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(54) **PRODUCTION OF RECOMBIANT PRODUCTS USING CAPILLARY MEMBRANES**

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(57) **ABSTRACT**

The invention provides for a method of producing at least one recombinant product under aerated conditions. The method includes providing a porous substrate having a first side and a second side and which has a biofilm of microorganisms attached to the first side thereof, the substrate being configured to allow passage of a nutrient solution therethrough and to prevent the passage of microorganism cells therethrough. The method further includes causing a nutrient solution to flow through the substrate and the biofilm in a direction from the second side thereof to the first side thereof under aerated conditions, at a rate which is sufficiently low for a nutrient gradient having a concentration differential to be established across the biofilm, and the microorganisms being induced to produce recombinant product.

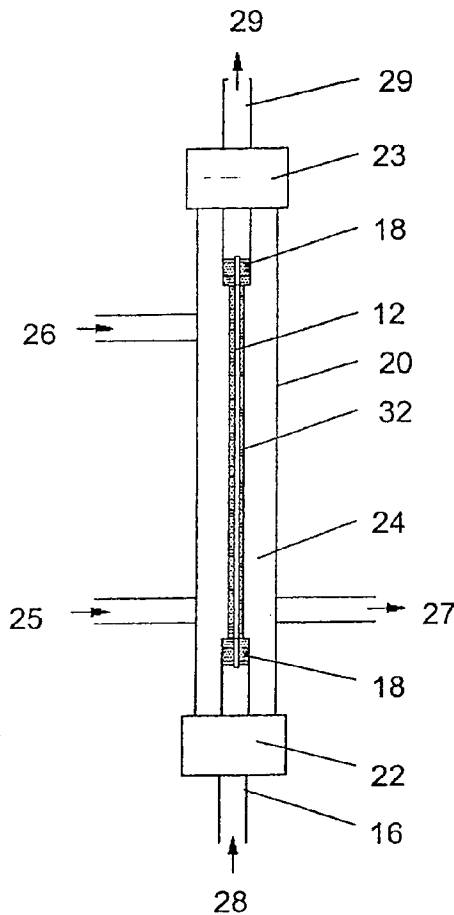
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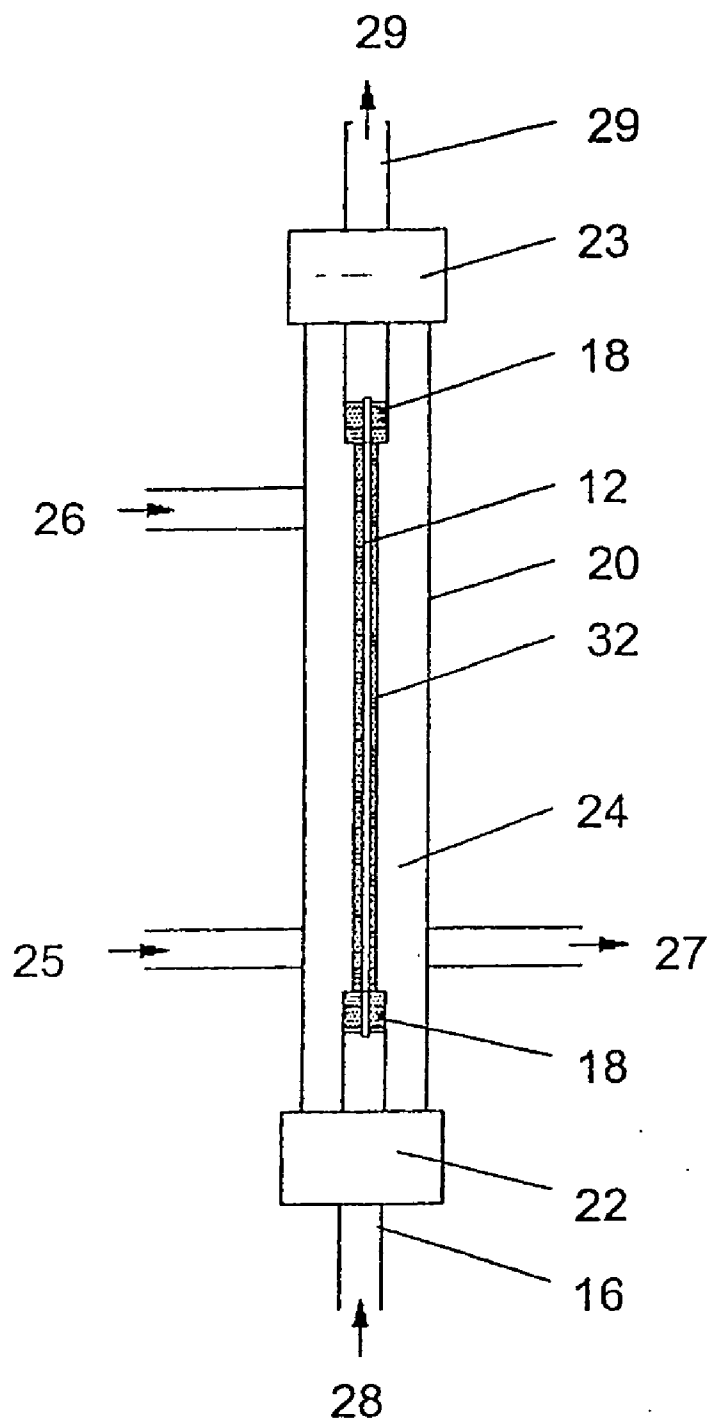


