly different even though they are very close in TAG profiles (Fig. 19).

In Fig. 18, the reaction is reversible and the equilibrium should be reached at the end. The reaction is different from other reactions discussed above such as hydrolysis, esterification, acidolysis, or alcoholysis. All other reactions often lead to the formation of "side-products" such as glycerol, water, released fatty acids, and released al-

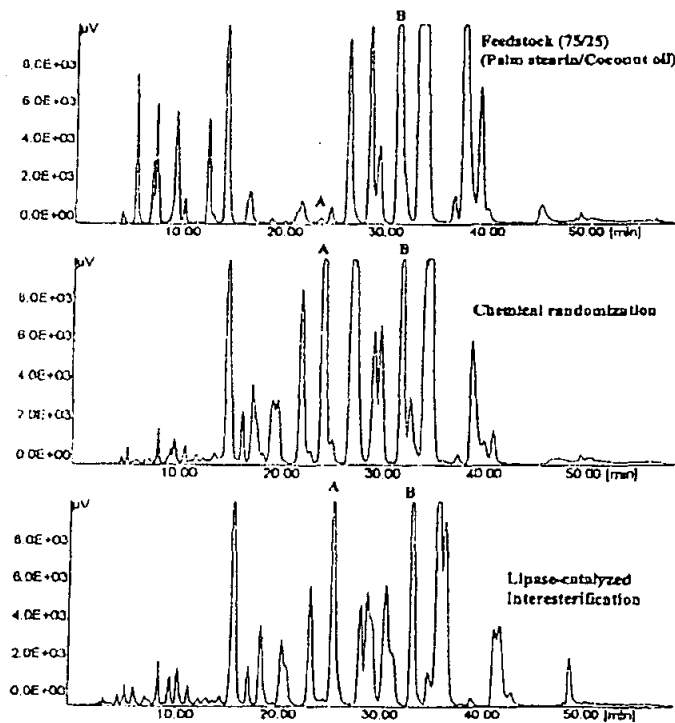


Fig. 19. A comparison between HPLC chromatograms of the palm stearin and coconut oil blend (feedstock), and products from chemical randomization and lipase-catalyzed interesterification. Peaks A and B are particularly different and can be used as markers.

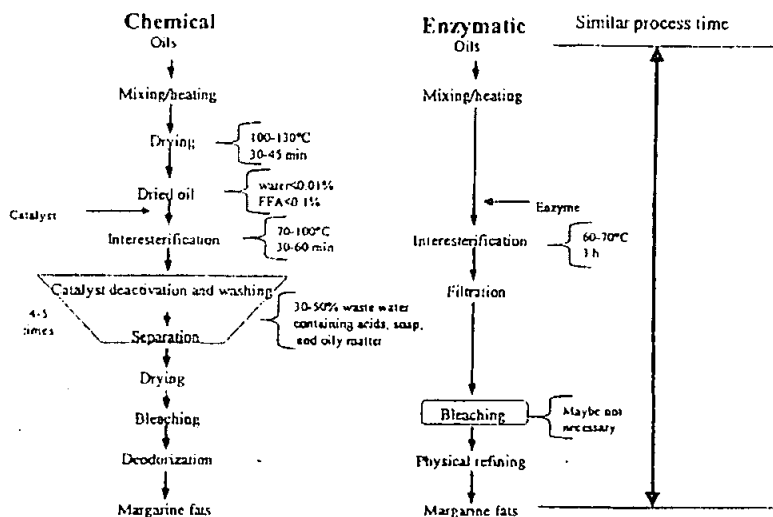


Fig. 20. A process comparison between chemical and enzymatic ester-ester exchange in stirred tank reactors.

cohol, respectively. These products can be removed, since they have other properties than the reaction substrates. This operation can often lead to higher product yields by breaking the reaction equilibrium and driving the reaction towards the product side. In the TAG-TAG reaction in Fig. 18 from the left to the right side, the consideration is different from the points depicted above and the purpose is to minimize the difference between "products" and the starting oils and fats. Therefore it is not so easy to apply any special operation to drive the reaction towards the right side by breaking the reaction equilibrium.

On the other hand the reaction can be driven to the left side to form solid fat and liquid oil fractions by reducing the reaction temperature to a certain low level depending on the starting oils and fats. During the reaction catalyzed by a lipase, the solid fraction, XXX for example, will be solidified to move out of the system. This leads to the formation of more XXX through the ester-ester exchange among different TAG species. The reaction can lead to the final formation of two main fractions, a solid fraction (XXX) and a liquid fraction (YYY for example). This operation is named "directed interesterification" and has been used for the chemical modification of oils and fats as well.

As applied in the chemical modification of oils and fats, the major application of ester-ester exchange has been focused on the production of margarine fats, which have a big and strong market. Margarine fats are daily-used but usually low-priced products. The uses of enzyme technology will meet further challenge with respect to economical balance of the costs and turnovers, comparing to the applications for those higher-valued products. The benefits and shortcomings of the technology should be clearly ex-

plored and weighted against each other. Among them, process and side reactions, which are the topics of this paper, are two aspects that should be concerned to make the enzyme method a benefits-weighted technology. Enzyme processes are simpler in general than chemical interesterification. A simple comparison between chemical and enzymatic processes using batch STRs is given in Fig. 20. For continuous operations, enzyme process is even simpler in which a PBR can be used as shown in Fig. 14. The product or the effluent can then be pumped to the deodorizer for physical refining.

Side reactions of hydrolysis in the enzyme process are a companion since enzymes, in general, require a certain amount of water in the system. This often leads to the formation of FFA and DAGs into the final products. The amount of water required for the active reaction depends on the type of lipases. Lipozyme TL IM for example is less affected by the amount of water and therefore the amount of water in the system can be reduced to a very low level [30]. The formation of FFA can thus be minimized. In general it is believed that water affects the rate of reaction. The reaction can be described as the hydrolysis-esterification mechanism. For the hydrolysis step, water is actually required acting as a reactant. The formed DAG can esterify with another FFA to form a new TAG. DAGs are therefore the intermediates to allow the exchange of acyls between hydroxyl groups (-OH) in the glycerol backbone. The rate of DAG formation is related to the water content in the system. However, water in the system will result in the formation of FFA and DAGs at the end. For the consideration of both, a high reaction rate and a low FFA content, the latter period of the reaction can be conducted at a reduced water

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any mathematical manipulations like any other theoretical models, for example, to predict responses at any given variable conditions in the defined ranges. (ii) Response surfaces and contour plots can be generated to characterize the shape of the surfaces and locate the optimum. (iii) An evaluation of the significance of factors can be performed. The main effect of each factor on the response can also be evaluated.

The shortcoming of using RSM for experimental optimization should be always kept in mind. Since the model is derived from the defined ranges of each factor, extrapolation is impossible. The model can only be used in the defined ranges for optimization or prediction [31].

8 Product development

RSM is an experimental strategy consisting of a particular set of mathematical and statistical methods. Many commercial programs have been developed and can be used to assist the optimization of a system. This has made optimization easier and accessible to common researchers who may not be a mathematician.

- Process development of conventionally available products with improved quality
- Product and process development of new products, especially products impossible to be produced by chemical or traditional methods

In principle, many chemical methods can be replaced by enzymatic ones. Therefore lipid products that can be produced by enzymatic routes are numerous and difficult to be summarized here. For acylglycerols associated with fatty acids, many different products can be produced through enzymatic re-structuring. The possibilities are depicted in Fig. 21. In the first row, alkyl esters can be produced via alcoholysis of oils and fats. Fatty acid fractionation is suitable for the enrichment of particular fatty acids by using the selectivity of lipases toward different fatty acids. Particular applications for the enrichment of PUFA such as EPA and DHA, GLA (gamma-linolenic acid), and CLA (conjugated linoleic acid) have been studied. Different reactions have been used to achieve the targets and

Optimization using RSM is very useful for enzymatic modification systems. One reason is that there are no true theoretical models able to fully predict such a complex system including the effects of enzyme characteristics, mass and heat transfer, factors, stability, water thermodynamics, etc. Another reason is that several factors have interactions and jointly and simultaneously influence the desired responses. A satisfactory RSM optimization can provide a lot of valuable information for the applied system. (i) Optimization can provide a statistical mathematical model between responses and factors. Usually quadratic polynomial models are used to approximate the responses. After the response from experiments is fitted, a mathematical relationship is set up between the response and all factors. The model obtained can therefore be used for

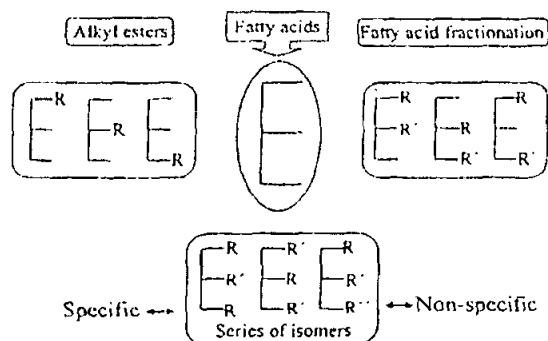


Fig. 21. Routes to re-structure acylglycerols.

efficiency, such as hydrolysis, esterification, alcoholysis, etc. [32–33].

Partial acylglycerols are located in the second row of Fig. 21. MAGs are traditional lipid products used as food emulsifiers or surfactants in other applications. DAGs are also intensively discussed for the reduction of fat accumulation in human body. Both MAGs and DAGs are the intermediates between TAGs and glycerol/FFA, which can be synthesized by different reaction routes [24–25]. A summary of these routes is depicted in Fig. 22. As shown in the figure, DAGs and MAGs are part of the reaction equilibrium. The only way to generate more products is to break the equilibrium and force the equilibrium to the formation of these products. This has been discussed in the early sections.

Structured TAGs are located in the bottom row of Fig. 21. For those specific structured TAGs, enzymatic methods are only feasible ways for large-scale productions. This has been the major focus of attention for decades due to the following considerations:

- products are valuable and high-priced referring to nutritional and functional properties,
- chemical methods are impossible for wide uses for the production of food related ingredients,
- market demands of related products are in high concern,
- economical balance can be maintained even with high cost of lipases.

