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RECOMBINANT PROTEIN PRODUCTION AND  
INSECT CELL CULTURE AND PROCESS  
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**RECOMBINANT PROTEIN PRODUCTION AND  
INSECT CELL CULTURE AND PROCESS**

The subject invention relates to *in vitro* culture of animal cells, specifically as hosts for an assortment of recombinant protein biotherapy products useful in the treatment of disease.

Insect cells, specifically Sf9 cells, are cultivated in the NASA horizontally rotating High Aspect Ratio Vessel to produce a metabolically transformed new cell line. The cells are selected and transformed to include a selected DNA sequence encoding the desired polypeptide product. The insect cells cultured by this process recombinantly produce the selected polypeptide which can then be collected and purified.

The novelty of the invention appears to lie in the large scale growth of aggregated insect cells of much higher concentration and viability, without support matrices.

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Recombinant Protein Production and  
Insect Cell Culture and Process

ORIGIN OF THE INVENTION

5 The jointly made invention described herein was made by employees of the United States Government and may be manufactured and used by or for the Government of the United States of America for governmental purposes without the payment of any royalties thereon or therefor.

10 The invention described herein was also made by inventors in the performance of work under a NASA contract with Krug Life Sciences and a memorandum of understanding with Tulane University and is subject to the provisions of Section 305 of the National Aeronautics and Space Act of 1958, Public Law 85-568 (72 Stat. 435; 42 U.S.C. 2457).

15 BACKGROUND OF THE INVENTION

20 *In vitro* cultures of animal cells are hosts for an increasing assortment of recombinant protein products. This is a relatively new phenomenon, beginning in the mid 1980's. In this short period of time, these proteins have made a substantial contribution to the U.S. economic market. In the pharmaceutical industry alone, biotherapies, which are primarily recombinant proteins, generated \$1.2 billion in U.S. sales in 1991 and will grow to nearly \$8.0 billion in 2001 as

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more proteins become commercially available [Thayer, A. M.,  
*C & EN*, Feb. 25, 27 (1991)]. The market size and its capacity  
for growth demonstrate the importance of recombinant protein  
production to the fields of biotechnology and bioengineering.

5           Recombinant proteins derived from animal cells are  
employed in a range of disciplines from agriculture to  
medicine to basic research. During the past decade in  
medicine, progress has been made in genetic engineering and  
animal-cell cultivation to produce an increasing assortment of  
10 recombinant proteins in sufficient quantities for the  
development of new drug therapies. The proteins themselves  
can serve as therapies, or they can be used to identify and  
test chemotherapies that regulate protein function *in vivo*.  
Recombinant tissue plasminogen activator is an example of a  
15 protein therapy. It is given to patients with evolving  
myocardial infarction to dissolve coronary thrombus and  
restore blood flow to the ischemic area [Longridge, D. J.,  
Follenfant, M. J., Maxwell, M. P., Ford, A. J., and Hughes B.,  
*Cardiovasc. Res.*, 24, 697 (1990)]. In clinical trials,  
20 another protein therapy, erythropoietin, has been used to  
treat anemia in patients with progressive renal failure  
[Eschbach, J. W., Kelly, M. R., Haley, N. R., Abels, R. I.,  
and Adamson, J. W., *N. Engl. J. Med.*, 321, 158 (1989)] and  
sickle cell disease [Goldberg, M. A., Brugnara, C., Dover, G.  
25 J., Schapira, L., Charache, S., and Bunn, H. F., *N. Engl. J.*  
*Med.*, 323, 366 (1990)]. Examples of protein-regulating  
chemotherapies include enalapril, an inhibitor of angiotensin-  
converting enzyme, for the treatment of sickle cell disease.  
[Falk, R. J., Scheinman, J., Phillips, G., Orringer, E.,  
30 Johnson, A., and Jennette, J. C., *N. Engl. J. Med.*, 326, 910-  
915 (1992)]. A chemotherapy derived from azole regulated  
cytochrome P-450 dependent testosterone biosynthesis [Bossche,  
H. V., Lauwers W., Willemsens, G., Cools, W., *Prog. Clin.*  
*Biol. Res.*, 185A, 187 (1985)].

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At present, both procaryotes and eucaryotes are utilized as hosts for commercial production of recombinant proteins. The choice of one over the other is based on the structural complexity of the protein being produced and the desired yield. If a protein can be produced in a biologically active form from either host, procaryotes are preferred: they grow faster and express more protein than animal cells [Marino, M. H., *BioPharm*, July/August, 18 (1989); Bebbington, C. and Hentschel, C., *Trends Biotechnol.*, 3, 314 (1985)]. Doubling times are in hours rather than days. Similarly, yields are in grams of protein per liter media rather than milligrams per liter. Eucaryotes are chosen as hosts when procaryotes are unable to produce functional protein [Marino, M. H., *BioPharm*, July/August, 18 (1989); Bebbington, C. and Hentschel, C., *Trends Biotechnol.*, 3, 314 (1985)]. This typically occurs when the protein requires post-translational modification (e.g., glycosylation, phosphorylation or macromolecular assembly) to be functional. Bacteria cannot perform post-translational modifications at all; simple eucaryotes such as yeast do so to a limited extent; but complex eucaryotes such as animal cells, with few exceptions, perform the entire complement of post-translational modifications.

Commercial production of recombinant proteins from animal cells requires that the production process be reliable, yielding consistent amounts of product with reproducible biological activity. Such stringency has been achieved in vitro from animal cells cultured in a bioreactor which provides a controlled environment for cell growth.

