EXHIBIT 7

McGRAW-HILL DICTIONARY OF SCIENTIFIC AND TECHNICAL TERMS

Sixth Edition

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On the cover: Representation of a fullerene molecule with a noble gas atom trapped inside. At the Permian-Triassic sedimentary boundary the noble gases helium and argon have been found trapped inside fullerenes. They exhibit isotope ratios quite similar to those found in meteorites, suggesting that a fireball meteorite or asteroid exploded when it hit the Earth, causing major changes in the environment. *(Image copyright © Dr. Luann Becker. Reproduced with permission.)*

Over the six editions of the Dictionary, material has been drawn from the following references: G. M. Garrity et al., *Taxonomic Outline of the Procaryotes,* Release 2, Springer-Verlag, January 2002; D. W. Linzey, *Vertebrate Biology,* McGraw-Hill, 2001; J. A. Pechenik, *Biology of the Invertebrates,* 4th ed., McGraw-Hill, 2000; *U.S. Air Force Glossary of Standardized Terms,* AF Manual 11-1, vol. 1, 1972; F. Casey, ed., *Compilation of Terms in Information Sciences Technology,* Federal Council for Science and Technology, 1970; *Communications-Electronics Terminology,* AF Manual 11-1, vol. 3, 1970; P. W. Thrush, comp. and ed., *A Dictionary of Mining, Mineral, and Related Terms,* Bureau of Mines, 1968; *A DOD Glossary of Mapping, Charting and Geodetic Terms,* Department of Defense, 1967; J. M. Gilliland, *Solar-Terrestrial Physics: A Glossary of Terms and Abbreviations,* Royal Aircraft Establishment Technical Report 67158, 1967; W. H. Allen, ed., *Dictionary of Technical Terms for Aerospace Use,* National Aeronautics and Space Administration, 1965; *Glossary of Stinfo Terminology,* Office of Aerospace Research, U.S. Air Force, 1963; *Naval Dictionary of Electronic, Technical, and Imperative Terms,* Bureau of Naval Personnel, 1962; R. E. Huschke, *Glossary of Meteorology,* American Meteorological Society, 1959; *ADP Glossary,* Department of the Navy, NAVSO P-3097; *Glossary of Air Traffic Control Terms,* Federal Aviation Agency; *A Glossary of Range Terminology, White Sands Missile Range, New Mexico,* National Bureau of Standards, AD 467-424; *Nuclear Terms: A Glossary,* 2d ed., Atomic Energy Commission.

McGRAW-HILL DICTIONARY OF SCIENTIFIC AND TECHNICAL TERMS, Sixth Edition

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FENNEL

Fennel (*Foeniculum vulgare*). (*USDA*)

fenestrated membrane [HISTOL] One of the layers of elastic tissue in the tunica media and tunica intima of large arteries. { 'fen·ə,strād·əd 'mem,brān }

fenestration [ARCH] The arrangement of openings, especially windows, in the wall of a building. [BIOL] **1.** A transparent or windowlike break or opening in the surface. **2.** The presence of windowlike openings. { ,fen·ə'strā·shən }

fenitrothion [ORG CHEM] $C_9H_{12}NO_5PS$ A yellow-brown liquid, insoluble in water; used as a miticide and insecticide for rice, orchards, vegetables, cereals, and cotton, and for fly and mosquito control. { ,fen·ə·trō'thī,ān }

fennel [BOT] *Foeniculum vulgare*. A tall perennial herb of the family Umbelliferae; a spice is derived from the fruit. { 'fen·əl }

fennel oil [MATER] The essential oil obtained from fennel; a colorless liquid with aromatic scent and bitter taste, insoluble in water and boiling at 160–220°C; used in medicine, perfumes, and liqueurs. Also known as oil of fennel. { 'fen·əl ,ȯil }