There are a number of products available with a specific structure, which have been developed in the last 20 years. Some of them have been commercialized. The typical products are depicted in Fig. 23 for their targeted structure. The production of these structured TAGs mainly involves the acidolysis reaction or the TAG-ethyl ester exchange reaction in order to exploit the regiospecificity of lipases [5]. Steps of other reactions can also lead to the formation of specifically structured TAGs [27–28]. This has been discussed in the above sections. For the non-spe-

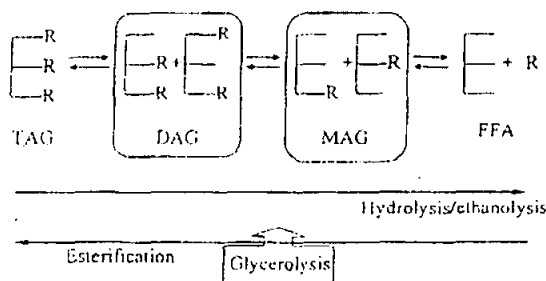


Fig. 22. Diacylglycerol (DAG) and monoacylglycerol (MAG) are intermediates of all the reaction methods including hydrolysis, alcoholysis, esterification, and glycerolysis in the equilibrium systems.

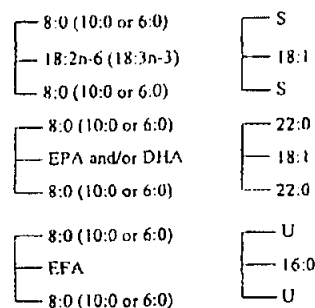


Fig. 23. Typical specific structured triacylglycerols. EFA – essential fatty acid, S – saturated fatty acids, U – unsaturated fatty acids.

cific TAG products, the research has been focused on margarine fats, which nearly exclusively involves the ester-ester exchange reaction.

9 Process technology

Enzyme reactors are central part of enzyme processes. Many different reactors have been studied and used for the enzymatic modification of lipids, such as STRs, PBRs, membrane reactors, gas-lifting reactors, fluidized reactors, simple circulating reactors, spraying reactors, foam reactors, etc. with/without internal or external extractive or separation steps or other enforcing steps [34–37]. This list is certainly not a scientific classification. Some of those typical reactors or process configurations or their combinations have been discussed in some of the above sections.

There are no universal criteria for the choice of a reactor. The following can be used for the evaluation of a suitable reactor for a particular system and process:

- reaction efficiency (homogeneity, dispersion, enzyme activity, etc.),
- product quality (yield and purity),
- enzyme reuses or stability,
- process feasibility and practicality,
- process productivity,
- process investment and availability,
- process and product change flexibility,
- easiness of process operation, control, and monitoring.

The influence of each above-mentioned criterion can be arbitrary from case to case, however in general, factors are listed by decreasing significance from scientific point of view. An efficient reaction is the most essential requirement for a particular system.

PBRs are one of the most interesting reactor types that have currently received a lot of attention and applications in the lipid modification industry. The particular concerns for the application of PBRs in lipid modification can be:

- simple process and operation,
- no filtration of enzyme necessary,
- less damage for the enzyme particle size and structure,
- continuous process easy and enzyme reuses simple,
- low investment,
- less reaction time and in turn leading to less side reactions in those specific reactions (better quality),
- efficient reaction under optimal operation.

A few things on the other hand should be carefully controlled or avoided, such as:

- internal and external mass transfer limitations,
- channeling under either upward or downward flow,
- pressure drops under smaller size of enzyme particle or higher flow rate,
- water content control and regulation,
- fouling of enzyme bed during the process running.

All these issues require a thorough understanding of the process and system. In general the design of the unit PBR should limit to a certain capacity in order to achieve above issues under optimal operations. In general a smaller size of the unit PBR has many advantages such as: Easy to change, low pressure drop, easy to avoid channeling, low cost for optimization, easy to control mass/heat transfer, easy to maintain a stable productivity, easy to maintain a suitable linear flow rate, easy to expand the capacity by parallel connection of a few PBRs.

Considering the reduction of enzyme activity, flow rates should be constantly adjusted and reduced to obtain a stable product quality and yield in a single PBR system. This is often not practical for the implementation of the technology in the industrial plants since stable process capacity cannot be maintained, even though the automatic adjustment of flow rates can be conducted in the present electronic modern world. The way to maintain a stable production capacity for a practical process is to make a series PBR design in which a number of unit PBRs are set in series along the flow line and the ages of the enzyme in each PBR are different (Fig. 24). The used-up PBR can be moved out of the series from the first step and the new one is then inserted into the series in the last step. In this way a stable production capacity can be maintained with little fluctuation if the number of unit PBRs is sufficient. In a PBR system for the

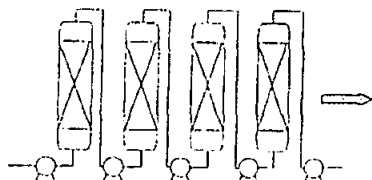


Fig. 24. Series setup of packed bed reactors.

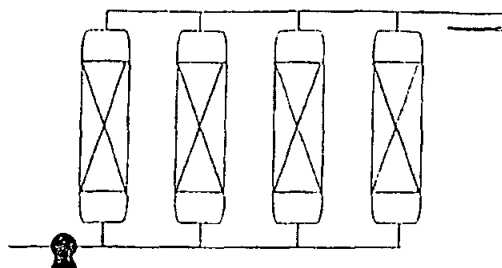


Fig. 25. Parallel setup of packed bed reactors.

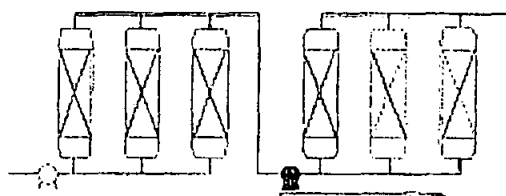


Fig. 26. Series and parallel setup of packed bed reactors.

production of structured lipids from sunflower oil and caprylic acid with the reaction of acidolysis, four columns in a series resulted in a 2–3 mol-% fluctuation from the average incorporation of caprylic acid into sunflower oil (47 mol-%) compared to the two columns in series with 4–5 mol-% fluctuation from the average (43 mol-%). In principle, seven columns in series can lead to a production yield or capacity with the fluctuation less than 5% with whatever the enzyme activity and stability.

With reference to the benefits of using a smaller size PBR, process capacity can be enlarged by parallel connections of more than one PBR (Fig. 25). Considering the necessity of series connection setup for a stable production capacity, parallel and series connections can be implemented in the same process setup (Fig. 26). By this operation, the process can provide the required flexibilities with a stable and practical production.

Acknowledgements

This paper has been inspired by the questions from my students. It is the author's hope that this paper is useful as a brainstorming for those who are working on their thesis study or other research projects. Project grants and financial supports from STVF, MFF/Fotek, and LMC are acknowledged. Discussion among the project groups and project partners are appreciated. The paper is not fully referred in order to simplify the writing and reduce the paper length. The initial work for those used in this paper are acknowledged. Partial contents have been presented in a few international conferences.

12

- 12

United States Patent [19]

Brouillard

[11] 4,029,546

[45] June 14, 1977

[54] COLUMN APPARATUS AND PROCESS FOR IMMOBILIZED ENZYME REACTIONS

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[73] Assignee: Penick & Ford, Limited, Cedar Rapids, Iowa

[22] Filed: Mar. 29, 1976

[21] Appl. No.: 671,063

[52] U.S. Cl. 195/31 R; 195/31 F; 195/63; 195/68; 195/116; 195/139; 195/DIG. 11

[51] Int. Cl.² C12D 13/02; C12B 1/00; C07G 7/02

[58] Field of Search 195/63, 68, DIG. 11, 195/31 R, 31 F, 127, 139, 116

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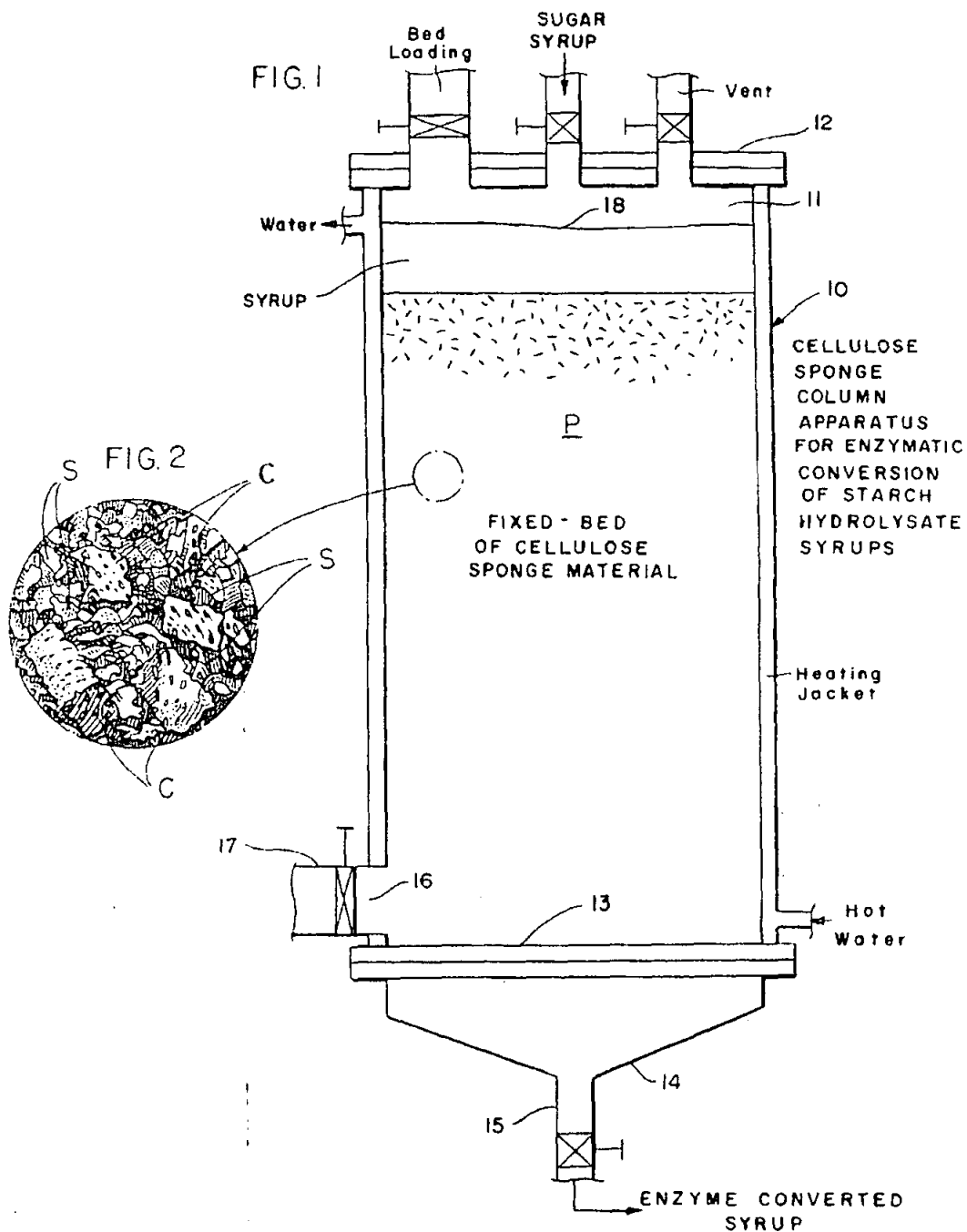
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Primary Examiner—Lionel M. Shapiro
Attorney, Agent, or Firm—Tilton, Fallon, Lungmus, Chestnut & Hill