Several bioreactor designs have been employed in the past for the cultivation of animal cells [Merten, O. W., *Trends Biotechnol.*, 5, 230 (1987); Nelson, K.L., *BioPharm.*, February, 42 (1988); Tramper, J., Smit, D., Straatman, J., and Vlak, J. M., *Bioprocess Eng.*, 3, 37 (1988)]. These include a stirred-tank reactor, a hollow-fiber reactor containing porous fiber

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5 bundles in which cells grow, and an airlift reactor in which  
gas bubbles rise through a draft tube lifting the culture  
fluid to the top of the reactor where it returns to the bottom  
through the annular space between the draft tube and outer  
10 shell of the reactor. [See Inlow et al., U.S. Pat. No.  
5,024,947, entitled "Serum Free Media For the Growth on Insect  
Cells and Expression of Products Thereby", issued June 18,  
1991]. Bioreactors up to 10,000 liters in size are used in  
industry for animal cells [Rhodes, M., *Genetic Engineering*  
15 *News*, 10, March, 7 (1990)]. Choosing the size and type of  
bioreactor for a particular process depends on a number of  
factors such as market demand, cell properties and yield.  
Hollow-fiber reactors are currently limited to volumes on the  
order of one liter [Cracauer et al., U.S. Pat. No., 4,804,628,  
15 entitled "Hollow Fiber Cell Culture Device and Method of  
Operation" issued Feb. 14, 1989; Nelson, K.L., *BioPharm.*,  
March, 34 (1988)]. Airlift reactors can not readily keep  
microcarrier cultures (attachment-dependent cells growing  
bound to beads that are typically 100  $\mu\text{m}$  in diameter) well  
20 mixed [Merten, O. W., *Trends Biotechnol.*, 5, 230 (1987)]. The  
stirred-tank reactor is the system of choice for many  
companies because of its flexibility: it can support the  
growth of both suspension and anchorage-dependent cells, can  
be operated in different feed modes, and can be scaled up to  
25 very large volumes (10,000 liters) [Nelson, K.L., *BioPharm.*,  
March, 34 (1988); Nelson, K.L., *BioPharm.*, February, 42  
(1988)].

30 In bioreactors, mixing is essential for cell  
proliferation: it supplies cells with nutrients and oxygen,  
maintains a homogenous environment throughout the reactor, and  
prevents cells from settling. But in conventional  
bioreactors, mixing can also cause cell damage from  
sufficiently large hydrodynamic forces. In a stirred tank,  
for example, cell damage has been attributed to two mixing

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5 phenomena: bulk-fluid turbulence and gas/liquid interfaces [O'Connor, K. C. and Papoutsakis, E. T., *Biotechnol. Tech.*, 6, 323 (1992); Kunas, K. T. and Papoutsakis, E. T., *Biotechnol. Bioeng.*, 36, 476-483 (1990)]. Typically, these interfaces  
10 arise during cultivation as a result of sparging, vortex formation, turbulent eddies, fluid-wall shear gradients and surface oxygenation. Cell damage from bulk-liquid turbulence has been correlated to the ratio of the Kolmogorov-scale eddy size to bead diameter for microcarrier cultures [Cherry, R. S. and Papoutsakis, E. T., *Bioprocess Eng.*, 1, 29-41 (1986); Croughan, M. S., Hamel, J. F., and Wang, D. I. C., *Biotechnol. Bioeng.*, 29, 130-141 (1987)] and cell diameter for suspension cultures [Kunas, K. T. and Papoutsakis, E. T., *Biotechnol. Bioeng.*, 36, 476-483 (1990); McQueen, A., Meilhoc, E., and Bailey, J. E., *Biotechnol. Letters*, 9, 831 (1987)]. Damage  
15 initiates as the ratio approaches unity and intensifies at lower values. It has been proposed that eddies of the same size or smaller than cell particles (microcarrier beads or individual cells) cause high shear stresses on the cell surface, interparticle collisions and reactor-particle collisions [Cherry, R. S. and Papoutsakis, E. T., *Bioprocess Eng.*, 1, 29-41 (1986)]. For larger eddies, shearing and collisions are minimized as cell particles move in eddy streamlines.  
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25 Because animal cells are not enclosed in a cell wall like bacteria, they are susceptible to hydrodynamic forces within a bioreactor. Agitation, shear and other hydrodynamic phenomena have a profound effect on cell morphology and physiology which can result in cell damage and death. From a  
30 morphological perspective, hydrodynamic forces alter cell shape, adhesion, membrane integrity and spreading [O'Rear, E. A., Udden, M. M., McIntire, L. V., and Lynch E. C., *Biochim. Biophys. Acta*, 691, 274 (1982); Lawrence, M. B., McIntire, L. V., and Eskin, S. G., *Blood*, 70, 1284 (1987); Ives, C. L.,

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5 Eskin, S. G., and McIntire, L. V., *In Vitro Cell. Dev. Biol.*  
22, 500 (1986)]. These morphological changes are accompanied  
by and related to physiological changes in DNA synthesis, mRNA  
synthesis and cytoskeletal rearrangement to name a few  
10 [O'Connor, K. C. and Papoutsakis, E. T., *Biotechnol. Tech.*, 6,  
323 (1992); Diamond, S. L., Sharefkin, J. B., Dieffenbach, C.,  
Frasier-Scott, D., McIntire, L. V., and Eskin, S. G., *J. Cell.*  
*Physiol.*, 143, 364-371 (1990); Sato, M., Levesque, M. J., and  
Nerem, R. M., *Arteriosclerosis*, 7, 276 (1987)]. There are  
several mechanisms by which these hydrodynamic effects can  
cause cell death, including lysis from loss of membrane  
integrity, detachment of anchorage-dependent cells from  
surfaces, and reduced metabolic activity [O'Connor, K. C. and  
15 Papoutsakis, E. T., *Biotechnol. Tech.*, 6, 323 (1992);  
Croughan, M. S., Hamel, J. F., and Wang, D. I. C., *Biotechnol.*  
*Bioeng.*, 33, 731-744 (1989); Petersen, J. F., McIntire, L. V.,  
and Papoutsakis, E. T., *J. Biotechnol.*, 7, 229 (1988)].