fen peat *See* low-moor peat. { 'fen ,pēt }

Fenske equation *See* Fenske-Underwood equation. { 'fen·skē i,kwā·zhən }

Fenske-Underwood equation [CHEM ENG] Equation in plate-to-plate distillation-column calculations relating the number of theoretical plates needed at total reflux to overall relative volatility and the liquid-vapor composition ratios on upper and lower plates. Also known as Fenske equation. { ¦fen·skē 'ən·dər,wůd i,kwā·zhən }

fenster *See* window. { 'fen·stər }

fensulfothion [ORG CHEM] $C_{11}H_{17}S_2O_2P$ A brown liquid with a boiling point of 138–141°C; used as an insecticide and nematicide in soils. { ,fen,səl·fō'thī,ān }

fentinacetate [ORG CHEM] $C_{20}H_{18}O_2Sn$ A yellow to brown, crystalline solid that melts at 124–125°C; used as a fungicide, molluscicide, and algicide for early and late blight on potatoes, sugarbeets, peanuts, and coffee. Also known as triphenyltinacetate. { ,fent·ən'as·ə,tāt }

fenuron [ORG CHEM] $C_9H_{12}N_2O$ A white, crystalline compound with a melting point of 133–134°C; soluble in water; used as a herbicide to kill weeds and bushes. { ,fen'yu,rän }

fenuron-TCA [ORG CHEM] $C_{11}H_{13}Cl_3N_2O_3$ A white, crystalline compound with a melting point of 65–68°C; moderately soluble in water; used as a herbicide for noncrop areas. { ,fen'yu,rän ¦tē¦sē¦ā }

FEP resin *See* fluorinated ethylene propylene resin. { ¦ef¦ē¦pē 'rez·ən }

ferbam [ORG CHEM] $C_9H_{18}FeN_3S_6$ [iron(III) dimethyldithiocarbamate] A fungicide for protecting fruits, vegetables, melons, and ornamental plants. { 'fər·bəm }

ferberite [MINERAL] $FeNO_4$ A black mineral of the wolframite solid-solution series occurring as monoclinic, prismatic crystals and having a submetallic luster; hardness is 4.5 on Mohs scale, and specific gravity is 7.5. { 'fər·bə,rīt }

ferghanite [MINERAL] $U_3(VO_4)_2·6H_2O$ Sulfur-yellow mineral composed of hydrated uranium vanadate, occurring in scales. { fər'gä,nīt }

fergusonite [MINERAL] $Y_2O_3·(Nb,Ta)_2O_5$ Brownish-black rare-earth mineral with a tetragonal crystal form; it is isomorphous with formanite. { 'fər·gə·sə,nīt }

Fermat numbers [MATH] The numbers of the form $F_n = (2^{(2^n)}) + 1$ for $n = 0, 1, 2, \ldots$. { 'fer·mä ,nəm·bərz }

Fermat's last theorem [MATH] The proposition, proven in 1995, that there are no positive integer solutions of the equation $x^n + y^n = z^n$ for $n \geq 3$. { fer'mäz ¦last 'thir·əm }

Fermat's principle [OPTICS] The principle that an electromagnetic wave will take a path that involves the least travel time when propagating between two points. Also known as least-time principle; stationary time principle. { fer'mäz 'prin·sə·pəl }

Fermat's spiral [MATH] A plane curve whose equation in polar coordinates (r,θ) is $r^2 = a^2\theta$, where a is a constant. { fer'mäz ,spī·rəl }

Fermat's theorem [MATH] The proposition that, if p is a prime number and a is a positive integer which is not divisible by p, then $a^{p-1} - 1$ is divisible by p. { 'fer,mäz ,thir·əm }

ferment [BIOCHEM] An agent that can initiate fermentation and other metabolic processes. { ¦fər¦ment }

fermentation [MICROBIO] An enzymatic transformation of organic substrates, especially carbohydrates, generally accompanied by the evolution of gas; a physiological counterpart of oxidation, permitting certain organisms to live and grow in the absence of air; used in various industrial processes for the manufacture of products such as alcohols, acids, and cheese by the action of yeasts, molds, and bacteria; alcoholic fermentation is the best-known example. Also known as zymosis. { ,fər·mən'tā·shən }