[57] ABSTRACT

A column apparatus and process is provided for immobilized enzyme reactions using a bed of regenerated sponge material chemically modified to incorporate enzyme-immobilizing groups. The sponge material selected for use in column apparatus has a flow of direction length of 2 to 5 feet or more, a density of less than 1 gram for each 18 cc. of bed volume (e.g. 1 g./20–70 cc. bed vol.), and a water flow porosity such that over 0.5 (preferably over 1.0) gal. water/min./ft.² flows through the bed. The apparatus and process can be used with a wide variety of enzymes, but has particular applicability to the enzymatic conversion of starch hydrolysates. Starch-derived sugar syrups having D.E.'s of 40 to 97 can be produced. Also, isomerization of glucose syrups can be carried out to produce a syrup of enhanced sweetness, comprising a mixture of glucose and fructose.

21 Claims, 2 Drawing Figures



COLUMN APPARATUS AND PROCESS FOR IMMOBILIZED ENZYME REACTIONS

BACKGROUND AND PRIOR ART

The properties of ion exchange celluloses have been studied for chromatographic separations and immobilization of enzymes. See, for example, Guthrie et al, *Ind. Eng. Chem.*, 52, 915-916 (Nov. 1960); Barker et al, *Carbohyd. Res.*, 8, 491-497 (1968); and Wilson et al, *Biotech. J. Bioeng.*, XI, 349-361. Natural celluloses modified to incorporate cationic groups, such as tertiary amine or quaternary ammonium groups, are available commercially in microgranular and fibrous forms. The di- and tri-ethylaminoethylated celluloses are referred to, respectively, as DEAE-cellulose and TEAE-cellulose, while the cationic derivative of cellulose prepared by reaction with epichlorohydrin and triethanolamine is known as ECTAEOLA-cellulose. Companies which are understood to have produced cationic celluloses for commercial use include Brown Company, New York, N.Y., W. & R. Balston, Ltd., Kent, England, and Servi Co., Heidelberg, Germany.

As far as is known, however, the only commercial application of enzymes immobilized on an ion exchange cellulose has been in connection with the partial conversion of starch-derived glucose to fructose, thereby obtaining a syrup of increased sweetness. For this purpose, glucose isomerase enzyme is bound to DEAE-cellulose (or similar cationic cellulose), and the glucose syrup is passed through a series of very thin beds of the isomerase-containing cellulose. See, Snyder, B. J., "Continuous Isomerization of Glucose to Fructose on a Commercial Basis", *Die Starke*, 26, 409-412 (1974); and Thompson et al U.S. Pat. No. 3,788,945, granted Jan. 29, 1974. This technology, as applied commercially by Standard Brands Incorporated and A.E. Staley & Company, involves the use of contact beds of 1 to 5 inches in thickness. This is necessary so that the pressure drop across each bed is small and the compaction of the bed is minimal. But because of the thinness of the beds, in order to avoid the effects of substrate channeling, it is essential to employ a series of such beds. Consequently, fixed-bed columns, such as are used for processes involving ion-exchange resins, cannot be employed. Instead, pressure leaf filters are used. The glucose isomerase bound to the cellulose carrier is pumped as an aqueous slurry through the pressure leaf filter in such a manner as to cover each leaf evenly with a thin layer of the cellulose material. As disclosed in U.S. Pat. No. 3,788,945, the depth to width ratio of the beds is preferably limited to from about 0.02 to 0.05.

Because of the expense and inconvenience of carrying out glucose isomerization in pressure leaf filters, the corn syrup industry has been actively searching for alternative processes where the glucose isomerase enzyme can be immobilized on a material usable in commercial-size fixed bed columns. Such column materials must effectively immobilize the enzyme; be chemically and physically stable, resisting disintegration under conditions of use; and being sufficiently porous while minimizing channeling effects, so that there is adequate and uniform contacting but with no excessive pressure drop across the bed. Some microorganisms which produce isomerase contain this enzyme in the cell, and the enzyme is bound therein, or can be bound by a heat treatment. For example, a column material can be

prepared for *Arthrobacter* cells. One process is described in U.S. Pat. No. 3,821,086. Such natural column material shows considerable promise, and provides advantages over the use of shallow beds of cellulose-immobilized isomerase. However, the need is great for a column material of general utility, which can be used for immobilizing soluble isomerase, as well as other soluble enzymes, such as those used for the conversion of starch oligosaccharides to dextrose and maltose. Such a column material could be used to immobilize alpha amylase, glucoamylase, or mixtures thereof, for commercial production of corn starch-derived syrups having D.E.'s from 40 to 97.

In particular, to minimize capital investment, increase plant capacity and reduce production costs, there has been a manifest need for a column process to produce intermediate D.E. corn syrups, such as the syrups now produced by enzymatic hydrolysis of corn starch to obtain syrups having a D.E. in the range of about 40 to 70. As far as is known, all present commercial processes for producing this type of syrup utilize a final stage batch enzyme treatment in which the soluble enzymes are dissolved in the syrup. This is a one-time enzyme use. Usually, the final stage involves treatment simultaneously with an alpha-amylase and a glucoamylase. Therefore it would be desirable to provide a column material to which a mixture of these enzymes can be chemically bonded and immobilized therein. Such a column material and column apparatus would also have many other applications, and could be used, in general, wherever batch enzyme treatments of substrates with soluble enzymes are now used.

SUMMARY OF INVENTION

The novel column apparatus and process for immobilized enzyme reactions of this invention is based in part on the discovery that an efficient and relatively inexpensive column material for immobilizing enzymes can be prepared from regenerated cellulose in sponge form. It is the physical properties of the selected cellulose sponge material, which primarily characterize the novelty of the column apparatus and process of this invention. Although regenerated celluloses are known to have substantially lower molecular weights and degrees of polymerization than native cellulose, it has been found that regenerated cellulose in sponge form can be readily modified by known chemical processes to incorporate effective amounts of enzyme-immobilizing groups. In fact, it is one of the surprising aspects of the present invention that all of the chemistry needed to practice the invention has been well-known for many years, being described in detail in the chemical literature and prior patents for application to natural cellulose. For example, as has been done commercially for native cellulose, regenerated cellulose sponge material can be chemically modified by known processes to produce DEAE-, TEAE-, ECTEOLA-sponge cellulose.

The regenerated cellulose sponge can be used as a starting material for the purpose of the present invention and is readily available, being manufactured by a number of companies for sale as household or industrial cleaning sponges. Moreover, the scrap or waste from the preparation of cellulose cleaning sponges can be used. One type of such waste material is known as "D-slab". It consists of the trim ends of the cellulose sponge sheets as formed before cutting into sponge blocks. Heretofore, this trim end material has had relatively little commercial value, although it has had some

use, such as filling for inexpensive pillows and similar upholstering uses. It is therefore unexpected that D-slab trim sponge wastes can be used for immobilized enzyme columns.

The preparation and characteristics of cellulose sponge material for use in the column apparatus and immobilized enzyme reaction processes of this invention will subsequently be described in detail. However, certain important features may be indicated here. By selecting and processing the cellulose sponge material so that it has a density under conditions of use of less than a certain maximum, and preferably within a specified range, the sponge material may be employed in deep beds of the kind conventionally used for fixed-bed columns. Moreover, high flow-through rates can be employed without substantial liquid pressure drop across the beds. As will be appreciated, therefore, the resulting column apparatus and immobilized enzyme reaction process represents a great improvement over the prior art of thin-bed enzyme-immobilizing cellulose materials.

As far as is known, it is broadly new to employ cellulose sponge in a column apparatus or process for immobilized enzyme reactions. With respect to a column material in sponge form, a recent U.S. Pat. No. (3,919,048) describes the use of ground natural sponge for enzyme immobilization, and proposes the natural sponge as a column filling. However, natural sponge differs both chemically and physically from regenerated cellulose sponge. Natural sponge is formed of spongin, which consists mainly of protein. In large pieces, it has large openings forming channels there-through. When ground it is as dense as powdered natural cellulose.

Experiments have also been conducted of other natural proteinaceous materials for insolubilization of enzymes, namely collagen powder and collagen sponge. Silman et al, *Biopolymers*, 4, 441-448 (1966). The purpose was to study the effect on papain, and no suggestion is made to use collagen sponge for fixed-bed column-type enzyme reactions.

It is therefore quite surprising and unexpected that cellulose sponge, which is available as a scrap material, and which, as far as is known, has not heretofore been used in any enzymatic processes, can be employed as the key element of a column apparatus and process for immobilized enzyme reactions.

THE DRAWING

The accompanying drawing in FIG. 1 illustrates a fixed-bed column apparatus utilizing a bed of regenerated cellulose sponge material in accordance with the present invention, the apparatus being illustrated in conjunction with a preferred process, namely, the enzymatic conversion of syrups.

FIG. 2 is a greatly enlarged view of the material of the bed, which, in the illustration given, consists of a mixture of rectilinear pieces of sponge and sponge shreads.