FIG 1

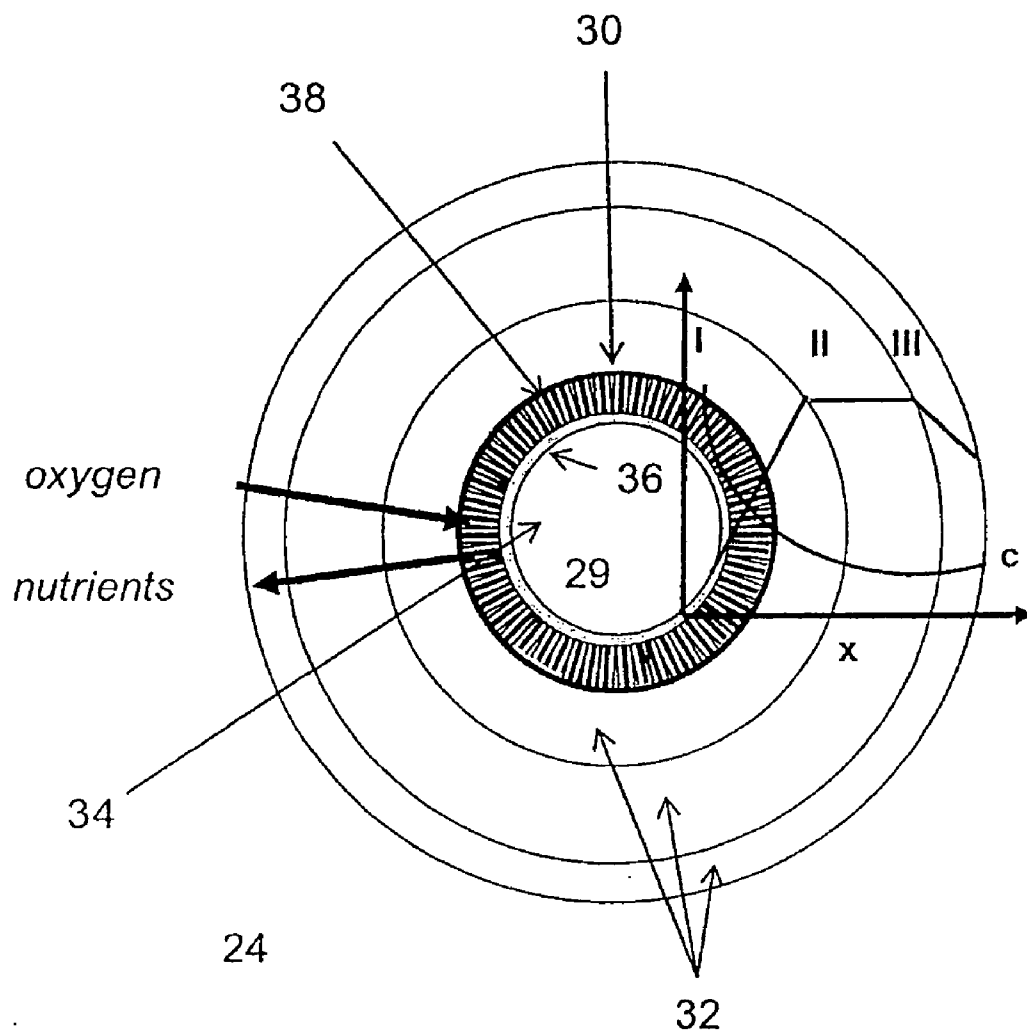


FIG 2

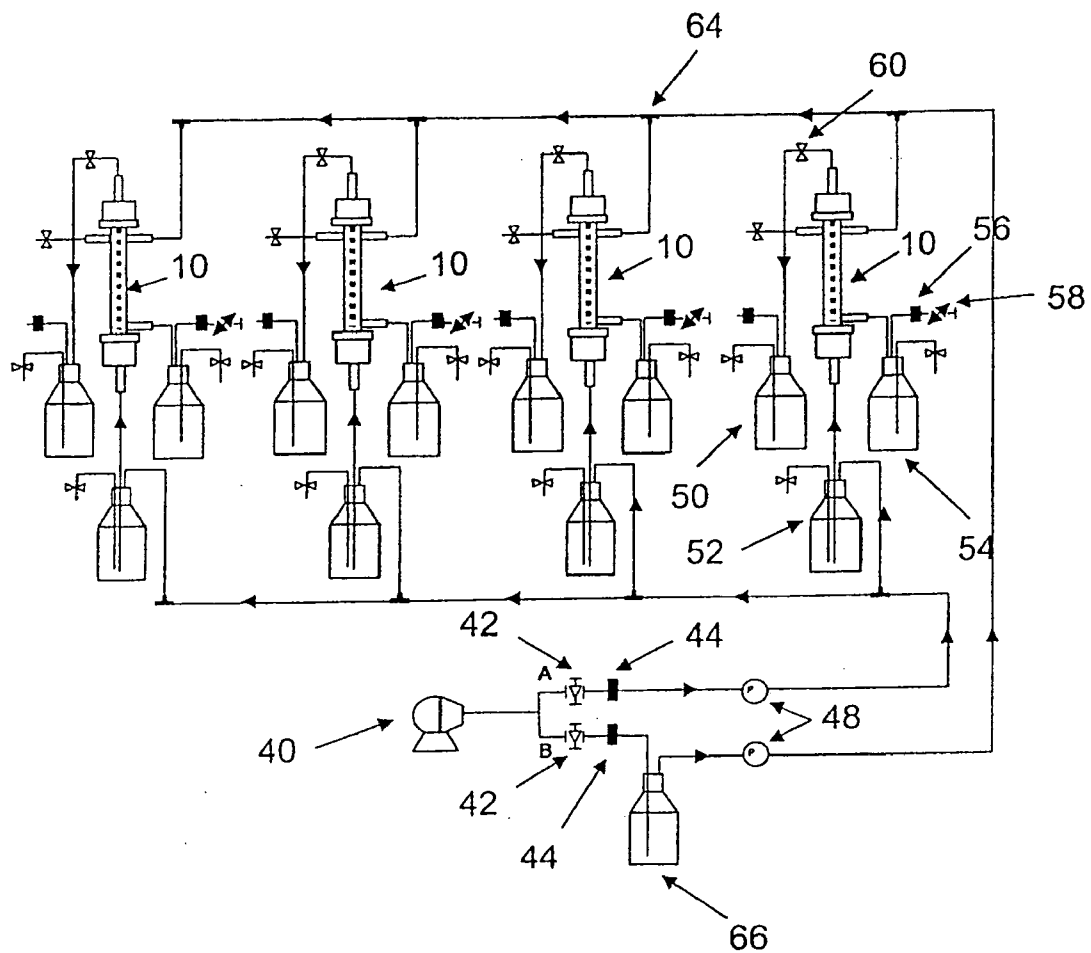


FIG 3

PRODUCTION OF RECOMBLANT PRODUCTS USING CAPILLARY MEMBRANES

FIELD OF INVENTION

[0001] THIS INVENTION relates to the production of recombinant products. In particular, it relates to a method of producing recombinant products under aerobic conditions, and to apparatus for producing such recombinant products under aerobic conditions.