Insect cells are distinctly different from animal cells.  
Very little is known about insect cell metabolism. There is  
20 a relatively small body of literature on the subject. Insects  
and vertebrate animals are classified in totally different  
phylogenetic systems. Consequently, insect cells have  
undergone a metabolic evolution that was completely  
independent compared to animal cell metabolic evolution.  
25 Insects have a unique life cycle and, as such, have distinct  
cellular properties. One of these is the lack of  
intracellular plasminogen activators in insect cells which are  
present in vertebrate cells. These differences were  
highlighted when investigators began to express recombinant  
30 intact human plasminogen in Sf9 cells after unsuccessful  
attempts in vertebrate cells. The metabolic differences that  
enabled expression are unknown. [Whitefleet-Smith, J., Rosen,  
E., McLinden, J., Ploplis, V. A., Fraser, M. J., Tomlinson, J.  
E., McLean, J. W., and Castellino, F. J., *Arch. Biochem.*



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5 *Biophys.*, 271, 390 (1989)]. In the latter, plasminogen is rapidly converted by the activators to plasmin. Other differences include high expression levels of protein products ranging from 1 to greater than 500 mg/liter [Marino, M. H., *BioPharm*, July/August, 18 (1989)] and ease at which DNA can be cloned into the cells [Fraser, M. J., *In Vitro Cell. Dev. Biol.*, 25, 225 (1989); Summers, M. D. and Smith, G. E., *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experiment Station Bulletin No. 1555 (Texas A & M University, College Station, Texas, 1988)], both of which make insect cells exceptional hosts for protein production.

15 Cultivation of insect cells is difficult particularly on a large scale or at a high cell density since they are more sensitive to hydrodynamic forces in a bioreactor [Wu, J., King, G., Daugulis, A. J., Faulkner, P., Bone, D. H., and Goosen, M. F. A., *Appl. Microbiol. Biotechnol.*, 32, 249 (1989); Tramper, J., Williams, J. B., and Joustra, D., *Enzyme Microb. Technol.*, 8, 33 (1986)] and have a greater oxygen uptake rate than most vertebrate cells [Maiorella, B., Inlow, D., Shauger, A., and Harano, D., *Bio/Technology*, 6, 1406 (1988)]. The oxygen uptake rate has been calculated to be  $4 \times 10^{-10}$  mmole cell<sup>-1</sup> hr<sup>-1</sup> for *Trichoplusia ni* cells [Maiorella, B., Inlow, D., Shauger, A., and Harano, D., *Bio/Technology*, 6, 1406 (1988)] as compared to 0.1 to  $2 \times 10^{-10}$  mmole cell<sup>-1</sup> hr<sup>-1</sup> for typical vertebrate cells [Nelson, K.L., *BioPharm.*, March, 34 (1988)]. The sensitivity to hydrodynamic forces is so severe that growth of a Lepidopteran cell line, the fall armyworm ovary *Spodoptera frugiperda* (Sf9), is completely inhibited in bench-scale sparged stirred tanks or airlift bioreactors unless surfactant is added to protect the cell membrane [Murhammer, D. W. and Goochee, C. F., *Biotechnol. Prog.*, 6, 391 (1990)]. The combination of enhanced respiration and hydrodynamic sensitivity places severe

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constraints on cell oxygenation: sufficient oxygen must be transported to the insect cells for respiration without inducing hydrodynamic damage to the cells. Conventional methods of oxygenation, such as sparging, supply sufficient oxygen but do so by increasing mixing of growth medium and gas flow rate, resulting in cell destruction from hydrodynamic forces in the medium and at the gas/liquid interface. Developing a bioreactor environment which achieves favorable conditions for growth through a reduction in hydrodynamic forces is a topic of much interest today. One of the features of this invention is the specific non-animal cell culture processes stemming from shear reduction and the discovery of novel in vitro metabolism.

Much of the cultivation and production research on insect cells has been performed with Sf9 cells [Wu, J., King, G., Daugulis, A. J., Faulkner, P., Bone, D. H., and Goosen, M. F. A., *Appl. Microbiol. Biotechnol.*, 32, 249 (1989); Maiorella, B., Inlow, D., Shauger, A., and Harano, D., *Bio/Technology*, 6, 1406 (1988); Godwin, G., Belisle, B., De Giovanni, A., Kohler, J., Gong, T., and Wojchowski, D., *In Vitro*, 25, 17a (1989)]. This cell line is frequently chosen because it grows more robustly than other insect cells, is an immortal cell line, can be adapted from attachment-dependent to attachment-independent growth, is an exceptional host for recombinant protein production as described in the following paragraph, can grow in serum-free media and is commercially available [Fraser, M. J., *In Vitro Cell. Dev. Biol.*, 25, 225 (1989); Wu, J., King, G., Daugulis, A. J., Faulkner, P., Bone, D. H., and Goosen, M. F. A., *Appl. Microbiol. Biotechnol.*, 32, 249 (1989); Godwin, G., Belisle, B., De Giovanni, A., Kohler, J., Gong, T., and Wojchowski, D., *In Vitro*, 25, 17a (1989)]. Individual Sf9 cells have diameters from 10 to 20  $\mu\text{m}$  and can be maintained by following standard published protocols known to those skilled in the art of animal cell culture with the

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following exceptions that are distinctly different from typical animal culture protocols: they grow optimally at 27 rather than 37 °C, external CO<sub>2</sub> is not required for growth since the cells generate significant quantities of the gas as a metabolic end product, and the pH for growth media is 6.2 instead of 7.4 because the cells raise pH as they grow rather than lower it [Fraser, M. J., *In Vitro Cell. Dev. Biol.*, 25, 225 (1989); Godwin, G., Belisle, B., De Giovanni, A., Kohler, J., Gong, T., and Wojchowski, D., *In Vitro*, 25, 17a (1989)].