fermentation accelerator [MATER] Substance that speeds chemical fermentation (as for wines) without participating in the resulting chemical changes; can be an enzyme or other catalytic agent. { ,fər·mən'tā·shən ak'sel·ə,rād·ər }

fermentation tube [MICROBIO] A culture tube with a vertical closed arm to collect gas formed in a broth culture by microorganisms. { ,fər·mən'tā·shən ,tüb }

fermenter [FOOD ENG] A vessel used for fermenting, such as a vat for fermenting mash in brewing. { fər'ment·ər }

ferment oil [MATER] A volatile oil formed by the fermentation of plant material in which the oil was not present originally. { ¦fər¦ment ,ȯil }

fermi *See* femtometer. { 'fer·mē }

Fermi age [NUCLEO] The value calculated for the slowing-down area in the Fermi age model; it has the dimensions of area, not time. Also known as age; neutron age; symbolic age of neutrons. { 'fer·mē ,āj }

Fermi age equation [NUCLEO] An equation in the Fermi age model which states that the Laplacian of the slowing-down density equals the partial derivative of the slowing-down density with respect to the Fermi age. { 'fer·mē ¦āj i,kwā·zhən }

Fermi age model [NUCLEO] A model used in studying the slowing down of neutrons by elastic collisions; it is assumed that the slowing down takes place by a very large number of very small energy changes. { 'fer·mē ,āj ,mäd·əl }

Fermi beta-decay theory [NUC PHYS] Theory in which a nucleon source current interacts with an electron-neutrino field to produce beta decay, in a manner analogous to the interaction of an electric current with an electromagnetic field during the emission of a photon of electromagnetic radiation. { 'fer·mē ¦bād·ə di¦kā ,thē·ə·rē }

Fermi constant [NUC PHYS] A universal constant, introduced in beta-disintegration theory, that expresses the strength of the interaction between the transforming nucleon and the electron-neutrino field. { 'fer·mē ,kän·stənt }

Fermi derivative [RELAT] A generalization of covariant differentiation along a curve that reduces to covariant differentiation when the curve is geodesic; an orthonormal tetrad constructed at each point along a timelike curve such that the Fermi derivative of the tetrad along the curve is zero has (1) its timelike basis vector equal to the curve's unit tangent vector and (2) its spatial basis vectors nonrotating along the curve. { 'fer·mē də,riv·əd·iv }

Fermi-Dirac distribution function [STAT MECH] A function specifying the probability that a member of an assembly of independent fermions, such as electrons in a semiconductor or metal, will occupy a certain energy state when thermal equilibrium exists. { ¦fer·mē di¦rak ,dis·trə'byü·shən ,fəŋk·shən }

Fermi-Dirac gas *See* Fermi gas. { ¦fer·mē di¦rak ,gas }

Fermi-Dirac statistics [STAT MECH] The statistics of an assembly of identical half-integer spin particles; such particles have wave functions antisymmetrical with respect to particle interchange and satisfy the Pauli exclusion principle. { ¦fer·mē di¦rak stə'tis·tiks }

Fermi distribution [SOLID STATE] Distribution of energies of electrons in a semiconductor or metal as given by the Fermi-Dirac distribution function; nearly all energy levels below the Fermi level are filled, and nearly all above this level are empty. { 'fer·mē ,dis·trə,byü·shən }

Fermi energy [STAT MECH] **1.** The average energy of electrons in a metal, equal to three-fifths of the Fermi level. **2.** *See* Fermi level. { 'fer·mē ,en·ər·jē }

Fermi gas [STAT MECH] An assembly of independent particles that obey Fermi-Dirac statistics, and therefore obey the Pauli exclusion principle; this concept is used in the free-electron theory of metals and in one model of the behavior of the nucleons in a nucleus. Also known as Fermi-Dirac gas. { 'fer·mē ,gas }

Fermi hole [SOLID STATE] A region surrounding an electron in a solid in which the energy band theory predicts that the

EXHIBIT 8

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Porro et al. (43) **Pub. Date: Jun. 21, 2007**