BACKGROUND TO INVENTION

[0002] Recombinant products, including but not limited to enzymes, hormones, antibodies, antibody fragments, virus like particles, peptides, DNA and RNA fragments are produced by a variety of microbial expression systems. Products may also include chemical products resulting from expression of recombinant synthetic enzymes.

[0003] The market for recombinant products for the pharmaceutical as well as other industries has increased in recent years. Commercial products include both recombinant products expressed in recombinant hosts as well as chemicals produced by recombinant biosynthetic enzymes.

[0004] The efficient production of such recombinant products typically relies on the use of efficient expression hosts as well as good bioprocess technology. Expression hosts include a number of bacterial species of which *Escherichia coli* is most prominent, as well as fungi, protozoa, plant, insect and mammalian cells. Some of the products are intracellular, but it is preferred that products be excreted into the medium for easy purification. Most expression systems are aerobic, requiring considerable input of energy to increase oxygen mass transfer in submerged culture bioreactors. Most bioprocess technology is focused on obtaining very high cell concentrations in the bioreactor, typically by fed-batch culture mode. Production hosts are either engineered to produce recombinant products of interest constitutively or once sufficient biomass is present the production hosts are induced to express recombinant products of interest. This induction is typically a molecule that is added at the appropriate time or is a metabolite produced by the production host as a metabolic waste product or a secondary metabolite.

[0005] The strategy of inducing recombinant protein production once the culture is in the stationary phase is typical since:

[0006] 1) It allows for the production of products that are toxic to the producing organism.

[0007] 2) Volumetric productivity is improved if the biomass concentration in the reactor is very high.

[0008] 3) Precursors for the formation of the product are typically primary metabolites, which accumulate during the primary growth phase.

[0009] In submerged batch culture it is often difficult to time the addition of recombinant protein inducers optimally so that inducers are added when the culture reaches stationary phase and not too soon or too late. It is particularly difficult when multiple cultures are run in parallel under different conditions during optimization experiments.

[0010] Although most recombinant protein expression hosts are aerobic organisms, high density culture is limited because of oxygen mass transfer limitations and toxicity or solubility constraints of nutrients supplied in growth medium. Factors that affect the volumetric productivity of bioreactors

for recombinant protein production include: low shear environment, high biomass concentration, extended production phase, good nutrient mass transfer.

[0011] Typically, in high density culture oxygen and nutrient mass transfer is influenced by culture morphology and/or rheology. In submerged culture, expression of recombinant products in filamentous organisms such as *Aspergillus* are particularly affected by morphological differentiation and pellet formation and productivity has been enhanced through immobilization of the recombinant organism. Immobilization not only limits shear stress and reduces broth viscosity, improving oxygen supply and nutrient mass transfer, but was also shown to inhibit extracellular protease production in *Aspergillus niger* thereby limiting degradation of recombinant proteins produced in this organism (Wang et al. 2005). In most immobilized systems oxygen mass transfer limitations remain a rate limiting step in productivity.

[0012] Any reference herein to a "microorganism" must be interpreted to mean a reference to organisms which are members of the bacteria, fungi, protozoa and includes plant or mammalian cells that have been engineered to produce recombinant products under aerobic conditions.

[0013] Any reference herein to a "stationary phase" must be interpreted to mean a period of limited growth or growth and death substantially in equilibrium which typically follows immediately after the primary growth phase in batch or continuous cultivation of microorganisms.

[0014] Any reference herein to "recombinant protein" must be interpreted as synonymous with "recombinant product" and to mean any product or the product thereof if it is a biosynthetic product, homologous or heterologous to the expression host, the production of which may be induced by some mechanism at the onset of—or during the stationary phase of a typical batch process. The product can either be secreted by the expression host into the extracellular environment or retained intracellularly. Recombinant products are not limited to proteins.

[0015] Any reference herein to a "nutrient solution" must be interpreted to mean a liquid solution containing one or more nutrients required for microorganism growth and which includes at least one nutrient which is growth limiting. The nutrient solution may also carry an inducer required for initiating the expression of recombinant products.

[0016] Any reference herein to an "inducer" must be interpreted to mean a biological or synthetic chemical included in the nutrient solution or a biosynthetic product produced by the immobilized expression host during growth and used as a means of regulating the production of recombinant products or proteins under the control of a relevant promoter regulating expression of the said recombinant products or proteins.

[0017] Any reference herein to "permeate" must be interpreted to mean spent nutrient solution or product stream that has passed through a substrate and biofilm and may carry secreted product, metabolic waste products and/or the remainder of nutrients not metabolized by the microorganism during passage of nutrient solution through the biofilm.

[0018] Any reference herein to "aerated conditions" must be interpreted to mean any cultivation condition in which a gas, which contains oxygen, is passed through a bioreactor across a biofilm surface, and which supplies microorganisms with oxygen for growth.