Sf9 shows great promise as an animal-cell host for the production of recombinant proteins. One of the reasons is the ease at which proteins can be cloned, expressed and purified relative to vertebrate animal cells. Sf9 more readily accepts foreign genes coding for recombinant proteins than many vertebrate animal cells because it is very receptive to viral infection and replication [Bishop, D. H. L. and Possee, R. D., *Adv. Gene Technol.*, 1, 55, (1990)]. Expression levels of recombinant proteins are extremely high in Sf9 and can approach 500mg/liter [Webb, N. R. and Summers, M. D., *Technique*, 2, 173 (1990)]. The cell line performs a number of key post-translational modifications; however, they are not identical to those in vertebrates and, therefore, may alter protein function [Fraser, M. J., *In Vitro Cell. Dev. Biol.*, 25, 225 (1989)]. Despite this, the majority of recombinant proteins that undergo post-translational modification in insect cells are immunologically and functionally similar to their native counterparts [Fraser, M. J., *In Vitro Cell. Dev. Biol.*, 25, 225 (1989)]. In contrast to animal cell culture, Sf9 facilitates protein purification by expressing relatively low levels of proteases and having a high ratio of recombinant to native protein expression [Goswami, B. B. and Glazer, R. O. *BioTechniques*, 10, 626 (1991)].

There has been an explosive growth in the number of proteins that have been expressed in Sf9 with less than 10 by

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1985 to over 100 by 1990. These include  $\beta$ -interferon [Smith, G. E., Summers, M. D., and Fraser, M. J., *Mol. Cell. Biol.*, 3, 2156 (1983)], interleukin-2 [Smith, G. E., Ju, G., Ericson, B. L., Moschera, J., Lahm, H., Chizzonite, R., and Summers, M. D., *Proc. Nat. Acad. Sci. USA*, 82, 8404 (1985)], chimeric plasminogen activators [Devlin, J. J., Devlin, P. E., Clark, R., O'Rourke, E. C., Levenson, C., and Mark, D. F., *Bio/Technology*, 7, 286 (1989)] and macrophage colony stimulating factor [Chiou, C. J., and Wu, M. C., *FEB*, 259, 249 (1990)] to name a few.

Baculoviruses serve as expression systems for the production of recombinant proteins in insect cells. These viruses are pathogenic towards specific species of insects, causing cell lysis [Webb, N. R. and Summers, M. D., *Technique*, 2, 173 (1990)]. They are, and have been, a natural part of the ecosystem where they control the population size of their hosts [Miltenburger, H. G. and Kreig, A., *Advances in Biotechnological Processes*, 3, 291 (1984)]. Some 300 species of baculovirus have been isolated. They are nonhazardous to humans, other vertebrates and indeed most invertebrates. After acute exposure of baculovirus from *Autographa californica*, *Mamestra brassicae* and *Cydia pomonella*, NMRI mice and Chinese hamsters had no chromosomal aberrations or health disturbances [Miltenburger, H. G. and Kreig, A., *Advances in Biotechnological Processes*, 3, 291 (1984)]. Similarly, physical examinations and laboratory tests failed to detect any abnormalities in humans given various baculoviruses orally [Heimpel, A. M. and Buchanan, L. K., *J. Invertebr. Pathol*, 9, 55 (1967)].

Baculoviruses are desirable alternatives to conventional chemical insecticides for agricultural pest control because of their selective pathogenicity towards targeted insects, non-pathogenicity towards vertebrates and compatibility with the ecosystem. At least three baculoviruses have been registered

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by the Environmental Protection Agency for commercial distribution as insecticides: the baculoviruses of *Heliothis zea*, *Orgyia pseudotsugata* and *Lymantria dispar* [Miltenburger, H. G. and Kreig, A., *Advances in Biotechnological Processes*, 3, 291 (1984)]. Baculovirus has been successfully tested to control insect populations in Sweden, the Soviet Union, Italy, Canada and the United States [Miltenburger, H. G. and Kreig, A., *Advances in Biotechnological Processes*, 3, 291 (1984)]. At present, chemical insecticides are the primary means of controlling pest populations. Their use, however, is facing growing opposition--they pollute the environment, and insects may become resistant to their effects. It is estimated that 30% of the agricultural pests in the Western Hemisphere can be controlled by insect viruses [Falcon, L. A., in *Viral Pesticides: Present Knowledge and Potential Effects on Public and Environmental Health*, Summers, M. D. and Kawanishi, C. Y., eds. (Health Effects Research Laboratory, Office of Health and Ecological Effects, U.S. Environmental Protection Agency, Research Triangle Park, NC, 1978), p.11]. This is a significant figure. Substituting baculovirus for its chemical counterpart in these cases could substantially reduce the total amount of chemical insecticides used in the environment.

Recombinant protein expression is achieved by cloning the desired gene into baculovirus at the site of the wild-type polyhedrin gene [Webb, N. R. and Summers, M. D., *Technique*, 2, 173 (1990); Bishop, D. H. L. and Possee, R. D., *Adv. Gene Technol.*, 1, 55, (1990)]. The polyhedrin gene is nonessential for infection or replication of baculovirus. It is the principle component of a protein coat in occlusions which encapsulate virus particles. When a deletion or insertion is made in the polyhedrin gene, occlusions fail to form. Occlusion negative viruses produce distinct morphological differences from the wild-type virus. These differences enable a researcher to identify and purify a recombinant

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5 virus. In baculovirus, the cloned gene is under the control of the polyhedrin promoter, a strong promoter which is responsible for the high expression levels of recombinant protein that characterize this system. Expression of recombinant protein typically begins within 24 hours after viral infection and terminates after 72 hours when the Sf9 culture has lysed.