(54) **INCREASE IN STRESS TOLERANCE WITH ASCORBIC ACID DURING FERMENTATION**

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(21) Appl. No.: **11/546,951**

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Related U.S. Application Data

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(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**

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C12N 1/21	(2006.01)
C12N 15/74	(2006.01)

(52) **U.S. Cl.** **435/139**; 435/252.3; 435/254.2; 435/483

(57) **ABSTRACT**

A method of increasing stress tolerance in recombinant organisms that have been engineered for industrial production is described. Stress tolerance is increased by making L-ascorbic acid available to the recombinant organism, either by exogenous addition to the culture medium or by endogenous production from D-glucose by the recombinant organism. To enable endogenous production, the recombinant organism is transformed with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a D-arabinono-1, 4-lactone oxidase (ALO). The recombinant organism may be further transformed with a myoinositol phosphatase (MIP).

Figure 1

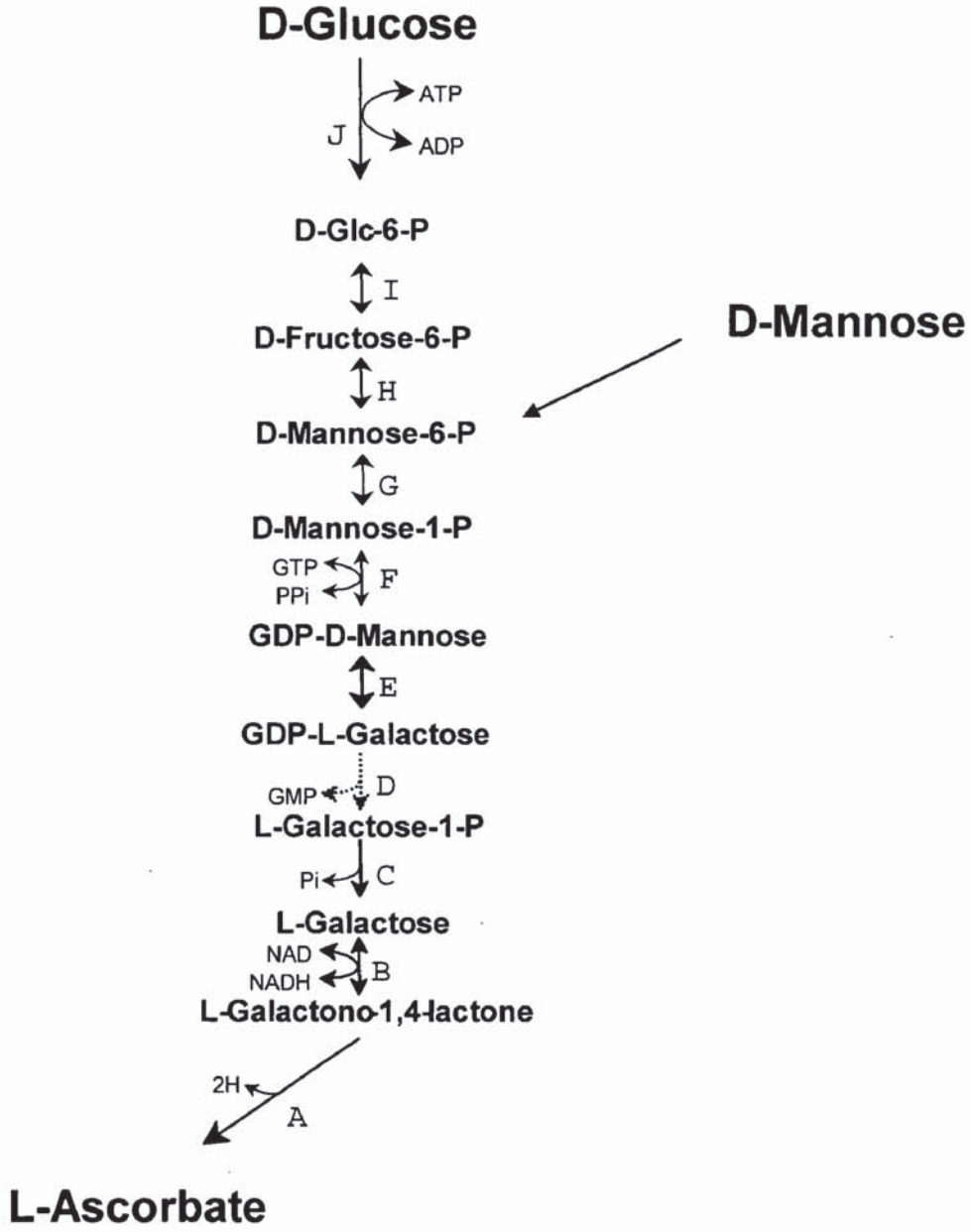

D-Glucose
ATP
J
ADP
D-Glc-6-P
I
D-Fructose-6-P
D-Mannose
H
D-Mannose-6-P
G
D-Mannose-1-P
GTP
PPi
F
GDP-D-Mannose
E
GDP-L-Galactose
GMP
D
L-Galactose-1-P
Pi
C
L-Galactose
NAD
NADH
B
L-Galactono-1,4-lactone
2H
A
L-Ascorbate