SUMMARY OF INVENTION

[0019] According to one aspect of the invention, there is provided a method of producing at least one recombinant product under aerated conditions, which method includes:

[0020] providing a porous substrate having a first side and a second side and which has a biofilm of microorganisms attached to the first side thereof, the substrate being configured to allow passage of a nutrient solution therethrough and to prevent the passage of microorganism cells therethrough; and

[0021] causing a nutrient solution to flow through the substrate and the biofilm in a direction from the second side thereof to the first side thereof under aerated conditions, at a rate which is sufficiently low for a nutrient gradient to be established across the biofilm wherein the nutrient concentration is relatively higher closer to the substrate and sufficiently high to support primary growth of the microorganisms and wherein the nutrient concentration is relatively lower further from the substrate and sufficiently low to enter and sustain a stationary phase of the microorganisms, and the microorganisms being induced to produce recombinant product.

[0022] Induction may be caused in various ways such as by nutrient depletion, addition of an inducer molecule, accumulation of a primary metabolic product or secondary metabolite produced under nutrient limiting conditions, auto-induction, or the like. In other words, the method may use derepression and/or induction of recombinant gene expression. In other words the induction may occur through the addition of an inducer molecule to the nutrient solution or through the accumulation of a primary metabolic product or secondary metabolite produced under nutrient limiting conditions.

[0023] The induction may occur through the addition of an inducer molecule to the nutrient solution such that the flow of the nutrient solution from the second side through the substrate and the biofilm to the first side carries said inducer to the microorganism thereby to induce production of at least one recombinant protein or product by the microorganisms. Induction may also occur through the accumulation of primary or secondary metabolites within the biofilm such that as they are carried through the biofilm in the nutrient solution resulting in a concentration gradient wherein the inducer concentration is relatively low closer to the substrate and relatively high further from the substrate and sufficiently high to induce expression of recombinant products.

[0024] The inducer may also be a primary or secondary metabolites that accumulate within the biofilm such that as they are carried through the biofilm in the nutrient solution resulting in a concentration gradient wherein the inducer concentration is relatively low closer to the substrate and relatively high further from the substrate and sufficiently high to induce expression of at least one recombinant products by the microorganism.

[0025] The recombinant product may be collected in the permeate from the first side if secreted by the microorganisms or may be harvested from the biofilm if retained intracellularly by the microorganisms.

[0026] The inducer may be any suitable inducer such as isopropyl b-D-thiogalacto-side (IPTG) (0.1-1 mM), methanol (0.5-1.5%) or organic acids produced by the microorganism that interact with the specific promoter with which the recombinant expression system is engineered. Concentration of inducer may be varied in order to optimize the expression of soluble, correctly folded protein products.

[0027] The method may include contacting the biofilm with a gas. The gas may be blown over an outside surface of the biofilm thereby to carry away spores and dead cells of the

microorganisms as well as to remove spent nutrient solution which may contain secreted product. The gas may provide oxygen for metabolism. The gas may be air.

[0028] The microorganisms may be a substantially pure culture of a strain of a microorganism or a mixed culture of different microorganisms. The microorganisms may be selected from the group consisting of *Aspergillus* sp., *Trichoderma* sp., *Mucor* sp., *Pichia* sp., *Fusarium* sp., *Neurospora* sp., *Penicillium* sp., *Streptomyces* sp., *Chryso sporium luc knowense*, *Mortierella alpinis*.

[0029] The method of producing the recombinant product may be carried out at a temperature within the range of growth and product formation of the microorganisms. The method of producing the recombinant product may be carried out at a temperature of 2-109° C., preferably 20-40° C., e.g. 30° C.

[0030] The method of producing the recombinant product may be carried out at an initial pH within the range of growth and product formation of the microorganisms. The method of producing the recombinant product may be carried out at an initial pH of 1-13, preferably 4-8, e.g. 7.

[0031] The method of producing the recombinant product may be carried out for a period for as long as the biofilm or culture remains viable and productive and not too dense so as to prevent product flow through the membrane. The method of producing the recombinant product may be carried out for a period of 2-60 days, preferably 2-30 days, e.g. 25 days.

[0032] The recombinant product may be produced intracellularly or extracellularly by the microorganisms. The recombinant product may be collected from the first side if secreted by the microorganisms or may be harvested from the biofilm if retained intracellularly by the microorganisms. Intracellular metabolites may be retained within the cytoplasm or secreted to the periplasmic space and extracted using cell lysis or cell permeabilization procedures such as osmotic shock.

[0033] Nutrient solution may be passed through the biofilm and substrate at a flow rate that is sufficient to sustain and immobilize the microorganism while maintaining a nutrient gradient across the biofilm. Flow rates may differ depending on the nutrient content of the nutrient solution and or the type of microorganism cultured. Flow rates may range from 0.001-10 volumes nutrient solution per reactor volume per hour.

[0034] Biofilm thickness may depend on the nutrient content of the nutrient solution and the type of microorganism cultured. The biofilm may have a thickness of 0.1-10 mm and should be limited such that optimal mass transfer of oxygen between humidified air and the biofilm is maintained within a suitable range for the biofilm or culture to remain viable and productive.

[0035] According to a second aspect of the invention there is provided apparatus for producing a recombinant product under aerated conditions, which apparatus includes:

[0036] at least one porous substrate having a first side and a second side; and

[0037] a feeding arrangement for supplying a nutrient solution under aerated conditions into contact with the microorganisms which become attached to and form, in use, a biofilm on the first side of the substrate, the feeding arrangement being operable to cause the nutrient solution to flow through the substrate and the biofilm in a direction from the second side thereof to the first side thereof at a rate sufficiently low for a nutrient gradient having to be established across the biofilm wherein the nutrient concentration is relatively higher closer to the substrate and sufficiently high to support primary

growth of the microorganisms and wherein the nutrient concentration is relatively lower further from the substrate and sufficiently low to enter and sustain a stationary phase of the microorganisms, with the microorganisms being induced to produce recombinant product. Induction may also occur through the accumulation of primary or secondary metabolites within the biofilm such that as they are carried through the biofilm in the nutrient solution resulting in a concentration gradient wherein the inducer concentration is relatively low closer to the substrate and relatively high further from the substrate and sufficiently high to induce expression of at least one recombinant product by the organism.