10 **SUMMARY OF THE INVENTION**

15 Insect cells have shown promise for recombinant protein product. This is dependent upon a large scale production system that provides the proper environment. The subject of this invention is recombinant protein production using a unique process for insect cell culture. Higher expression rates and greater yields that can be realized with the new production systems will increase the variety of protein products and lower their cost to consumers. This can result in, for example, new biotherapies to treat disease, more sensitive diagnostic agents that will detect life-threatening illness sooner, and natural insecticides that have greater species specificity. Because recombinant proteins are germane to many disciplines, this invention will have a fundamental impact in many areas of investigation. This invention can be used for commercial protein production.

25 A new process system has been discovered for *in vitro* cultivation of shear-sensitive, invertebrate cells, specifically insect cells, and for the production of recombinant proteins from these cells. Insect cells were cultivated in a bioreactor which is a horizontally rotating culture vessel designed to create low shear by modulating the rotation, preferably in a bioreactor called the High Aspect Ratio Vessel (HARV) described in the published patent application by the National Aeronautics and Space

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Administration in the Scientific and Technical Aerospace Reports Volume 29/Number 9, May 8, 1991, ACC NOS. N91-17531, U.S. Serial No. 625,345 now U.S. Patent No. 5,153,131, entitled "A Culture Vessel With Large Perfusion Area to Volume Ratio," invented by David A. Wolf, Clarence F. Sams and Ray P. Schwarz and filed on December 11, 1990 and issued October 6, 1992, which patent is incorporated by reference in its entirety in this specification.

The insect cells were used for production of recombinant polypeptides herein defined as a type of polypeptide, protein or virus. For the purposes of this invention the terms polypeptide and protein are not intended to limit the scope of production of any selected amino acid sequence. The insect cells are selected and transformed to include a selected DNA sequence encoding the desired polypeptide product. The cells are then cultured in the HARV which is a horizontally rotating culture vessel designed to create low shear conditions by modulating the rotation. The insect cells cultured by this process recombinantly produce the selected polypeptide which can then be collected and purified. A preferred method for transformation of the insect cells utilizes an insect pathogenic virus, specifically a baculovirus, cloned with the selected DNA sequence. If desired, selected DNA sequences can be used in the transformation process that promote the expression of the DNA sequence encoding the polypeptide. Selective initiation methods for producing the recombinant polypeptide may also be utilized. For instance, a regulation sequence that can turn on protein production by exposure to certain conditions or in the presence of certain compounds can be included in the DNA sequence used to transform the insect cells. In an alternative process, the insect cells are used as hosts for virus production. The virus may be wild type or transformed. Lepidoptera cells are a preferred cell for transformation, and other insect cells that can be cultured by

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the process of this invention may be used. The insect cells may be cultured in a HARV or other vessel prior to transformation.

5 The insect cells cultured by the process of this invention produce a cell line with characteristic metabolism regardless of the genetic transformation to produce a new cell line. The metabolically transformed Lepidoptera cell line of this invention has been designated RES. Specifically, both non-infected and baculovirus-infected RES utilize an alternate  
10 metabolic pathway as compared to their Sf9 counterparts characterized by pH change, glucose and amino acid consumption, and lactic acid production. Transformations can be viral or metabolic or both.

15 An alternative process of this invention utilizes an insect pathogenic virus to produce recombinantly the selected polypeptide using the insect cells as a host. The preferred virus is a baculovirus. The virus is prepared to include the incorporation of the selected DNA sequence encoding the polypeptide. Other DNA sequences to promote or enhance the  
20 expression and production of the polypeptide may also be included in the incorporated DNA sequence. The insect cells are cultured as described herein and the selected virus is transferred into the culture vessel inoculating the insect cells. The virus infects the insect cells and uses the insect  
25 cells as a host to replicate and produce the encoded polypeptide. The polypeptide may be collected and purified as desired.

In the preferred process using the HARV, insect cells were grown in a liquid medium suspension without support  
30 matrices such as microcarrier beads. In this environment, the cells aggregated into 3 mm spherical structures that retained high viability in excess of 95%. Maximum cell densities in the HARV range from  $5 \times 10^6$  to  $1 \times 10^7$  viable cells/ml. In contrast, insect cells remain as a single-cell suspension when



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5 cultivated with conventional animal-cell bioreactors such as stirred tanks. Moreover, maximum cell densities ranged between  $1.5 \times 10^6$  to  $4 \times 10^6$  total cells/ml and are on a total rather than a viable cell basis. [Murhammer, D. W. and Goochee, C. F., *Biotechnol. Prog.*, 6, 391 (1990)]. Similar values were reported by Ogonah et al. [1991]. [Ogonah, O., Shuler, M. L., and Granados, R. R., *Biotechnol. Lett.*, 13, 265 (1991)].

10 Further distinctions between the process of this invention and conventional cultivation were observed after insect cells were infected with recombinant baculovirus coding for the protein  $\beta$ -galactosidase ( $\beta$ -gal). Using the process of the present invention, the cultures infected at a concentration of  $1.3 \times 10^6$  viable cells/ml with a multiplicity of infection (MOI) of 10 continued to grow for a period of 35  
15 hours after infection to  $1.7 \times 10^6$  while retaining a viability that exceeded 90%. Under the same infection conditions, conventional cultivation in shaker flasks resulted in a loss in viable cell concentration within 24 hours after infection.  
20 In fact, viable cell concentration remained higher in the HARV throughout the 180 hours of cultivation by approximately a factor of 2 or greater. These higher concentrations can result in a greater yield of baculovirus and recombinant protein from the HARV culture. Moreover, these results are  
25 illustrative of alternate metabolic pathway usage. Not only does this represent a new process for expressing protein, but enables the production of pathogenic virus for use as an insecticide.

30 Production of recombinant  $\beta$ -gal using the present invention surpassed that achieved in shaker flasks. For the infection conditions described in the previous paragraph, the amount of  $\beta$ -gal present was greater by a factor of 2 to 7 during cultivation, resulting in a total yield of protein that ranged from 8 to 33 units of  $\beta$ -gal/ml medium. A unit of  $\beta$ -gal

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is defined as the amount of the enzyme required to hydrolyze 1.0  $\mu$ mole/min o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) at pH 7.3 and 37 °C.