Figure 2A

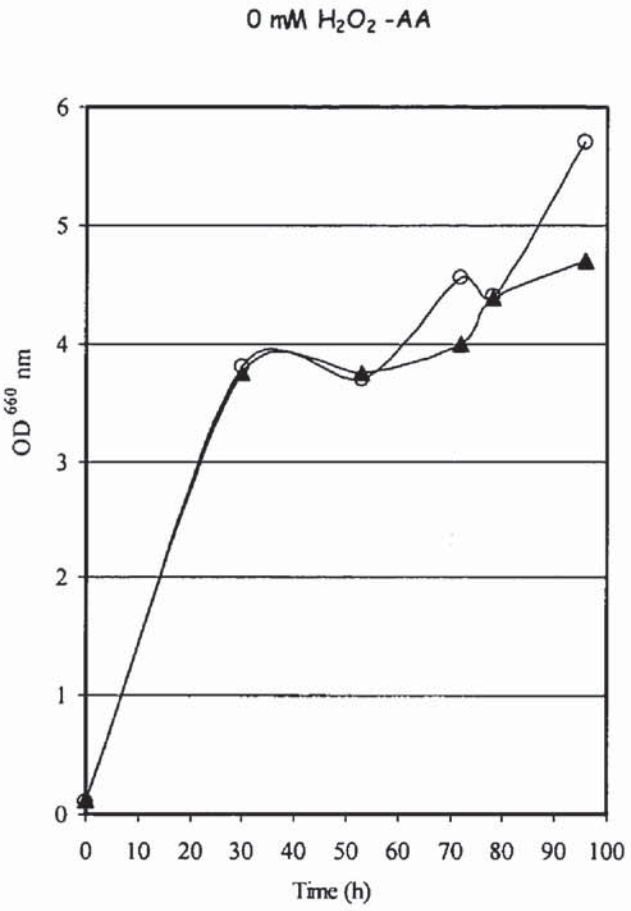

0 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 2B

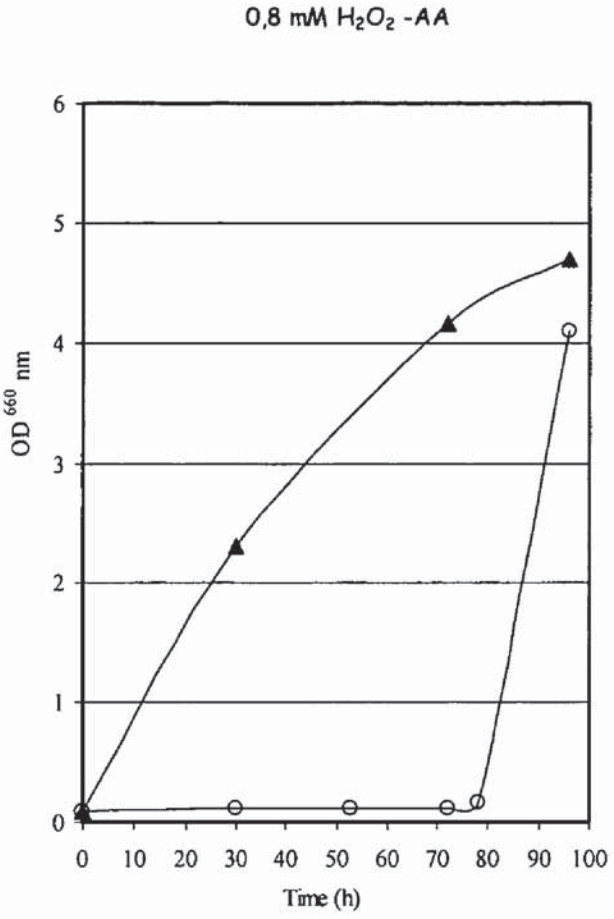

0,8 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 2C