[0038] The feeding arrangement may include at least one of a mechanical source such as a pump and a pneumatic source such as compressed air, for feeding the nutrient solution into contact with the substrate at the second side and causing the nutrient solution to flow through the substrate and the biofilm to the first side.

[0039] The apparatus includes a pneumatic source such as compressed air supplied at the first side which may flow across and be directed into contact with the outside of the biofilm. The gas should include oxygen for growth and metabolism and may also be used to carry away spores and dead cells of the microorganism and to remove permeate which may contain secreted product.

[0040] The apparatus may include product collection means for collecting the gas and the permeate, which may contain product, from the first side of the biofilm. The product collection means may also include an outlet including a back-pressure creating means such as, but not limited to, a mechanical restriction, which may be used to regulate the pressure of the gas at the first side thereby altering oxygen transfer of the gas to the biofilm under the resultant applied backpressure.

[0041] The substrate may comprise a porous membrane having apertures sufficiently large to permit the flow of the nutrient solution therethrough, but which apertures are sufficiently small to prevent passage or growth of microorganism cells therethrough. The exact configuration of the membrane may vary greatly. In particular embodiments, the membrane may be in the form of a hollow-fibre membrane, a capillary membrane or may have a tubular or flat sheet configuration.

[0042] The apparatus may include a housing for the substrate, the housing being spatially separate from the substrate. The housing may include an inlet for the nutrient solution, an inlet for the gas solution and an outlet for discharging permeate from the housing.

[0043] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will override terms commonly understood by one of ordinary skill in the art. Preferred methods and apparatus are described below, although methods and apparatus similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The methods, apparatus and examples disclosed herein are for illustrative purposes only and not intended to be limiting.

BRIEF DESCRIPTION OF DRAWINGS

[0044] Further features of the invention are described hereinafter by way of a non-limiting example of the invention,

with reference to and as illustrated in the accompanying diagrammatic drawings. In the drawings;

[0045] FIG. 1 shows a schematic side elevation of an apparatus in accordance with the invention;

[0046] FIG. 2 shows a schematic sectional plan view of the porous substrate and biofilm, sectioned I, II and III, supplied with nutrients from the second side to the first side and oxygen supplied at the first side such that a nutrient gradient is established across the biofilm that is highest at the second side and lowest at the first side, and an oxygen gradient is established that is highest at the first side and lowest at the second side; and

[0047] FIG. 3 shows a schematic view of a plurality of single-fibre membrane bioreactors forming a scaled-up embodiment of the invention.

BRIEF DESCRIPTION OF THE PREFERRED EMBODIMENT

[0048] With reference to FIGS. 1 to 2 of the drawings, an apparatus for producing a recombinant protein under aerated conditions, in accordance with the invention, is designated generally by the reference numeral 10. The apparatus is in the form of a bioreactor 10 shown in FIG. 1 of the drawings at a laboratory scale, however, it will be appreciated that the principles embodied in the bioreactor 10 can be applied to a scaled-up or commercial embodiment.

[0049] The bioreactor 10 includes a ceramic hollow fibre capillary membrane 12 with ends of the membrane being potted into plastic inserts 14, 16 with epoxy 18. The housing or reactor shell 20 of glass is arranged coaxially with the capillary membrane 12 and is provided with end caps 22 and 23 which thread onto the glass housing 20. The housing 20 includes an air inlet 26 for introducing an oxygen into the space 24 between the capillary membrane 12 and the housing 20, inoculation inlet 25 for introducing an inoculum into the space 24 between the housing 20 for attachment to the membrane 12. The housing further includes a permeate and air outlet 27 for discharging the feed solution and flowing air from the housing.

[0050] In use, a biofilm 32 is established on an external surface 30 of the capillary membrane 12. This is achieved by introducing a spore or vegetative inoculum of the desired microorganism into the space 24 between the membrane surface 30 and the glass housing 20 at the first side and filtering the inoculum through the capillary membrane 12 from the external surface 30 towards the membrane lumen 34 at the second side, and draining any permeate out of the lumen 34 and through the outlet 29. The lumen 34 is thus in flow communication with the outlet 29. The inoculum is thus immobilized on the membrane surface 30. The membrane 12 has a relatively thin, porous skin 36 on the inside and a relatively large, porous, externally unskinned void structure 38 radiating outwardly from the skin 36. Typically, the membrane 12 has an outside diameter of about 2 mm.

[0051] During operation an appropriate nutrient solution for the microorganism is introduced into the housing via the medium inlet 28. The nutrient solution is caused to flow through the membrane 12 in a direction from the second side or lumen of the membrane 34 to the first side or outer surface thereof 30. Air is displaced from the membrane lumen 34 or second side through a medium outlet 29 which is closed off during operation to facilitate permeation of the nutrient feed

from the lumen side, through the capillary membrane wall **36** and **38** to the space **24** between the membrane surface **30** and the glass housing **20**.

[0052] In FIG. 3, a plurality of bioreactors **10** are illustrated. A compressed air supply is indicated by reference numeral **40** is connected via two regulator valves **42** to two 0.22 m filters **44**. A humidification vessel **46** is provided and pressure is measured through pressure gauges **48**. Priming vessels **50** and medium supply vessels **52** are shown connected to the bioreactors **10**. Permeate collection vessels **54** are connected to vent filters **56** and flow regulators **58**.