DETAILED DESCRIPTION

Cellulose sponge material for use in practicing the present invention is available commercially, and include the cellulose sponge scrap known commercially as "D-slab" trim ends. When the sheets of cellulose sponge are molded in the forming boxes, it is necessary to trim the ends of the sheets before they are cut into sponge blocks for sale as cleaning sponges. However, there is a variation in the density of cellulose sponge

material as supplied commercially, both in the form in which it is shipped, and after being wet with water to expand the sponge to its maximum volume. Some sponge material is compressed for compactness in marketing, and in that form may have a density as great as 1 gram (dry basis) of sponge material per 3 to 4 cubic centimeters (cc.). When such sponges are wet and fully expanded, their volume increases to a stable volume of about 25 to 32 cc. per g. Other cellulose sponge material as shipped by the manufacturer have a density in the range of 13 to 16 cc/g., and on being wet with water expand to a maximum stable volume having a density as low as 1 g./25 cc. As will be discussed below, selecting a sponge material which provides the proper density under conditions of column use is important. If necessary, cellulose sponge materials can be prepared to a specified density; the porosity and thereby the density, being controllable by the relative proportion of water-soluble salt mixed with the viscose prior to the molding operation.

The process for the manufacture of cellulose sponges parallels the manufacture of rayon by the viscose process but does not require the more elaborate finishing steps. Instead of being pumped to the spinning machine, the viscose is mixed with a minor proportion of a fibrous reinforcing material consisting of cotton, hemp, ramie, flax, jute, coco, staple fiber, etc. (either in a natural or alkali swollen state), and a crystalline pore-forming material capable of inducing coagulation of the viscose on heating, usually a hydrated salt with a low melting point such as Glauber's salt, $\text{Na}_2\text{SO}_4 \cdot 10 \text{H}_2\text{O}$. The content of fibrous material is usually about 5 to 10% by weight basis on the cellulose content.

The mixture of viscose, fibrous reinforcing material and crystalline pore-forming material is then formed and the cellulose coagulated and regenerated. The finished sponge is washed free of salts and other contaminants. Modern processes incorporate colored pigments into the viscose/fiber/crystalline mixture, and generally also add softening agents, usually polyhydric alcohols, and bleaching agents such as hydrogen peroxide to the regenerating baths to improve the physical properties of the sponge and increase the saleability of the product. Sodium sulfate and carbon disulfide are recovered in the process. The fibrous content of the regenerated cellulose sponge is desirable but not essential, for the purposes of this invention. The physical stability of the cellulose sponge material for column use, particularly when the material is employed in subdivided condition, as preferred, is believed to be improved by the content of fibrous reinforcing material. Water soluble additives, such as softening agents, are removed by washing the sponge material with water prior to use for enzyme immobilization. Such washing can be carried out either before the sponge material is charged to the columns, or the washing can be carried out in the columns.

Detailed procedures for the manufacture of cellulose sponges are set in the patent literature, such as the patents of E. I. duPont de Nemours & Company, a major commercial producer of cellulose sponges. See, for example, U.S. Pat. Nos. 2,133,810 and 3,284,229. DuPont cellulose sponge as sold commercially for cleaning sponges, or the D-slab trim ends of such DuPont sponge material, has been found to have excellent physical properties for the purposes of the present invention. Another suitable sponge material is sold commercially by a division of General Mills, Tona-

wanda, N.Y., as the "O-Cel-O" cellulose sponge. For such sponge materials, hemp is a particularly suitable reinforcing fiber, and may be incorporated in an amount of about 6 to 8% by weight (dry basis) based on the weight of the regenerated cellulose. As indicated above, however, although the incorporation of a minor amount of a fibrous reinforcing material in the cellulose sponge material is desirable, the amount and kind of reinforcing material can be varied widely. Desirably, however, the reinforcing material is in the range of 5 to 10% by weight (dry basis) based on the regenerated cellulose, and is itself a cellulosic material, so that its fibrous form is not affected by enzymes other than cellulases.

While the cellulose sponge material can be employed in the form of large sheets, pads, or blocks, it is preferably used in a subdivided form, which facilitates the filling and uniform packing of a fixed-bed column. For example, D-slab cellulose sponge scrap can be shredded to produce a mixture of shreds having a size in the range of 0.263 to 0.525 Tyler Equivalent Screen Size. Oversized material is not objectionable, that is pieces of material having a size greater than 1.050 screen size (Tyler), but it will usually be desirable to separate the ultra-fine material, which may result from the shredding operation, namely, the particles having a screen size less than 0.185 (Tyler). Alternatively, or additionally, the cellulose scrap or other cellulose sponge starting material can be cut into small generally rectilinear chunks or blocks having approximate average dimensions of from $0.25 \times 0.25 \times 0.25$ inches up to at least $0.75 \times 0.75 \times 0.75$ inches. These sizes are not critical, but simply advantageous. Further, it has been found desirable to mix the sponge shreds with the sponge blocks, such as from 80 to 120 parts by weight of the sponge shreds per 100 parts of the small blocks.

The objective is to produce a column packing material having a density under conditions of use such that less than 1 gram of sponge material (dry basis) fills 18 cubic centimeters of bed volume or more. For example, the density of the sponge material can be such that 1 gram of the material (dry basis) fills from 20 to 70 cubic centimeters of bed volume. For certain processes, densities within the range of 1 gram of sponge material (dry basis) per at least 25 cc., such as 30 to 60 cc. of bed volume are preferred. The sponge material need not be compressed in the column bed, but can be allowed to occupy its natural stable volume after being thoroughly wet with water. Once expanded by water-wetting, the volume of the cellulose sponge material does not change appreciably. Further, the contacting operations are carried out with aqueous solutions, so that during contacting, the volume of the sponge material per unit weight remains substantially unchanged. Since the sponge material is modified, as will be subsequently described, to incorporate enzyme immobilizing groups therein, it will be understood that the density will be on the basis of the modified sponge material, such as sponge material in cationic form, but that all water soluble components of the sponge as supplied by the manufacturer, have been removed, and are not included in calculating the column density.

With cellulose sponge beds of the character described, there will be little or no pressure drop across the beds due to the flow resistance of the sponge material. Deep beds can therefore be employed. Even if a series of beds are used, the bed depths will be at least 2 feet in the direction of flow, and preferably at least 5

feet. The maximum bed depth or height, where the column extends vertically, can extend for as much as 40 to 50 feet, although typical columns may range from 5 to 20 feet in the direction of flow. The beds may be used in column apparatus having round, square, or rectilinear cross-sections. Usually the cross-sectional area will be substantially uniform throughout the length of the column. There is no limit on the lateral extent of the beds, although usually the diameter or lateral extents of the beds will range from about 5 to 15 feet. A typical column apparatus, therefore, might comprise a vertically-extending column, for either upflow or downflow contacting, having a height sufficient that a bed can be incorporated therein with some head room thereabove, having a height of from 8 to 20 feet, and a diameter of from 5 to 10 feet.

Columns prepared as described, will have a high degree of liquid porosity while avoiding channeling effects. More specifically, water can be flowed through beds of over 10 feet in height at flow rates in excess of 0.5 and preferably in excess of 1.0 gallon per minute per square foot of bed cross-sectional area. If the cross-section of the bed varies the smallest cross-section is used to determine flow rate. When an enzyme conversion is being conducted in the column, the rate of flow is only limited by the contact time necessary to achieve the desired conversion. There will be substantially no liquid pressure drop across the bed due to the flow resistance of the sponge material. For example, the solution to be contacted can be pumped into the top of a column having a head space above the fixed-bed of cellulose sponge material, at a rate maintaining a liquid level above the top of the bed. The outflow rate from the bottom of the bed can be controlled correspondingly, so that the liquid, in effect, percolates through the bed without any pump pressure being required. The difference in static head between the top and the bottom of the bed, will be due to the height of the liquid in the column, and not to the resistance of the cellulose bed. Alternatively, the solution to be contacted can be pumped into the bottom of the column flowing upwardly through the bed, the head space above the bed being maintained substantially full of the contacted liquid. The outflow will be controlled in relation to the in-flow, and the pump pressure required for the operation being only that necessary to move the liquid against its own static head, and not due to the resistance of the sponge material itself. As will be understood, the beds can also be used so that the flow direction is horizontal, although more commonly, the beds will be used in vertically-extending columns with a downwardly direction of flow.

In preparing the column material, it has been found undesirable to grind the cellulose sponge to a very fine state of subdivision, where the sponge structure is largely eliminated. As the extent of subdivision increases to that of a fine powder, the density of the ground sponge material increases until it is comparable to that of present commercial cationic cellulose powders, such as DEAE-cellulose prepared from natural cellulose. In one test, ground cellulose sponge was found to have a density after wetting and full expansion corresponding to 1 gram of sponge material per 18 cc. The material was still much more permeable to water than natural cellulose powder, but the porosity for use in deep bed columns was not as satisfactory as where the sponge material under conditions of use has a density (dry basis) of less than 20 cc. per gram of sponge

material. In general, column-fill densities under conditions of use of less than 18 cc. per gram (dry basis) of sponge material are undesirable.

For the purpose of the present invention, the cellulose sponge material is chemically modified to incorporate an effective amount of enzyme-immobilizing groups. In general, reagents bonding immobilizing groups to the regenerated cellulose chains through ether linkages can be employed. Cationic groups are preferred but enzyme-immobilizing anionic groups are also known, and could be used for some purposes. A wide variety of cationic etherifying agents can be employed, such as those described in U.S. Pat. Nos. 3,823,133 and 3,809,605. Further, where required to chemically stabilize the cellulose, cross-linking procedures can be used to permit a higher degree of substitution to be obtained, thereby increasing the capacity of the regenerated cellulose. Suitable procedures for this purpose are described in the literature, for example, in Guthrie et al, *Ind. & Engr. Chem.*, 52, 915-917 (1960). As is known in the art, tertiary amine and quaternary ammonium groups are particularly effective for immobilizing enzymes. The regenerated cellulose may therefore be modified to incorporate the di- and triethylaminoethyl groups, providing DEAE-regenerated cellulose and TEAE-regenerated cellulose, respectively. The regenerated cellulose can be reacted according to known procedures with epichlorohydrin and triethanolamine to form ECTOLA-regenerated cellulose. It will be understood that all of these derivatives are formed while the regenerated cellulose material remains in sponge form.