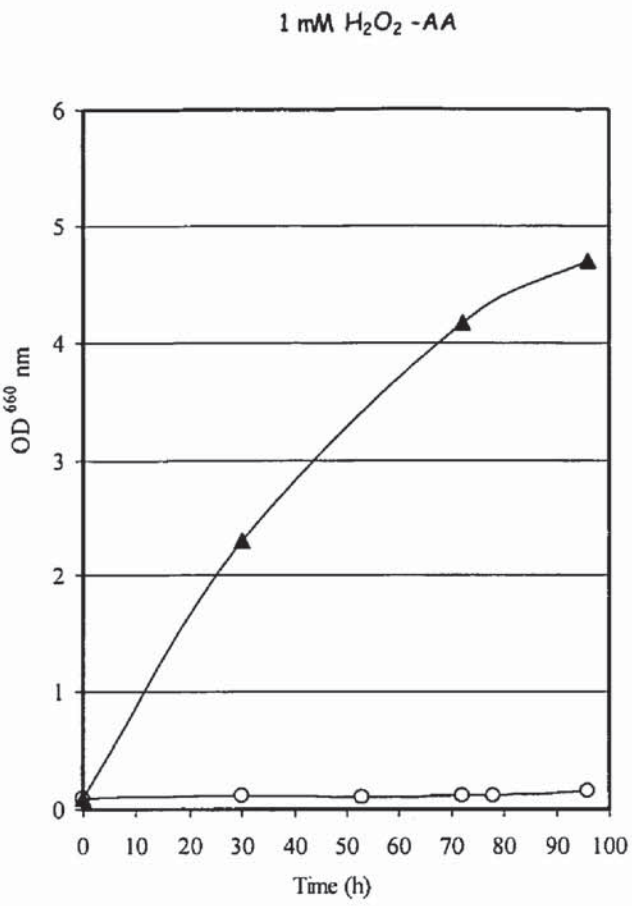

1 mM H_2O_2 -AA
OD 660 nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 3A

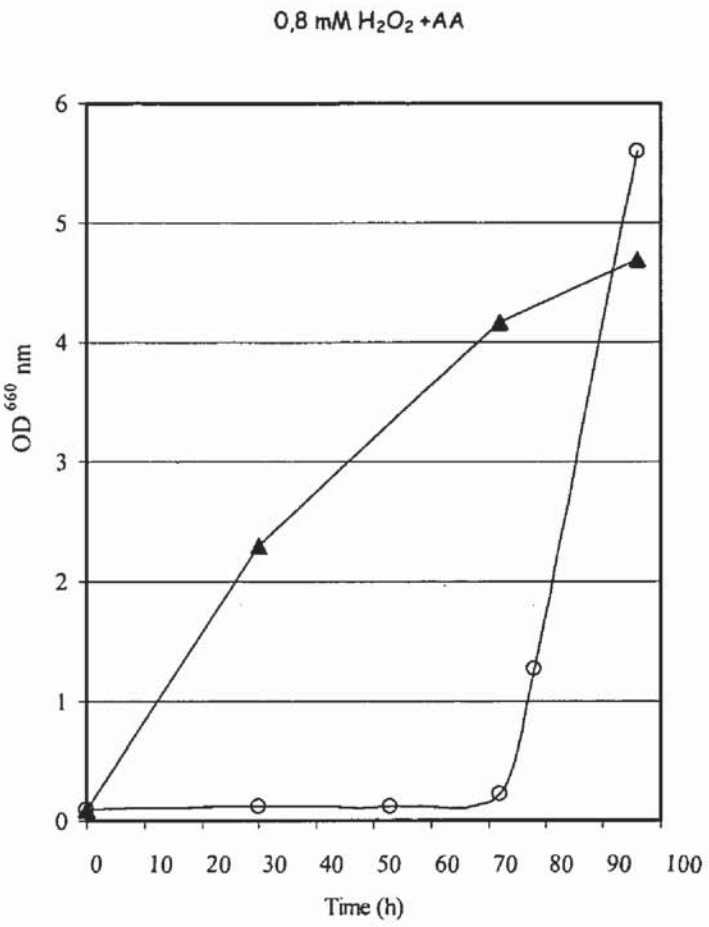