[0053] With reference to FIG. 3, the line to the permeate collection vessel **54** is mechanically sealed by means of a clamp **60** before the microorganism is introduced directly into the space between the membrane surface and the glass housing of each bioreactor **10**, such that the space between the membrane surface and glass housing is filled with inoculum. The inoculum is immobilized onto the membrane surface under pressure, supplied from the compressor **40** through line B. The pressure of compressed air is controlled by the pressure regulator valve **42** and monitored using the pressure gauge **48**. T-pieces **64** distribute the pressure evenly between the manifolded bioreactors **10**. The pressurized air from line B is sterilized by passing the gas through the 0.22 μ m filter **44** and then humidified by bubbling the air stream through sterile distilled water in a humidification vessel **66** prior to entering the bioreactor **10**, in order to prevent desiccation of the microorganism and to improve the oxygen transfer rate between the air stream and the biofilm. The inoculation filtrate is collected from the lumen side into the priming vessel **50**. Once complete the line to permeate collection vessel **54** is opened and the air pressure and the air flow rate from line B is set at the air outlet of the permeate collection vessel **54** using the flow regulator **58**.

[0054] During operation nutrient solution is supplied from the medium supply vessel **52** under pressure supplied by from the compressor **40** through line A. The pressure of compressed air is controlled by the pressure regulator valve **42** and monitored using a pressure gauge **48**. T-pieces **64** distribute the pressure evenly between the manifolded bioreactors **10**. The pressurized air from line A is sterilized by passing the gas through the 0.22 μ m filter **44**. The nutrient solution is fed pneumatically from the medium supply vessel **52** into the lumen of the capillary membrane, displacing air from the lumen into the priming vessel **50**. During operation the line to the priming vessel **50** is mechanically sealed with the clamps **60**. The rate at which nutrient solution is supplied from the membrane lumen at the second side to the biofilm immobilized on the membrane surface at the first side is determined by the pressure differential between the compressed air supply of line B and the compressed air supply of line A. Permeate can be removed at intervals from the permeate collection vessel.

[0055] Fundamental to the production of recombinant proteins is the regulation of gene expression by molecules that either repress or induce gene expression by acting on a promoter element controlling expression of the foreign gene. In addition to better known inducers such as IPTG and methanol, nutrients such as carbon or nitrogen may serve to either down-regulate (repress) or up-regulate (induce) the expression of a recombinant product. Through nutrient starvation the promoter element may be de-repressed and or induced, thus initiating gene expression and recombinant protein production. This is achieved with the membrane-immobilized

biofilm bioreactor **10** by the production of radial nutrient concentration gradients through the biofilm **32**. As a result, the nutrient concentration at the membrane/biofilm interface is high, whereas the nutrient concentration at the exposed outer surface of the biofilm **32** is low. In FIG. 2 of the drawings, for the purposes of illustration, a graph depicts the nutrient concentration level C (y axis) vs. distance from the membrane surface (x axis) while nutrient concentration zones from high to low are designated by the reference numbers I to III, respectively. In zone I the nutrient concentration is sufficiently high to support primary growth of the microorganisms, in zone II the nutrient concentration is sufficiently low to cause nutrient starvation which stresses the microorganisms thereby to induce and sustain a stationary phase of the microorganisms, while in zone III the nutrient concentration may be depleted resulting in cell death and lysis.

[0056] Due to the toxicity of many recombinant products and the high cell densities required for increased productivity most expression systems have been engineered to produce recombinant products during stationary phase growth through either auto-induction or the addition of an inducer during stationary phase growth. Auto-induction may result from

[0057] 1) the nutrient gradients described herein; or

[0058] 2) as the concentration of metabolic waste product increases as the nutrient solution perfuses the biofilm, which may also induce product formation in cultures where the accumulation of metabolic waste induces product formation;

[0059] 3) this could also result in the production of a secondary metabolite which induces the production of a recombinant product. It will be appreciated that after the establishment of the nutrient gradient, product formation may also be induced by altered pH as a result of organic acid accumulation.

[0060] In this invention optimal conditions for expression may be maintained while limiting exposure of the recombinant host to toxic products. When operated the bioreactor allows for extended production of recombinant proteins and as the biomass is retained, allows for the recovery of a relatively high purity, biomass free product. In addition, degradation and decay of recombinant products by proteolytic enzymes is reduced through the continuous removal of product.

[0061] In a recombinant host where expression is auto-regulated by nutrient conditions or metabolic products produced by the host organism itself, recombinant protein production may be maintained in zone II e.g. Xylanase production in *Aspergillus niger* under the control of the glyceraldehydes-6-phosphate dehydrogenase (gpd) promoter (Rose and van Zyl, 2002).

[0062] In a recombinant host where expression is regulated by a synthetic or biosynthetic chemical, recombinant protein production may be induced throughout the biofilm in the absence of an inhibitor or recombinant protein production may be maintained in zone II where such an inhibitor may be metabolized by the microorganism e.g. Recombinant production in *Pichia pastoris* under the control of the AOX promoter is induced by methanol under carbon limiting conditions. In this expression system albumin is a secreted product and β -galactosidase is an intracellular product.

[0063] It will be appreciated that the regulation of recombinant protein production is host and expression system specific and that the means by which recombinant protein pro-

duction is achieved within the immobilized biofilm may differ while still incorporating the essential features of the nutrient, metabolite and/or inducer gradients described herein.

[0064] It will also be appreciated that the exact configuration of the bioreactor and of the fluid flow regime may vary greatly while still incorporating the essential features defined hereinabove.

[0065] Methods of secondary metabolite production described in patents U.S. Pat. No. 5,945,002 and EP9606333.4 (incorporated herein by reference thereto) may be adapted and used to produce recombinant products in accordance with the invention.

Example 1

Aspergillus niger Expression System

[0066] *Aspergillus niger* has been used routinely as a fungal expression host using a variety of expression vectors. In this example *A. niger* engineered to constitutively express the *Trichoderma reesei* β -1,4-xylanase gene (*xyn2*) under the transcriptional control of the glyceraldehydes-6-phosphate dehydrogenase (*gpd*) promoter from *A. niger* (Rose and van Zyl, 2002) is used to demonstrate the continuous production of recombinant xylanase by this stable, high yielding transformant using the apparatus described herein.