Where the cellulose sponge material is to be subdivided in preparing the column fill, this can be done first, the undersized fines removed by screening, and the grated subdivided sponge material washed with water to remove any soluble constituents. The subdivided sponge material can then be derivatized, using the procedures and reagents indicated above. A variety of such reagents and procedures are also illustrated in the following example. Since the chemistry involved in the reaction procedures are well-known, it is not believed it will be necessary to further discuss them herein.

Contrary to what might have been expected, it has not been found necessary to incorporate high levels of the enzyme-immobilizing groups in the cellulose sponge capacity in column use. In general, where cationic nitrogen groups are introduced as the immobilizing groups, the number of such groups on the basis of added nitrogen can correspond from 0.2 to 2.0 percent by weight nitrogen based on the dry weight of the cationic sponge material. Moreover, for many purposes, adequate capacity is obtained where the cationic nitrogen groups, such as tertiary amine groups or quaternary ammonium groups, are present in the cellulose sponge material in an amount corresponding to only 0.3 to 1.0 percent added nitrogen based on the dry weight of the cationic sponge material.

The column apparatus and column process of this invention for immobilized enzyme reactions is applicable to all enzymes which are immobilized by the functional groups and which do not attack or degrade the cellulose structure of the sponge. In general, enzymes as a class are adsorbed and immobilized by cationic nitrogen groups, such as tertiary amine or quaternary ammonium groups. Cellulase enzymes are not desirable, since their primary action is digest cellulose.

Cross-linking of the cellulose can increase its resistance to cellulolytic attack, and therefore enzymes containing trace amounts of cellulases may be employed. However, preferably, cellulose sponge material is not employed for reactions requiring the presence of immobilized cellulase, and, also, it is preferred that the enzymes applied to the column be substantially free of cellulase.

For enzymatic conversion of glucose, and mixtures of glucose with oligo-saccharides derived from natural starch, such as corn starch, the enzymes will be those which have heretofore been employed for such purposes, particularly alpha-amylase, glucoamylase, or mixtures of these enzymes. For conversions of glucose syrups to mixtures of glucose and fructose, glucose isomerase can be employed. For this purpose, a high D.E. feed stock is preferred, such as a starch derived glucose solution having a D.E. of 93 to 97, indicating that it is composed of large amounts of dextrose. For producing intermediate D.E. syrups from corn starch or other starch materials, the feed stock can be a starch derived mixture of glucose and oligosaccharides having a D.E. in the range of 15 to 45. For example, a mixture of alpha-amylase and glucoamylase enzymes can be applied to the cellulose sponge material, and employed with a feed stock of the character described to produce products having D.E.'s in the range of 42 to 70. These are important commercial products which are now produced entirely by batch-type enzyme conversions involving one-time use of the enzymes. With the process of the present invention, the useful life of the enzymes may be greatly extended, being usable for converting at least 10 to 20 times the amount of substrate to the intermediate D.E. corn syrup of commerce.

Other enzymes which can be used in the process and apparatus of this invention include trypsin, papain, chymotrypsin, lactase, urease, acylase, catalase, penicillin-amidase, beta-amylase, lipase, glucose oxidase, protease, gelatinase, hemicellulase, pectinase, lysozyme, pepsin, etc.

The following Experimental Examples are illustrative and provide further information about the enzyme-immobilizing cellulose sponge material prepared for use in the apparatus and process of this invention. The starting material was cellulose sponge or sponge scrap (D-slab) produced by E. I. duPont, Wilmington, DEL. The fiber content of the cellulose sponge material was not specifically determined, but is believed to comprise about 6 to 8 percent by weight of hemp fibers based on the dry weight of the regenerated cellulose. This material was found to be identical in properties to duPont cellulose sponges purchased in retail stores. In general, the duPont cellulose sponge as received has a density of about 1 gram per 13-16 cc. of material. On thorough wetting with water, the cellulose sponge, in the form of sheets or blocks expands to a stable density averaging 1 gram per 28 cc. When the cellulose sponge material is subdivided, such as by shredding, the density can be further decreased. Such shredded material on being thoroughly wet with water, expanded to a stable density averaging about 51 cc. per gram (dry basis). It will be understood that the density after expansion by water wetting corresponds with the density expected of the material under ordinary conditions of use. The density, however, is related to the dry weight of the cellulose sponge material, which includes the regenerated cellulose, the fibrous reinforcing material, and the added enzyme-immobilizing groups, but excludes water-solu-

ble substances that are removed from the sponge cellulose material before it is used in columns for immobilized enzyme reactions.

EXPERIMENTAL EXAMPLES

EXAMPLE 1

Derivatives of regenerated cellulose sponge (DuPont Cellulose Sponge) were prepared by reaction with cationizing reagents according to the following general procedure: The sponge was shredded to produce thin pieces ("shreds") of an average size of around 0.25 to 0.75 inches in length. The sponge shreds were washed in hot water, dried, and resuspended in water containing an amount of salt from a strong acid and a strong base representing supersaturation and a strong base consisting of an alkali metal hydroxide was added. The cationizing reagent was then added and reaction continued with stirring at an elevated temperature for about 2 hours. After this, the cationized cellulose sponge was recovered by neutralizing to about pH 4; with mineral acid, filtered, washed with water and dried to equilibrium moisture in an air oven.

Applying this procedure to a specific example 20 grams (0.125 mole) of washed, shredded cellulose sponge was suspended in 970 grams of water containing 580 grams of sodium sulfate. An amount of 12 grams of 50% sodium hydroxide solution (0.15 mole) was added followed by 10.3 grams (0.06 mole) of diethylamino-ethylchloride hydrochloride (DEC). The mixture was stirred at 45° C for 130 to 190 minutes. Thereafter, the mixture was adjusted to pH 4 with hydrochloric acid, held for 1 hour, filtered, and washed by resuspending in 6 successive 2-liter portions of water to remove the salts of reaction and the sodium sulfate present during the reaction. The final product was dried at 80° C. and allowed to equilibrate at room temperature to obtain diethylaminoethyl (DEAE) sponge cellulose, usable as a column material for immobilized enzyme reactions. Nitrogen was determined on the product to determine the amount added as shown in Table 1.

Also shown in Table 1 are the results of other experiments wherein the mole ratio of the reagent to the regenerated cellulose of the sponge was varied as well as the amount of alkali catalyst added, on a dry cellulose basis (d.b. cellulose).

Table 1

Run	Mole Ratio DEC/ Cellulose	Reaction in Minutes	% NaOH on d.b. Cellulose	% Nitrogen Added
A	1:2	120	30	1.36
B	1:2	130	60	1.20
C	1:2	130	30	1.61
D	1:5	190	30	0.85
E	1:1	130	60	2.04

The nitrogen added is a measure of the DEAE groups bonded to the sponge cellulose, which are available for enzyme immobilization.

EXAMPLE 2

Using the procedure described in Example 1, and following the details of Run A, a derivative of a cellulose sponge column material was prepared using as a reagent, 4-chloro-2-butenyl trimethylammonium chloride, except that the produce was adjusted to pH 1, held for 1 hour, and washed as before. After recovery in the manner previously described, the amount of nitrogen

added was found to be 0.66%. This is a measure of the cationic groups which are trimethyl ammonium butenyl-2-cellulose ether groups in chloride form. The resulting cationic sponge can be used as a column material for immobilized enzyme reactions.

EXAMPLE 3

Using the procedure of Example 1, and following the details of Run A, a derivative of cellulose sponge was prepared using as a reagent 2,3-epoxypropyl trimethylammonium bromide. After recovery in the manner previously described, the amount of nitrogen added was found to be 0.24%. This is a measure of the cationic groups which are 2-hydroxy-propyl trimethyl ammonium cellulose ether groups in bromide form. The resulting cationic sponge can be used as a column material for immobilized enzyme reactions.

EXAMPLE 4

A variant of the reagent described in Example 3 was reacted in the same manner, using 3-chloro-2-hydroxypropyl trimethylammonium chloride. The recovered product was found to contain 0.35% added nitrogen. This is a measure of the cationic groups which are 2-hydroxypropyl-trimethyl ammonium cellulose ether groups in chloride form. The resulting cationic sponge can be used as a column material for immobilized enzyme reactions.

EXAMPLE 5

Using the procedure described in Example 1, and following the details of Run A, a derivative of the regenerated cellulose sponge was prepared using as a reagent, 4-bromobutyl trimethylammonium bromide. After recovery in the manner previously described, the amount of nitrogen added was found to be 0.45%. This is a measure of the cationic groups which are butyl trimethyl ammonium cellulose ether groups in bromide form. The resulting cationic sponge can be used as a column material for immobilized enzyme reactions.

EXAMPLE 6

Cellulose sponge shreds cationized according to Run A in Example 1 with DEC to contain 1.26% added nitrogen, as DEAE cellulose sponge, was treated with an enzyme solution to cause the enzyme to be adsorbed onto the cationic sponge.

An amount of 10 grams d.b. of cationic sponge was mixed with 10 grams of glucoamylase (Diazyme L-100, Miles Laboratories, Elkhart, Ind.) in ion-exchanged water at room temperature for four hours, and filtered, yielding a mixture containing 770 IGU of enzyme activity. The sponge was separated from the liquid by filtering and analysis showed an activity of 35.4 IGU/gram. IGU = an immobilized glucoamylase unit and is the amount of enzyme which will catalyze, under the assay conditions, the production of one gram of glucose in 1 hour. Assay conditions constitute use of a solution of 30% aqueous maltodextrin solids maintained at pH 4.2 and 60° C. for 1 hour. Enzyme activity is measured by calculating the increase in glucose concentration.

Six grams of sponge containing glucoamylase was wetted with water, placed in a 1-inch diameter column, and 30% solids concentration syrup at 35% dextrose equivalent (D.E.; Standard Analytical Methods, Corn Refiners Association, Method E-26) flowed through the column at a rate of 4 ml./minute at pH 4.2 and 138°

F. The D.E. of the effluent syrup was maintained at 45%. After 7 days an amount of 14,870 grams of syrup (dry solids basis) had been passed through the column.

EXAMPLE 7

Cellulose sponge shreds cationized according to Run A in Example 1 with DEC to contain 0.33% added nitrogen, as DEAE cellulose sponge, was treated with Diazyme L-100 enzyme solution as in Example 6 and found on analysis to contain an activity of 24.3 IGU per gram.