### 5 DETAILED DESCRIPTION OF THE INVENTION

10 Insect cells for in vitro cultivation have been produced and several cell lines are commercially available. This process includes using insect cells capable of culture as described herein. The preferred cell line is Lepidoptera Sf9 cells. Other cell lines include Lepidopteran cell line High Five and cabbage looper "Trichoplusia ni" both available from Invitrogen Corporation. Sf9 insect cells from Invitrogen Corporation are the preferred cell line and were cultivated in 15 the bioreactor freely suspended in serum-free EX-CELL 401 Medium purchased from JRH Biosciences and maintained at 27°C. The preferred bioreactor is the HARV as described above. The metabolically transformed cell line RES derived from the Sf9 cells was cultivated in the HARV using the process of this 20 invention. To inoculate the HARV, it was first half-filled with medium to act as a cushion for the suspended Sf9 cells as they were pipetted into the vessel. The volume of suspended cells necessary for the desired cell density of  $1 \times 10^6$  cells/ml was added to this medium. Additional medium was used 25 to fill the vessel. Two 5-ml syringes half-filled with medium were attached to the small ports and manipulated to remove all air bubbles from the vessel. One syringe was removed, the open small port was capped, and one syringe remained attached for pressure equalization. The vessel was screwed into its 30 base. The initial rotation speed was set at 11 or 12 rpm, with adjustments made to modulate the rotation to create and maintain low shear conditions while maintaining cellular suspension.

The pH was monitored to determine the frequency with

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which to replace conditioned media. As fresh media has a pH of 6.4, the objective was to maintain the pH of the cultures as close to 6.4 as possible so as not to deplete the nutrients necessary for normal cell growth. At best, the cultures could be maintained within a pH range of 6.1 to 6.3. When the pH dropped to 6.1, the culture received a 20% change of medium. The vessel rotation was stopped, and the culture remained stationary for about 5 minutes until the medium near the top port had clarified. Conditioned medium (10 ml) was removed through the top port and replaced with fresh medium. Air bubbles were eliminated from the vessel, and rotation was resumed.

Cell density and viability of the HARV cultures were checked by staining a sample with trypan blue and examining with a hemocytometer under a microscope at low magnification. Glucose concentration of the cultures was monitored with a Yellow Springs Instruments (YSI) Model 27 Industrial Analyzer equipped with a glucose membrane kit (YSI 2365). The analyzer measured the amount of hydrogen peroxide produced in the enzymatic conversion of glucose (with glucose oxidase acting as the enzyme). The glucose concentration was maintained between 200 and 100 mg/dl. When it dropped to 100 mg/dl, the culture received a change of medium sufficient to increase the concentration to 200 mg/dl. The amount of time necessary for the cultures to deplete the concentration from 200 to 100 mg/dl was noted, and the rate of glucose metabolization was determined.

RES cells cultivated in the HARV in this manner, aggregated into spherical structures as large as 3 mm in diameter which readily dissociate with minimal agitation. In contrast, insect cells remain as a single-cell suspension when cultivated with conventional animal-cell bioreactors such as stirred tanks. Aggregated cultures retained high viability in the HARV - in excess of 95%. Maximum cell densities in the

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HARV range from  $5 \times 10^6$  to  $1 \times 10^7$  viable cells/ml. These values are higher and unanticipated in view of the reported findings by Murhammer and Goochee for a sparged bioreactors of the stirred-tank design and for airlift bioreactors. [Murhammer, D. W. and Goochee, C. F., *Biotechnol. Prog.*, **6**, 391 (1990)]. In these cases, maximum cell densities ranged between 1.5 and  $10^6$  to  $4 \times 10^6$  total cells/ml and are on a total rather than a viable cell basis. Similar values were reported by Ogonah et al. [Ogonah, O., Shuler, M. L., and Granados, R. R., *Biotechnol. Lett.*, **13**, 265 (1991)].

Conditioned medium in the HARV was also analyzed for amino acid, ammonia and lactic acid content during cultivation. A Beckman 6300 High Performance Amino Acid Analyzer containing a 10 cm lithium high performance column (Beckman 338051) measured the concentration of amino acids in the medium with ninhydrin detection (Beckman application notes A6300-AN-003, October 1985). Ammonia concentration was determined from a spectrophotometric assay monitoring the reductive amination of 2-oxoglutarate to glutamate in the presence of glutamate dehydrogenase (Sigma kit 171) on a Beckman Synchron CX5 Clinical Analyzer. A second spectrophotometric assay measured lactic acid concentration from the conversion of lactate to pyruvate catalyzed by lactate dehydrogenase (Sigma kit 826) on a Cobas MIRA Chemistry System (Roche Diagnostic System).

The RES cells cultured in the HARV have a different type of metabolism from the parent cell line, Sf9 cells cultured by methods typically used such as shaker cultures. The low-turbulence, low-shear environment of the HARV has a profound effect on cell metabolism relative to that obtained in shakers cultures. In shaker cultures, cells are mixed by orbital-bed rotation rather than impeller mixing, making the shaker culture one of the most gentle forms of conventional cultivation for suspension cells. Glucose utilization is less

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in the HARV; cells consume on average  $6 \times 10^{-6}$  mg/dl cell hr in the HARV as compared to  $3 \times 10^{-5}$  mg/dl cell hr in shaker cultures. The pH profile follows a different trend; pH changes more rapidly in the HARV at a rate of  $2 \times 10^{-7}$   $\Delta$  pH units/cell hr as compared with  $4 \times 10^{-8}$   $\Delta$  pH units/cell hr in shaker cultures. The Sf9 insect cell line with altered metabolism has been designed as cell line RES and is the preferred cell line for recombinant polypeptide production and viral production.