Strain and Cultivation Conditions

[0067] Recombinant strain *Aspergillus niger* D15 containing the xylanase II (*xyn2*) gene of *Trichoderma reesei* integrated into its fungal genome was obtained from Prof. W. H. van Zyl, Dept. of Microbiology, University of Stellenbosch, South Africa (Rose and van Zyl, 2002).

[0068] The xylanase producing *Aspergillus* was cultivated in 20% (v/v) molasses, 1 \times defined medium (Punt and van den Hondel, 1992), 2 \times defined medium (Punt and van den Hondel, 1992) or minimal medium (Record et al. 2002).

Bioreactor Setup

[0069] Single fibre membrane bioreactors were configured as illustrated in FIG. 3. Four banks of four bioreactor modules were operated, each bank under different nutrient conditions. Bioreactors were autoclaved and setup for aerobic operation according to standard operating procedures (SOPs). Sterile growth medium was aseptically dispensed into each of the medium supply vessels prior to starting the experiment.

Inoculation

[0070] All bioreactor were inoculated with 1 ml spore suspension generated by resuspending spores from a single agar plate in 20 ml sterile distilled water. Inoculum was aseptically injected directly into the extracapillary space (ECS) i.e. between the membrane surface and the glass housing of each bioreactor. Immobilization of inoculum on the outer surface of capillary membranes was completed by reverse flow, according to SOPs.

Operation

[0071] Bioreactors were operated under aerobic conditions according to SOPs. Oxygen in the form of 0.22 μ m filtered, humidified air was supplied from a compressor using a pressure regulator valve and monitored using a pressure gauge. Hoffman clamps were used at the air outlet of each bioreactor

to regulate airflow and create backpressure. The air pressure within the ECS i.e. the space between the membrane surface and the glass housing at the first side was monitored using a pressure gauge and maintained at 60 kPa and the airflow through the bioreactor was set to 1 vol air per minute per reactor volume using a rotameter.

[0072] Growth medium was supplied to each bioreactor separate medium supply vessels using 0.22 μ m filtered compressed air using a pressure regulator valve and monitored using a pressure gauge. Growth medium was fed to the biofilm from the lumen or second side of capillary membranes, through the membrane wall to the first side or outer surface of the capillary membrane. Medium flow rate from the second side to the first side was manually regulated according by maintaining a positive pressure differential set across the membrane surface from lumen or second side to outer membrane surface or first side of the bioreactor, thus controlling the rate of nutrient feed (flux) to the biofilm. Increased biofilm growth creates greater resistance to flow and the pressures should be adjusted accordingly to maintain constant flux. Under optimal flux conditions the biofilm thickness and flux are maintained substantially in equilibrium facilitating the nutrient gradients required for differentiation of the microorganism and continuous recombinant protein production.

[0073] Permeate was collected in permeate collection vessels and sampled daily for further analysis. The rate of permeate accumulation and pH was monitored daily. Samples were stored at -20° C. for further analysis.

Biofilm Development

[0074] Using different growth medium different biofilm thickness, differentiation and productivity were observed for the *A. niger* D15 transformant.

[0075] Biofilm growth developed rapidly in the minimal medium (Record et al. 2002) showing even, black pigmented and heavily sporulating growth as early as day 3.

[0076] Growth on 1 \times and 2 \times defined medium (Punt and van den Hondel, 1992) was similar, with slightly greater biofilm thickness when using 2 \times growth medium. Growth was slower than in defined medium and did not demonstrate the same dark pigmentation or sporulation. Biofilm pigmentation was mottled cream to light and dark brown, darkening from day 10 and sporulating from day 15. Growth in molasses was poor, showing only effuse biofilm growth with dark-pigmentation and sporulation by day 14.

[0077] With reference to Table 1, bioreactors were harvested after 25 days and the dry cell weights calculated for each bioreactor. Significantly higher biomass was obtained using 2 \times defined medium. The amount of xylanase produced by bioreactors over a 25 day period was strongly correlated with biomass yield ($r^2=0.92$).

TABLE 1

	Average biomass produced in bioreactors (n = 2) cultured with different growth medium			
	Dry cell weight (g)		Units xylanase per g biomass	
	Mean	SD	Mean	SD
Minimal medium	0.53	0.04	4843	3103
2 \times defined medium	1.71	0.08	4207	1095

TABLE 1-continued

Average biomass produced in bioreactors (n = 2) cultured with different growth medium				
	Dry cell weight (g)		Units xylanase per g biomass	
	Mean	SD	Mean	SD
20% molasses	0.12	0.05	8282	3232
1x defined medium	0.66	0.05	5647	94

Productivity

[0078] Enzyme activity was quantified using Megazyme's azo-xylan (birchwood) xylanase activity assay. The procedure was performed in sodium phosphate buffer, pH 6.0 at 55° C. The pH and incubation temperatures used were previously described as optimal for recombinant xyn2 produced in *Aspergillus niger* D15 (Rose and van Zyl, 1992). Separate controls (blanks) were prepared for each sample due to differences in medium composition between bioreactors. One unit of enzyme activity is defined as the amount of enzyme required to release one μ mole of D-xylose reducing-sugar equivalents from arabinozylan, per minute.

[0079] With reference to Table 2, defined growth medium showed greater yields of xylanase production, with 2x defined medium doubling the volumetric productivity of bioreactors in comparison to 1x defined medium. This was correlated with the increased amount of biomass produced when using a more concentrated growth medium (as above). Bioreactors cultured using minimal medium showed were marginally less productive than those cultured with 1x defined medium, while 20% molasses supported poor growth and xylanase production in bioreactors.