Eight grams of the sponge containing enzyme was placed in a 1-inch diameter column and room temperature. A 30% solids ion exchange syrup at 36% D.E. was flowed into the column at pH 4.5. The column was gradually heated to 140° F. and the effluent syrup flowing at a rate of 6 ml./minute yielded 62% D.E.

As flow was continued, the percent D.E. gradually dropped to 51% D.E. after 7200 grams by dry substance produce had been collected. The flow was then reduced to 3 ml./minute and the percent D.E. recovered to 60.

The column was briefly exposed to 152° F. which inactivated the enzyme present. An additional amount of a glucoamylase (G-zyme; Enzyme Development Corp., New York, N.Y.) was added to the column by adding it to the feed syrup and flowing it onto the column. The column was adjusted to 140° F. and 36% D.E. ion exchanged syrup flowed through the column at 3 ml./minute until a quantity of 60% D.E. syrup equivalent to 42,000 grams dry substance syrup had been produced over a 24-day period.

EXAMPLE 8

This example shows the capability of a column of cationized cellulose sponge to contain a mixture of enzymes, and vary the sugar balance of the resulting syrup product. Ten grams of cellulose sponge shreds cationized with DEC according to Run A in Example 1 to contain 0.85% nitrogen, as DEAE cellulose sponge, was treated with a blend of 1.16 grams of a fungal α -amylase (Asperzyme; Enzyme Development Corp.) and 0.60 gram of G-zyme (See Example 7). The enzymes were dissolved in 42% D.E. syrup at 30% solids and flowed through the column several times before beginning a flow of syrup at pH 5.5 at a rate of 3 ml./minute. The effluent syrup was 64.1% D.E., and showed on analysis, 40.3% dextrose and 30.6% maltose.

After 7 days, a quantity of 10,318 grams dry substance syrup had been produced with the D.E. gradually falling to 48% at the same flow rate.

EXAMPLE 9

This example shows a different method of mixing the enzyme with the cationized sponge.

Six grams of DEAE sponge material (identical to that in Example 8) were treated with the same quantity of Asperzyme and G-zyme (see Examples 7 and 8) and allowed to mix over night in 42% D.E. syrup at 35% solids concentration and pH 5.5 at 130° F. The column was then filled with ion exchanged syrup at 44% D.E. and at a flow rate of 4 ml./minute the effluent syrup was 66% D.E. Analysis showed a sugar balance of 47.6% dextrose and 22.3% maltose.

After observing that the pH of the effluent syrup began to drift downward to 4.7, the feed syrup was switched to carbon refined syrup. Hereafter, the pH of

the effluent syrup was the same as the influent syrup. At a flow rate of 3.4 ml./minute at pH 5.5, the syrup produced was 62.2% D.E., and the sugar balance was found to be 42.6% dextrose and 22.5% maltose.

EXAMPLE 10

This example shows the effect of varying the influent D.E. of a syrup and its conversion by a fungal alpha amylase. Eight grams of cationized regenerated cellulose sponge shreds (DEAE) containing 0.48% added nitrogen was treated with 2 grams of Asperzyme (see Example 8), a fungal alpha amylase dissolved in water. The mixture was added to a column and 35% D.E. carbon refined syrup containing 0.01% sodium bisulfite at pH 5.5 and 35% solids was flowed onto the column at room temperature. The column temperature was raised to 130° F.

The effluent syrup collected at a flow rate of 3 ml./minute was 50.7% D.E. and had a sugar balance of 20.2% dextrose and 37.7 maltose.

After a period of time, the influent syrup was changed to 19.4% D.E., all other parameters being the same. The effluent syrup had a sugar balance of 4.8% dextrose and 40% maltose.

EXAMPLE 11

This example is similar to Example 8 except that the cationized (DEAE) sponge material contained 0.38% added nitrogen. The same ratio of Asperzyme and G-zyme (see Examples 7 and 8) was added in the manner of Example 10. Influent syrup at 43.5% D.E., 35% solids concentration, pH 5.5, and containing 0.01% sodium bisulfite was flowed through the column at 130° F. at a rate of 3 ml./minute.

Effluent syrup contained 63.6% D.E. and had a sugar balance of 35.5% dextrose and 31.5% maltose.

EXAMPLE 12

Regenerated cellulose sponge cationized with DEC (see Example 1) to contain 0.85% added nitrogen was treated with an enzyme solution to cause the enzyme to be absorbed onto the cationic sponge.

An amount of 1 gram dry basis (d.b.) of cationic sponge was mixed with 1 gram of a purified invertase (Convertit; Wallerstein Company, Morton Grove, Ill.), in ion exchanged water at room temperature for 4 hours. The sponge was separated from the liquid by filtration and washed on the filter with ion exchanged water to remove unbound enzyme.

One-half gram d.b. of sponge containing invertase was mixed with 50 ml. of 10% sucrose solution at pH 5.0 and 55° C. for 30 minutes yielding 86% invert sugar, demonstrating the presence of invertase activity in the sponge.

EXAMPLE 13

One gram of DEAE cellulose sponge identical to that in Example 12 was mixed with 1 gram Glucose Oxidase (Worthington Biochemical Corporation, Freehold, N.J.) in ion exchanged water to produce a sponge containing glucose oxidase according to the procedure described in Example 12.

One-half gram d.b. of sponge containing glucose oxidase was mixed with 50 ml. of 1% glucose solution at pH 7.0 and 37° C. After 3 hours, 95% of the glucose had been oxidized as indicated by loss of reducing power of the solution. This result demonstrates the presence of glucose oxidase in the sponge.

EXAMPLE 14

One gram of DEAE-cellulose sponge identical to that in Example 12 was mixed with 1 gram Urease (Jack Bean, double strength, J. T. Bake Chemical Company, Phillipsburg, N.J.) in ion exchanged water to produce a sponge containing Urease according to the procedure described in Example 12.

One-half gram d.b. of sponge containing Urease was mixed with 0.1 gram urea in 250 ml ion exchanged water at pH 6.5 in a stoppered Kjeldahl flask at ambient temperature for 1 hour. A nitrogen determination (Urea and Ammonical Nitrogen; Methods of Analysis, 11th ed., AOAC, Method 7.027) showed complete decomposition of the urea. This result demonstrates the presence of urease activity in the sponge.

EXAMPLE 15

One gram of DEAE sponge identical to that in Example 12 was mixed with a cell-free extract of glucose isomerase (obtained from *Arthrobacter* cells, NRRL 3728) to produce a sponge containing glucose isomerase, according to the procedure described in Example 12.

A glucose isomerase assay of the sponge containing glucose isomerase showed an activity of 2.3×10^{-3} units/gram d.b. sponge, where one unit is defined as the amount of enzyme which catalyzes the formation of 1 gram of fructose per minute under the following conditions: 60° C., 1.67 molar glucose, pH 7.75, and 0.4 molar magnesium. This result demonstrates that the sponge contains immobilized glucose isomerase. This sponge can be used for column conversion of glucose to fructose.

EXAMPLE 16

One gram of DEAE sponge identical to that in Example 12 was mixed with 1 gram Catalase (Miles Laboratories, Elkhart, Ind.) in ion exchanged water to produce a sponge containing catalase, according to the procedure described in Example 12.

When one-half gram d.b. of sponge containing catalase was mixed with 50 ml. of 1% hydrogen peroxide, the peroxide was immediately decomposed. This result demonstrates the presence of catalase activity in the sponge. The sponge can be used in a column for continuous decomposition of H_2O_2 in an aqueous medium.

EXAMPLE 17

One gram of DEAE sponge identical to that in Example 12 was mixed with 0.5 gram Trypsin (Miles Laboratories, Elkhart, Ind.) in ion exchanged water to produce a sponge containing trypsin according to the procedure described in Example 12.

One-half gram d.b. of sponge containing trypsin was mixed with 50 ml. of 2% casein solution at pH 7.0 and 37° C. After 30 minutes, the sponge was removed from the solution and trichloroacetic acid was added to precipitate the protein. A Kjeldahl nitrogen determination after filtration to remove the precipitate showed that enzyme activity was retained by the sponge. The sponge can be used as column material for continuous treatment of soluble protein, the protein being proteolyzed by the immobilized trypsin.

EXAMPLE 18

This example describes the preparation of a cationized cross-linked cellulose sponge.

A DEAE cellulose sponge derivative was prepared as in Example 1 using DEC, and following the details of Run A. Then the reaction slurry was adjusted to pH 1 and 20% by weight of formaldehyde (based on d.b. cellulose of the sponge material and the formaldehyde) was added and the mixture stirred for 1 hour. Thereafter, the product was adjusted to pH 3.9 and washed as previously described. It contained 1.3% added nitrogen, as DEAE groups.

An amount of 8 grams of the resulting cross-linked sponge material was contacted with 1.16 grams of Asperzyme and 0.6 gram of G-zyme (see Examples 7 and 8) dissolved in water. The mixture was added to a column and 37% D.E. carbon refined syrup containing 0.01% sodium bisulfite at pH 5.5 and 35% solids was flowed into the column at room temperature. The temperature was raised to 130° F. and the flow rate adjusted to about 3.0 ml./minute.

The effluent syrup was 66.6% D.E. and had a sugar balance of 47.6% dextrose and 29.2% maltose.

EXAMPLE 19

A cationized cellulose sponge derivative was prepared as in Example 18 using DEC, except that 100% by weight of formaldehyde was reacted for 1 hour. The product was adjusted to pH 4.0 and washed until no odor of formaldehyde was detectable. The product contained 1.16% added nitrogen, and was usable as a cationic column material for immobilized enzyme reactions.

For example, an amount of 40 grams of the resulting cross-linked cationic sponge material was contacted with 5.8 grams of Asperzyme and 3.0 grams of G-zyme (see Examples 7 and 8) dissolved in water. The mixture was added to a column and 37% D.E. carbon refined syrup containing 0.01% sodium bisulfite at pH 5.4 and 35% solids was flowed into the column at room temperature. The temperature was raised to 130° F. and the flow rate adjusted to 11 ml./minute. The effluent syrup had a D.E. of 67%.

EXAMPLE 20

The cationic cellulose sponge material may be used in columns in several forms, in the foregoing examples the sponge was in the form of small pieces which resulted from a shredding operation. An alternative procedure involves the use of plugs cut from the sponge mass, which are slightly smaller than the internal column diameter. The plugs are allowed to swell within the column.