0,8 mM H_2O_2 +AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3B

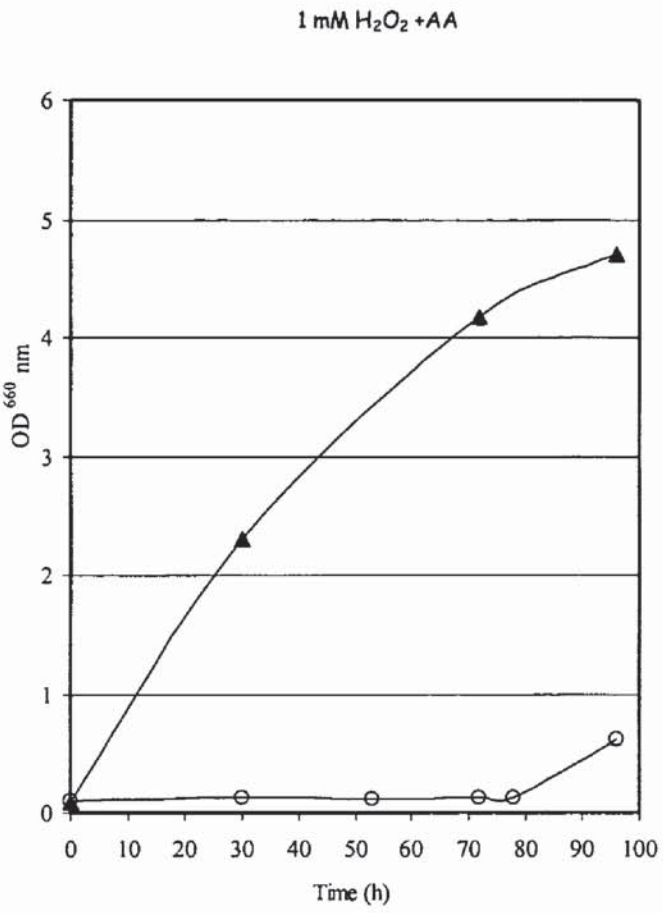
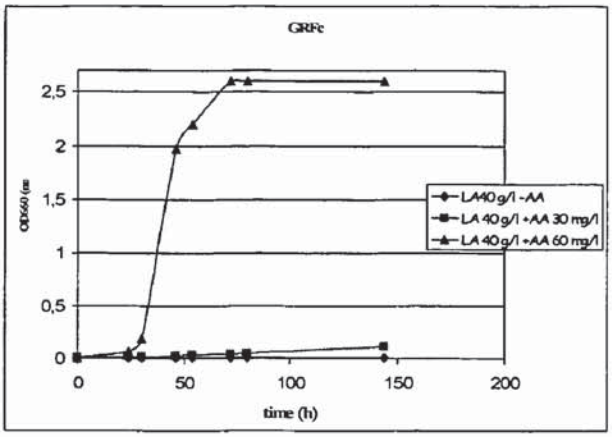
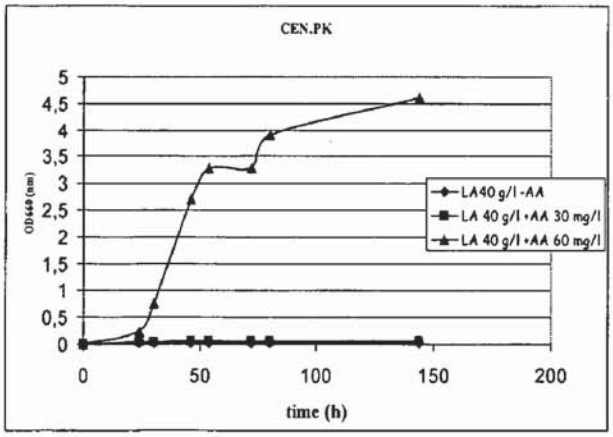