TABLE 2

Xylanase production by bioreactors.								
Bio-reactor	Xylanase (Units/l)			Space Time Yield (Units/h per l. reactor volume)			Xylanase (Units) Per 25 day period	
	Max.	mean	SD	Max.	mean	SD		
1	13.53	9.67	3.43	2.20	0.72	0.75	3498	
2	5.55	7.81	3.65	1.09	0.31	0.34	1470	
3	18.63	10.74	4.43	2.87	0.75	0.81	3621	
4	17.86	10.02	4.35	4.30	0.90	1.18	4311	
5	54.29	17.98	14.98	6.10	1.38	1.63	7042	
6	33.88	16.83	12.14	8.68	1.63	2.20	8250	
7	27.28	12.79	6.85	2.36	1.02	0.74	6059	
8	31.98	12.76	7.53	2.92	1.17	0.73	6940	
9	3.64	2.26	1.13	0.55	0.21	0.14	1135	
10	4.40	2.31	1.24	0.44	0.17	0.12	898	
11	3.23	2.06	1.02	0.30	0.18	0.13	965	
12	4.30	1.97	1.47	0.58	0.14	0.15	713	
13	21.99	12.38	6.00	1.11	0.65	0.36	3493	
14	30.20	15.70	8.39	1.44	0.72	0.43	3993	
15	22.76	13.51	6.19	1.28	0.74	0.42	4005	
16	23.51	15.95	7.74	1.78	0.81	0.46	4231	

REFERENCES

[0080] 1. Wang, L., Ridgway, D., Gu, T. and Moo-Young M. (2005) Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. *Biotech. Advances* 23, pp. 115-129

[0081] 2. Rose, S. H and van Zyl W. H. (2002) Constitutive expression of the *Trichoderma reesei* beta-1,4-xylanase gene (xyn2) and the beta-1,4-endoglucanase gene (egl) in *Aspergillus niger* in molasses and defined glucose media. *Appl. Microbiol. Biotechnol.* 58, pp. 115-129.

[0082] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications can be made without departing from the spirit and scope of the invention.

1. A method of producing at least one recombinant product under aerated conditions, the method comprising:

providing a porous substrate having a first side and a second side and which has a biofilm of microorganisms attached to the first side thereof, the substrate being configured to allow passage of a nutrient solution therethrough and to prevent the passage of microorganism cells therethrough; and

causing a nutrient solution to flow through the substrate and the biofilm in a direction from the second side thereof to the first side thereof under aerated conditions, at a rate which is sufficiently low for a nutrient gradient having a concentration differential to be established across the biofilm, wherein the nutrient concentration is relatively higher closer to the substrate and sufficiently high to support primary growth of the microorganisms, and wherein the nutrient concentration is relatively lower further from the substrate and sufficiently low to enter and to sustain a stationary phase of the microorganisms, and the microorganisms being induced to produce recombinant product.

2. The method of claim 1, wherein the microorganisms are selected from the group consisting of *Aspergillus* sp., *Trichoderma* sp., *Mucor* sp., *Pichia* sp., *Fusarium* sp., *Neurospora* sp., *Penicillium* sp., *Streptomyces* sp., *Chrysosporium lucknowense*, and *Mortierella alpinis*,

3. The method of claim 1, further comprising carrying out the method at a temperature between 2-109° C.

4. The method of claim 1, further comprising carrying out the method at an initial pH between 1-13.

5. The method of claim 1, further comprising carrying out the method for a period between 2-60 days.

6. The method of claim 1, wherein the flow rate of the nutrient solution from the second side to the first side is 0.001-10 volumes nutrient solution per bioreactor volume per hour.

7. The method of claim 1, wherein the biofilm has a thickness of 0.1-10 mm.

8. The method of claim 1, further comprising adding an inducer molecule to the nutrient solution such that the flow of the nutrient solution from the second side through the substrate and the biofilm to the first side carries said inducer to the microorganism thereby to induce production of at least one recombinant product by the microorganisms, wherein the inducer is a primary or secondary metabolite that accumulates within the biofilm such that as the metabolite is carried through the biofilm in the nutrient solution resulting in a concentration gradient, wherein the inducer concentration is relatively low closer to the substrate and relatively high further from the substrate and sufficiently high to induce expression of at least one recombinant products by the microorganism.

9. The method of claim 8, wherein the inducer is selected from at least one of isopropyl b-D-thiogalacto-side (IPTG) (0.1-1 mM), methanol (0.5-1.5%) and organic acids produced by the microorganism.

10. The method of claim 8, wherein the secondary metabolite is produced intracellularly by the microorganisms.

11. The method of claim 8, wherein the secondary metabolite is produced extracellularly by the microorganisms.

12. The method of claim 1, further comprising contacting the biofilm with a gas.

13. An apparatus for producing a recombinant product under aerated conditions, the apparatus comprising:

at least one porous substrate having a first side and a second side; and

a feeding arrangement for supplying a nutrient solution under aerated conditions into contact with the microorganisms which become attached to and form, in use, a biofilm on the first side of the substrate, the feeding arrangement being operable to cause the nutrient solution to flow through the substrate and the biofilm in a direction from the second side thereof to the first side thereof at a rate sufficiently low for a nutrient gradient having to be established across the biofilm, wherein the nutrient concentration is relatively higher closer to the substrate and sufficiently high to support primary growth of the microorganisms, and wherein the nutrient

concentration is relatively lower further from the substrate and sufficiently low to enter and sustain a stationary phase of the microorganisms, with the microorganisms being induced to produce recombinant product.

14. The apparatus of claim 13, further comprising a pump for feeding the nutrient solution into contact with the substrate at the second side and causing the nutrient solution to flow through the substrate and the biofilm to the first side.

15. The apparatus of claim 13, further comprising a source of pressurized gas provided at the first side and configured to allow flow of gas across and be directed into contact with an outside of the biofilm.

16. The apparatus of claim 15, further comprising product collection means for collecting the gas and spent nutrient solution from the first side.

17. The apparatus of claim 16, wherein the product collection means includes an outlet including a backpressure creating means to regulate the pressure of the gas at the first side thereby to regulate oxygen transfer of the gas to the biofilm under pressure.

18. The apparatus of claim 1, wherein the substrate comprises a porous membrane having apertures sufficiently large to permit the flow of the nutrient solution therethrough, but which apertures are sufficiently small to prevent passage or growth of microorganism cells therethrough.

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