By way of specific example, cylindrical plugs (height 1 foot, diameter 15/16 inch) of regenerated cellulose sponge were derivatized as described in Example 1 and dried. Ten of these plugs were inserted into a column (1 x 12 inch) and water down-flowed through the column until the sponge had been thoroughly wet and expanded. It then formed a tight seal against the walls and the height was 11 inches.

Five grams of Diazyme L-100 (see Example 6) in 50 cc. of water was added to the column. When all of the enzyme solution had been added, flow was stopped and the system maintained at 30° C. for 2 hours. Then corn syrup was added to the column, as described in Example 6. Similar results were obtained.

During the course of column operation, the flow rate was varied. D.E. of the effluent varied from 40 to 95, depending on the flow rate.

The density of the enzyme containing sponge in the column may be varied considerably by applying pressure to the top of the sponge layer with a screen, which can either prevent upward expansion when plugs are used; or can substantially reduce the apparent volume when shredded sponge is used, the screen being moved downwardly to compress the shredded material. Using the latter technique, it is feasible to densify the bed, as required, to reduce channeling. Such bed compaction does not appear to cause back-pressure or flow difficulties.

OTHER EXAMPLES

In addition to the foregoing, other procedures for preparing enzyme-immobilizing celluloses can be used. These include the following:

EXAMPLE 21

A carboxymethylchloro-S-triazinyl derivative of regenerated cellulose sponge can be prepared according to Example 1 of U.S. Pat. No. 3,278,392. It will absorb urease from an aqueous solution for immobilization thereof. When the enzyme containing sponge is put in a column and a urea solution is down-flowed the effluent will contain substantially no urea.

EXAMPLE 22

Similar enzyme absorption and immobilization results can be obtained when regenerated cellulose sponge is made cationic with a diazotized anthranilic ester (U.S. Pat. No. 3,647,630), or a p-diazophenoxyhydroxy propyl ether (U.S. Pat. No. 3,702,804), or a bromoacetyl derivative (U.S. Pat. No. 3,278,392), or by cyanogen bromide activation [*Biotechnology and Bioengineering*, Vol. XIV, p. 1039 (1972)]. The foregoing citations illustrate applicable reaction procedures.

EXAMPLE 23

A carbonate of regenerated cellulose sponge can be prepared from ethyl chloroformate, as described in Example 1 of U.S. Pat. No. 3,810,821. The anionic sponge will absorb anyloglucosidase from a water solution which cannot be removed by washing. The enzyme containing sponge, when put in a down-flow column can be used to give D.E.'s ranging from 60 to over 90 when the input corn syrup substrate has a D.E. of 34-38.

COMMERCIAL EXAMPLES

The following examples are intended to illustrate preferred commercial practice, as presently envisioned.

EXAMPLE 24

A column apparatus is employed of the kind conventionally used with ion exchange resins. Such an apparatus is illustrated diagrammatically in FIG. 1. The vertically-extending column 10 provides internally a space for receiving the fixed-bed of cellulose sponge packing P. Above the material there is provided an open head space 11. The column 10 is provided with a heating jacket, as indicated in FIG. 1, through which hot water can be circulated for maintaining a selected temperature during the enzyme reaction. The top or cover of the column 12, which may be removable, is provided with a series of connections, the larger one on the left, as indicated, being used for loading the cellulose

sponge material into the bed, the center one for supplying the solution to be converted, such as starch hydrolysate, and the one on the right to be used as an air vent. All of these inlets may be provided with suitable valves, as shown. The bottom of the bed is supported on a screen 13, which retains the cellulose material of the bed, while permitting liquid flow therethrough into the tapered bottom portion 14 which converges to outlet 15. The outflow through 15 is controlled by means of a valve, as indicated. Immediately above screen 13 on one side of the column, there is provided a clean-out opening 16 which communicates with a removal pipe 17 through a valve, as shown. With this arrangement when the cellulose sponge material is in sub-divided condition, as preferred, it can be pumped into the column in the form of a water slurry, the bed loading inlet at the top being used. After repeated use of the cellulose sponge bed, it may be desirable to remove the bed material, and replace it with a new cellulose sponge bed. This can be done by introducing water through the bed loading and/or syrup inlets at the top, the valve being opened on outlet pipe 17, and the bed material being pumped out as a slurry through pipe 17.

One advantageous form of the column material is illustrated in FIG. 2. It consists of a mixture of sponge shreds and small blocks or chunks, which are roughly cubical or rectilinear. The shreds preferably have a size such that they pass through a number 0.525 screen while being retained on a number 0.263 screen (Tyler). The "cubed" material can have an average size of $\frac{1}{4}$ to $\frac{1}{2}$ inches in each dimension. The blocks or chunks may be somewhat irregular in shape, since the sponge shreds fill the spaces between the larger pieces. This is indicated in FIG. 2 where the larger pieces are designated by the letter C, and the intervening shred by the letter S. Desirable proportions range from 50 to 60 parts by weight of the shredded material to 30 to 40 parts of the "cubed" material.

In the use of the apparatus of FIG. 1, such as for conversion of a syrup with an appropriate enzyme, the syrup is pumped into the head space 11 at the top of the column, and a level is maintained therein above the top of the bed of cellulose sponge material, as indicated at 18. The valve on outlet line 15 is opened to permit outflow at substantially the same rate as the inflow, the column 10 being maintained full of the aqueous syrup to be converted. In this operation, the syrup flows downwardly through the bed at a rate, which is sufficient to achieve the level of conversion desired. The height and diameter of the bed may be varied, as required, for the particular process. In the processes described in the following examples, the bed of cellulose sponge material, for example, may have a height of about 12 feet and a diameter of about 8 feet.

EXAMPLE 25

Batches of 26 pounds of regenerated cellulose sponge (duPont) in the form of approximate cubes varying in size from 0.75 inches are suspended in 500 pounds of water at 120° F. in a 175-gallon ribbon blender. After agitating for 20 minutes, the water is drained from the system. The washing process is repeated three times without removing the sponge from the blender to provide a sponge free of impurities.

To the washed wet sponge is then added 440 gallons of water, 125 pounds of anhydrous sodium sulfate, 12.5 pounds of 47% sodium hydroxide, and 3.9 pounds of

DEC.HCl. Additions are made while agitating and in the order given. The stirred mixture is agitated for 1.5 hours while maintaining the temperature at 50° C. The pH of the mixture is then adjusted to pH 4 with hydrochloric acid and agitated for one-half hour. The mixture is drained and then washed six times by adding water, agitating for 20 minutes, draining, and repeating the process.

The wet sponge is then dried at 38° C. This provides the derivatized cellulose sponge in cube form. The nitrogen content of the product is 0.60%.

The shredded form of the derivatized sponge is prepared by grinding the cube form above in a Fitz mill using a 2-mesh screen.

One hundred thirty-six pounds of homogeneous mixture of 65 parts cubes and 35 parts of the shreds is introduced into a 3 x 12 column (FIG. 1). Water is then introduced to swell the sponge and allow an excess of 1 foot of water above the bed. The cellulose bed is 6 feet in depth.

A solution of 20.5 pounds of Fungamyl (Novo) in 850 pounds water is then circulated through the column for 2 hours. Thereafter, a solution of 10.24 pounds dry basis of glucoamylase (G-zyme, Enzyme Development) is added to the water and is circulated through the column for 2 hours.

Filtered acid hydrolyzed corn starch hydrolyzate (syrup) with an average D.E. of 36-38 is then added to the top of the column. The syrup temperature is 130° F. and the pH adjusted to 5.5.

When column operation has stabilized, it produces 65 D.E. syrup containing 36% dextrose and 35% maltose at a flow rate of 4.5 gallons per minute.

EXAMPLE 26

The procedure of Example 25 is followed using only G-zyme (25 pounds) for attachment to the regenerated cellulose sponge. Other conditions are identical except that the pH is maintained at 4.0-4.5 and the temperature at 140° F. The syrup produced has a D.E. of 95.

EXAMPLE 27

The procedure of Example 26 is followed using cell-free enzyme from streptomyces ATCC 21175 prepared as described in Example 1 of U.S. Pat. No. 3,788,945. When used in a column operation with a 6 bed depth and a substrate syrup of 95 D.E. at pH 8, a product containing 42% fructose at commercially acceptable flow rates can be produced.

I claim:

1. Column apparatus for immobilized enzyme reactions, including column means providing a flow-through reaction chamber between the liquid inlet and outlet ends thereof, said reaction chamber extending for several feet in the direction of liquid flow, wherein the improvement comprises: a bed of regenerated cellulose sponge material in said reaction chamber for liquid passage therethrough, the regenerated cellulose of said material having been chemically modified to incorporate an effective amount of enzyme-immobilizing groups, said bed of sponge material being characterized by having (a) a flow direction length of at least 2 feet, (b) a density under conditions of use of less than 1 gram of said sponge material (dry basis) per each 18 cubic centimeters of bed volume, and (c) a water flow porosity such that over 0.5 gallons of water per minute per square foot of bed cross-section will flow there-

through without substantial liquid pressure drop due to flow resistance of the sponge bed.

2. The improved column apparatus of claim 1 wherein said reaction chamber and said bed of sponge material have a flow direction length of at least 5 feet.

3. The improved column apparatus of claim 1 wherein said sponge bed under conditions of use has a density of less than 1 gram of said sponge material (dry basis) per each 20 to 70 cubic centimeters of bed volume.

4. The improved column apparatus of claim 1 wherein said sponge bed has a water flow porosity such that more than 1.0 gallon of water per minute per square foot of bed cross-section will flow therethrough without substantial liquid pressure drop across the length of the bed due to the flow resistance of the sponge bed.

5. Column apparatus for immobilized enzyme reactions, including vertically-extending column means providing a flow-through reaction chamber between the vertically-spaced liquid inlet and outlets thereof, said reaction chamber having a vertical extent in the direction of liquid flow of more than 5 feet, wherein the improvement comprises: a bed of regenerated cellulose sponge material disposed in said reaction chamber for liquid passage therethrough the regenerated cellulose of said material having been chemically modified to incorporate an effective amount of enzyme-immobilizing groups, said sponge bed being characterized by (a) a vertical depth between said inlet and outlets of at least 5 feet, (b) a density under conditions of use of less than 1 gram of said sponge material (dry basis) per each 20 cubic centimeters of bed volume, and (c) a liquid flow porosity such that more than 1.0 gallons of water per minute per square cubic foot of bed cross-section flow therethrough without significant liquid pressure drop across the bed in the direction of flow due to the flow resistance of the sponge bed.