For viral infection, RES cells were exposed to a recombinant *Autographa californica* baculovirus from Invitrogen Corporation. Specifically, the HARV was seeded with Sf9 cells at a density of  $0.7 \times 10^6$  viable cells/ml. The cultivation protocol was followed until the cells saw a normal doubling time (the cell density increased from  $0.7 \times 10^6$  to  $1.3 \times 10^6$  in approximately 24 hours), confirming the onset of exponential growth. The reactor was inoculated with virus at the onset of exponential growth in order to maximize virus yield. For the inoculation, 20% of the conditioned medium was removed and replaced with an equal volume of fresh medium containing the calculated amount of virus for an MOI (multiplicity of infection) of 10. Thereafter, the infected culture received a 10-20% medium change as needed to maintain the pH between 6.1 and 6.3 and the glucose concentration between 100 and 200 mg/dl. The cell density and viability were checked daily, as the baculovirus is pathogenic towards Sf9 cells, causing eventual cell death and lysing. When the number of viable cells dropped to 10% of the total cell number, a maximum virus yield was reached, and the experiment was terminated. This transforms the insect cell line to include a DNA sequence encoding for a selected polypeptide. In this case the baculovirus and  $\beta$ -gal were used as a model system. The model system was then cultured in a horizontally rotating vessel modulated to create low shear conditions which

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facilitated recombinant polypeptide production.

5           Infected cultures were maintained in the HARV and in  
spinner flasks for 180 hours after the initial inoculation.  
During this time, there were profound differences in the  
response of the insect cells to virus infection with these two  
types of bioreactor. Both the HARV and spinner-flask cultures  
were infected at a concentration of  $1.3 \times 10^6$  viable cells/ml  
with a MOI of 10 as described above. The HARV culture grew  
for an unexpected period of 35 hours after infection to  $1.7 \times$   
10    $10^6$  viable cells/ml while retaining a viability that exceeded  
90%. After this initial period of growth, both viability and  
cell concentration dropped until they reached 8.2% and  $1.2 \times$   
 $10^5$  viable cells/ml, respectively, at the end of the  
experiment. In contrast, shaker cultures experienced a  
15   continuous reduction in viability and cell concentration  
throughout the experiment until these values reached 6.4% and  
 $4.0 \times 10^4$  viable cells/ml. It was discovered that over the  
entire 180 hours of cultivation, viable cell concentration  
remained higher in the HARV by approximately a factor of 2 or  
greater. The improvement is attributed to the unexpected  
20   altered metabolism. Metabolism is focused on viral products  
and not diverted to the repair damage results from detrimental  
shear forces. These higher concentrations can result in a  
greater yield of baculovirus and recombinant proteins from the  
HARV culture.

25           After infection with baculovirus, RES cells in the HARV  
retain a distinctive metabolic profile that distinguish them  
from Sf9 cells. Glucose utilization and change in the pH of  
conditioned medium are examples of this behavior. The pH of  
both shaker and HARV cultures changed at approximately the  
30   same rate during the initial 24 hours of infection:  $3.1 \times 10^{-9}$   
 $\Delta$  pH units/cell hr in shaker flasks and  $2.5 \times 10^{-9}$   $\Delta$  pH  
units/cell hr in the HARV. For the remainder of the  
experiment in shaker flasks, pH changed at a slightly higher

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rate of  $4.7 \times 10^{-9}$   $\Delta$  pH units/cell hr after 60 hours of infection and  $6.0 \times 10^{-9}$   $\Delta$  pH units/cell hr after 180 hours. Rather than an increase, RES cultures in the HARV experience a decrease in the rate of pH change as infection progressed to  
5  $0.85 \times 10^{-9}$   $\Delta$  pH units/cell hr after 60 hours and  $0.29 \times 10^{-9}$   $\Delta$  pH units/cell hr after 180 hours. There are analogous data for glucose. Glucose utilization in the HARV was less than in shaker flasks. In the HARV, the rate at which glucose was consumed was reduced from a value of  $1.4 \times 10^{-7}$  mg/dl cell hr  
10 after 35 hours of infection to  $0.67 \times 10^{-7}$  mg/dl cell hr after an additional 54 hours. For shaker flasks, the initial rate was higher at  $7.7 \times 10^{-7}$  mg/dl cell hr after 35 hours. This rate also was lowered as the infection progressed but only to  $2.0 \times 10^{-7}$  mg/dl cell hr.

15 To determine virus production as a function of time, infected cultures were titered daily using the following blue plaque assay from Invitrogen Corporation. Sf9 cells were seeded into four 60 x 15 mm animal cell culture plates at 80% confluency and allowed to attach for 30 to 45 minutes. The  
20 conditioned medium was removed from the attached cells after rocking the plates from side to side to pick up dead cells and debris. Each plate was washed with 1 ml fresh medium to remove any remaining debris, being careful not to pipet across the cell layer as this would have dislodged the attached  
25 cells.

Ten-fold dilutions of the virus inoculum were prepared. For the first dilution ( $10^{-1}$ ), 0.11 ml of cell-free virus sample was added to 0.99 ml of fresh medium. For the second dilution ( $10^{-2}$ ), 0.11 ml of the first dilution was added to  
30 0.99 ml fresh medium. This process was continued for subsequent dilutions through  $10^{-8}$ .

The virus dilutions were added to the prepared plates. One milliliter of a dilution was uniformly spread on each plate, making four plates, one each of  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$ , and

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10<sup>-5</sup> dilutions. The virus was allowed to infect the cells for one hour. After one hour, the virus inoculum was drawn off, leaving the attached, infected cells on the plate.