6. The improved column apparatus of claim 5 in which the enzyme-immobilizing groups chemically bonded to said regenerated cellulose are cationic nitrogen groups, the number of said groups on the basis of added nitrogen corresponding to 0.2 to 2.0 percent by weight nitrogen based on the dry weight of said cationic sponge material.

7. Column apparatus for immobilized enzyme reactions, including column means providing a flow-through reaction chamber between the liquid inlet and outlet ends thereof, said reaction chamber extending for several feet in the in direction of liquid flow, wherein the improvement comprises: a bed of regenerated cellulose sponge material in said reaction chamber for liquid passage therethrough, the regenerated cellulose of said material containing chemically bonded cationic nitrogen groups effective for enzyme-immobilization, said groups being selected from tertiary amine groups and quaternary ammonium groups, said groups being present in an amount corresponding to 0.3 to 1.0 percent added nitrogen based on the dry weight of the cationic sponge material, said sponge bed having a flow direction length of at least 5 feet, and a density under conditions of use such that 1 gram of said cationic sponge material (dry basis) fills from 20 to 70 cubic centimeters of bed volume.

8. Column apparatus for immobilized enzyme reactions, including vertically-extending column means providing a flow-through reaction chamber between vertically-spaced liquid inlet and outlet means, said

reaction chamber extending for more than 5 feet in the vertical direction of liquid flow, wherein the improvement comprises: a liquid-permeable bed of cationic regenerated cellulose sponge material in said reaction chamber, the cationic groups of said sponge material being of the kind and being present in an amount throughout said bed effective for immobilizing enzymes in said bed, said sponge bed having a vertical depth in the direction of flow of over 5 feet, and a liquid flow porosity such that more than 0.5 gallons of water per minute per square foot of bed cross-section will flow therethrough without substantial liquid pressure drop across said bed in the direction of flow due to the flow resistance of the sponge bed.

9. The improved column apparatus of claim 8 in which said sponge bed under conditions of use has a density such that 1 gram of said cationic sponge material (dry basis) fills from 30 to 60 cubic centimeters of bed volume.

10. The improved column apparatus of claim 7 in which said sponge bed has a liquid flow porosity such that more than 1.0 gallons of water per minute per square foot of bed cross-section will flow therethrough without substantial liquid pressure drop across the bed in the direction of flow due to the flow resistance of the bed.

11. The improved column apparatus of claim 7 in which said cationic nitrogen groups are selected from the class consisting of tertiary amine groups and quaternary ammonium groups, and the sponge material of said bed contains an amount of said groups corresponding to 0.3 to 1.0 percent by weight of added nitrogen based on the dry weight of the cationic sponge material.

12. The process for reacting a water-soluble enzyme-convertible substance with an enzyme other than cellulase, in which a water solution of the substance is passed through a porous bed of material containing immobilized therein at least one enzyme for the reaction, wherein the improvement comprises employing as said bed a regenerated cellulose sponge material which has been chemically modified to incorporate an effective amount of enzyme-immobilizing groups, said bed of sponge material having a flow direction length of at least 2 feet and a density under conditions of use of less than 1 gram of said sponge material (dry basis) per each 18 cubic centimeters of bed volume, and passing said water solution through said bed at a rate of over 0.5 gallon of solution per minute per square foot of bed cross-section.

13. The process of claim 12 in which said bed of sponge material has a flow direction length of at least 5 feet, and a density under conditions of use of less than 1 gram of said sponge material (dry basis) per each 25 cubic centimeters of bed volume.

14. The process for reacting a water-soluble enzyme-convertible substance with an enzyme other than cellulase, in which a water solution of the substance is passed through a porous bed of material containing immobilized therein at least one enzyme for the reaction, wherein the improvement comprises employing as said bed a regenerated cellulose sponge material which has been chemically modified to incorporate an effective amount of enzyme-immobilizing groups, said bed of sponge material having a flow direction length of at least 5 feet and a density under conditions of use such that 1 gram of said sponge material (dry basis) fills from 20 to 70 cubic centimeters of bed volume, and

passing said solution through said bed at a rate of more than 1.0 gallon of solution per minute per cubic foot of bed volume.

15. The process of claim 14 in which said sponge bed under conditions of use has a density such that 1 gram of said sponge material (dry basis) fills from 30 to 60 cubic centimeters of bed volume.

16. The process of claim 14 in which the enzyme-immobilizing groups chemically bonded to said regenerated cellulose are cationic nitrogen groups, the number of said groups on the basis of added nitrogen corresponding to 0.2 to 2.0 percent by weight nitrogen based on the dry weight of the cationic sponge material.

17. The process for reacting a water-soluble enzyme-convertible substance with an enzyme other than cellulase, in which a water solution of the substance is passed through a porous bed of material containing immobilized therein at least one enzyme for the reaction, wherein the improvement comprises employing as said bed a regenerated cellulose sponge material which has been chemically modified to incorporate an effective amount of enzyme-immobilizing groups, the regenerated cellulose of said material containing chemically bonded cationic nitrogen groups effective for enzyme-immobilization, said groups being selected from tertiary amine groups and quaternary ammonium groups, said groups being present in an amount corresponding to 0.3 to 1.0 percent added nitrogen based on the dry weight of the cationic sponge material, said sponge bed having a flow direction length of at least 5 feet and a density under conditions of use such that 1 gram of said cationic sponge material (dry basis) fills from 20 to 70 cubic centimeters of bed volume, and passing said solution through said bed at a rate of over 1.0 gallon of solution per minute per square foot of bed cross-section.

18. The process for enzymatically converting a sugar substrate selected from the group consisting of glucose, oligosaccharides derived from starch, and mixtures of glucose and said oligosaccharides, in which a water solution of said sugar substrate is passed through a porous bed of material containing immobilized therein at least one enzyme for said conversion selected from the group consisting of alphaamylase, glucoamylase, and glucose isomerase, wherein the improvement comprises employing in said bed a regenerated cellulose sponge material which has been chemically modified to incorporate an effective amount of enzyme-immobilizing groups therein, said bed of sponge material having a flow direction length of at least 5 feet and a density under conditions of use of less than 1 gram of said sponge material (dry basis) per each 20 cubic centimeters of bed volume, and passing said sugar solution through said bed at a rate of over 0.5 gallon of solution per minute per square foot of bed cross-section.

19. The process of claim 18 in which said bed of sponge material has a flow direction length of at least 5 feet and a density under conditions of use such that 1 gram of said sponge material (dry basis) fills from 30 to 60 cubic centimeters of bed volume, and in which said solution is passed through said bed at a rate of over 1.0 gallon of solution per minute per square foot of bed cross-section.

20. The process of claim 18 in which the enzyme-immobilizing groups chemically bonded to said regenerated cellulose are cationic nitrogen groups, the number of said groups on the basis of added nitrogen corresponding to 0.2 and 2.0 percent by weight nitrogen

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based on the dry weight of said cationic sponge material.

21. The process of claim 18 in which said enzyme-immobilizing groups chemically bonded to said regenerated cellulose are cationic nitrogen groups selected from tertiary amine groups and quaternary ammonium groups, said groups being present in an amount corre-

sponding to 0.3 to 1.0 percent added nitrogen based on the dry weight of the cationic sponge material, and said sponge bed having a density such that 1 gram of said cationic sponge material (dry basis) fills from 30 to 60 cubic centimeters of bed volume.

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Industria y Comercio
SUPERINTENDENCIA

**REQUISITOS MINIMOS PARA ADMISION A TRAMITE
PCT**

PATENTE DE INVENCION

☒MODELO DE UTILIDAD ☐

- ◆ Indicación de que se solicita la concesión de una patente ☒
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud ☒
- ◆ Descripción de la invención ☒
- ◆ Dibujos de ser estos pertinentes ☒
- ◆ Comprobante de pago de las tasas establecidas ☒

COMPLETA ☐INCOMPLETA ☐DISEÑO INDUSTRIAL ☐

- ◆ Indicación de que se solicita el registro de Diseño Industrial ☐
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud ☐
- ◆ Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño ☐
- ◆ Comprobante de pago de las tasas establecidas ☐

COMPLETA ☐INCOMPLETA ☐ESQUEMA DE TRAZADO ☐

- ◆ Indicación de que se solicita el registro de un Esquema de Trazado ☐
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud ☐
- ◆ Representación gráfica del esquema trazado ☐
- ◆ Comprobante de pago de las tasas establecidas ☐

COMPLETA ☐INCOMPLETA ☐

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Firma Responsable

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REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Bogotá,
2020

Doctor(a)
OLARTE GARCIA CARLOS REYNALDO

REFERENCIA	Radicación No.	8.115385
	Solicitud internacional	PCT/EP2007/002596
	Fecha inicio fase nacional	31/10/2008
	Trámite	11
	Evento	378
	Actuación	430
	Oficio No.	Nº 14097

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

1. La solicitud no contiene el poder debidamente otorgado, con el lleno de los requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente, deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando ésta fuere persona jurídica.

2. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante, conforme a lo establecido por el artículo 26 literal k) de la Decisión 486 de la Comisión de la Comunidad Andina. Cuando se trate de documentos otorgados en el extranjero, estos deberán legalizarse de conformidad con lo dispuesto por los artículos 259 y 260 del Código de Procedimiento Civil. Cuando se trate de las declaraciones a que hace referencia la Regla 4.17 ii) del Tratado de Cooperación en Materia de Patentes PCT., el alcance de las tales declaraciones se determinará según indiquen claramente una cesión de derechos del inventor al solicitante o su causante, de conformidad con lo dispuesto en el numeral 5.2.15 del Capítulo Quinto, Título X de la Circular Única de Superintendencia de Industria y Comercio.

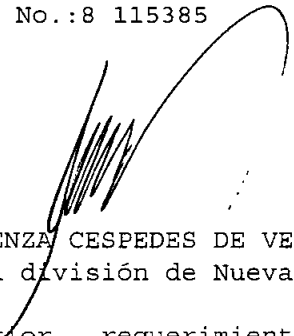
En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No.10 de 2001.

Continúa en la página siguiente...

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Radicación No.: 8 115385



ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la división de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista de
fecha _____
jfernand 07 NOV. 2008