5 The agarose was prepared during the hour in which the virus was infecting the cells. For four plates, 0.38 g agarose was added to 12.5 ml Millipore water and autoclaved at 120°C for 15 minutes. Once autoclaved, the agarose was maintained at 40°C. The 2X Hink's TNM-FH Medium (containing fetal bovine serum to compensate for the toxicity of the agarose) was warmed to 40°C. Three milligrams of X-gal were 10 mixed with 0.15 ml DMSO. To the warmed agarose, 0.125 ml of the X-gal-DMSO mixture and 12.5 ml of the 2X medium were added. Each plate received 5 ml of the agarose mixture, being careful not to dislodge the attached cells by pipetting across 15 the cell layer. Once the agarose had solidified, the plates were stored in a plastic bag with a damp paper towel to create a humid environment to prevent cracking of the agarose.

By the tenth day, blue recombinant plaques were visible. The virus titer was determined by taking the number of plaques 20 on a plate and dividing by the dilution factor of that plate.

$\beta$ -Galactosidase ( $\beta$ -Gal) served as a model protein to investigate recombinant protein production from baculovirus-infected insect cells in the HARV and baculovirus served as a model virus for pathogen production. This protein is frequently used in Sf9 studies since it is readily available 25 and can be easily assayed. A mutant form of the baculovirus *A. californica* (or a pathogen type in an alternate embodiment for virus production) containing the gene coding for  $\beta$ -Gal was used as a cloning vector to introduce and express the  $\beta$ -Gal gene in Sf9 cells. As the virus replicates, the  $\beta$ -Gal gene is 30 expressed along with native viral proteins. The effect that the HARV has on recombinant  $\beta$ -Gal production is two fold; it enhances both total and intrinsic production. Because the HARV cultures insect cells to larger cell densities than other



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conventional bioreactors, the overall production of the HARV culture on a per volume basis is larger than in conventional systems. On an intrinsic level, the HARV also raises the rate of recombinant protein synthesis through viral infectivity and/or cell metabolism. As for the former, the low-turbulence environment of the HARV facilitates adherence of viral particle to the cell surface. As for the latter, the changes that the HARV causes in cell metabolism may result in an increase in gene transcription, protein translation, post-translational processes and protein secretion from the cell.

The level of intracellular and extracellular recombinant  $\beta$ -galactosidase expression was measured with a spectrophotometric assay on a Thermomax microplate reader. The process for collecting the recombinantly produced polypeptide included pipetting spent cell culture media from the culture vessel into a test tube. The contents of the test tube were then assayed for the amount of  $\beta$ -gal present in the spent media. In an alternate embodiment, spent cell culture media with suspended cells was pipetted into a test tube. The test tube was centrifuged at 500 x g to pellet cells containing recombinantly produced polypeptide. The supernatant was removed and the cells were lysated by standard sonication or detergent preparations. The lysate was suspended in buffer and assayed for recombinantly produced polypeptide, for example  $\beta$ -gal. In an alternate embodiment the spent cell culture media containing recombinantly produced polypeptide or the lysate containing recombinantly produced polypeptide can be purified by standard molecular weight exclusion filtration, by column chromatography, by immunoprecipitation/absorption, or by centrifugation techniques known in the art for the specific recombinantly produced polypeptide. Specifically, the assay determined the amount of  $\beta$ -gal present based on the enzymatic conversion of o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) to o-nitrophenol

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and galactose. It measured the steady-state rate of ONPG consumption at 410 nm, 37°C, and pH 7.3 in 250  $\mu$ l of solution of 0.08 M NaPO<sub>4</sub>, 0.11 M 2-mercaptoethanol, 0.001 M MgCl<sub>2</sub>-6H<sub>2</sub>O, and 0.002 M ONPG and 8  $\mu$ l of enzymatic  $\beta$ -gal sample. At a  
5 wavelength of 410 nm, the molar extinction coefficient for ONPG is 3.5 mmole<sup>-1</sup>cm<sup>-1</sup>. Doubling the amount of  $\beta$ -gal present did not affect measurements of rate per unit amount of enzyme.

In the HARV,  $\beta$ -gal began to accumulate intracellularly 35 hours after the initial infection. Extracellular expression  
10 of the recombinant protein began 12 hours later. Using the infection protocol described above, the maximum  $\beta$ -gal concentration achieved in the HARV was 29 U/ml medium intracellularly and 7 U/ml extracellularly after 105 and 140 hours of infection, respectively. By definition, a unit of  $\beta$ -  
15 gal hydrolyzes 1.0  $\mu$ mole/min ONPG at pH 7.3 and 37°C. The maximum yield of  $\beta$ -gal (i.e., the combination of its intracellular and extracellular concentration) was 33 U/ml also at 105 hours. From 105 to 180 hours when the experiment was terminated, the  $\beta$ -gal yield dropped to 9 U/ml possibly as  
20 the result of denaturation or proteolytic activity. For comparison, the maximum intracellular and extracellular concentrations of  $\beta$ -gal in shaker cultures infected under identical conditions were 3.7 U/ml at 95 hours and 3.2 U/ml at 155 hours, respectively. The maximum yield occurred at 95  
25 hours and was 5 U/ml. As in the HARV, the yield dropped to 4.3 U/ml as the experiment progressed to 180 hours. During the course of the experiment it was discovered that the yield of  $\beta$ -gal was greater in the HARV by a factor ranging between 2 to 7.

30 The disclosure herein will be understood by those skilled in the art. It is not intended to limit the claimed invention to those processes and products expressly discussed. Alternative methods to practice this invention will be recognized by those skilled in the art.

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ABSTRACT

A process has been developed for recombinant production of selected polypeptides using transformed insect cells cultured in a horizontally rotating culture vessel modulated to create low shear conditions. A metabolically transformed insect cell line is produced using the culture procedure regardless of genetic transformation. The recombinant polypeptide can be produced by an alternative process using the cultured insect cells as host for a virus encoding the described polypeptide such as baculovirus. The insect cells can also be a host for viral production.