1 mM H_2O_2 +AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3C


GRFe
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l


CEN.PK
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l

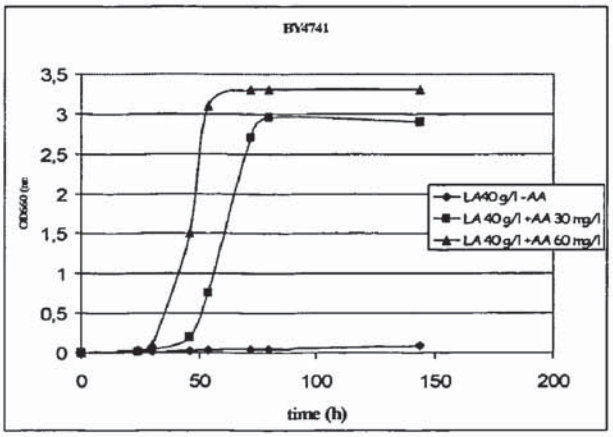

BY4741
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l

Figure 4A

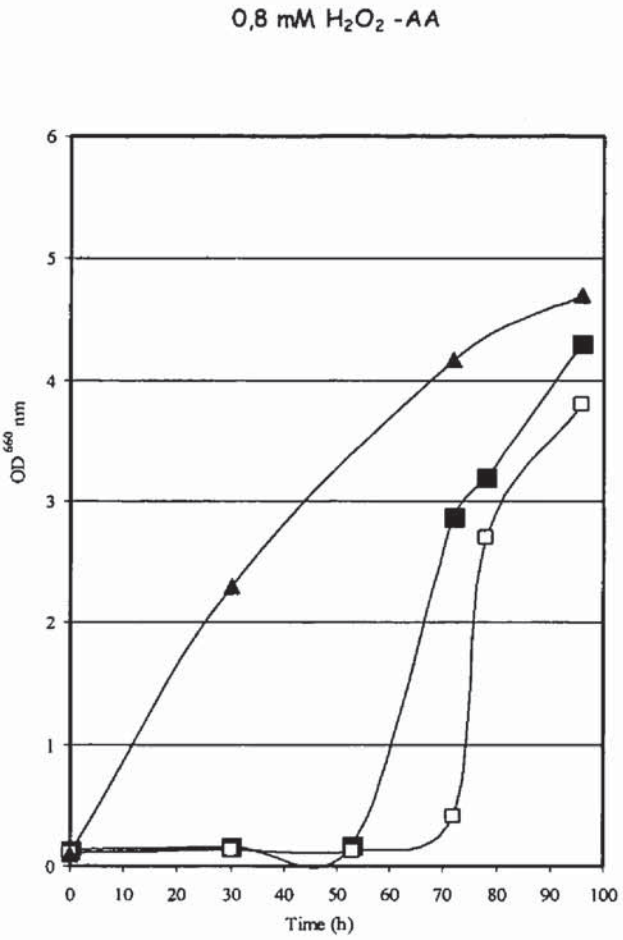

0,8 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 4B

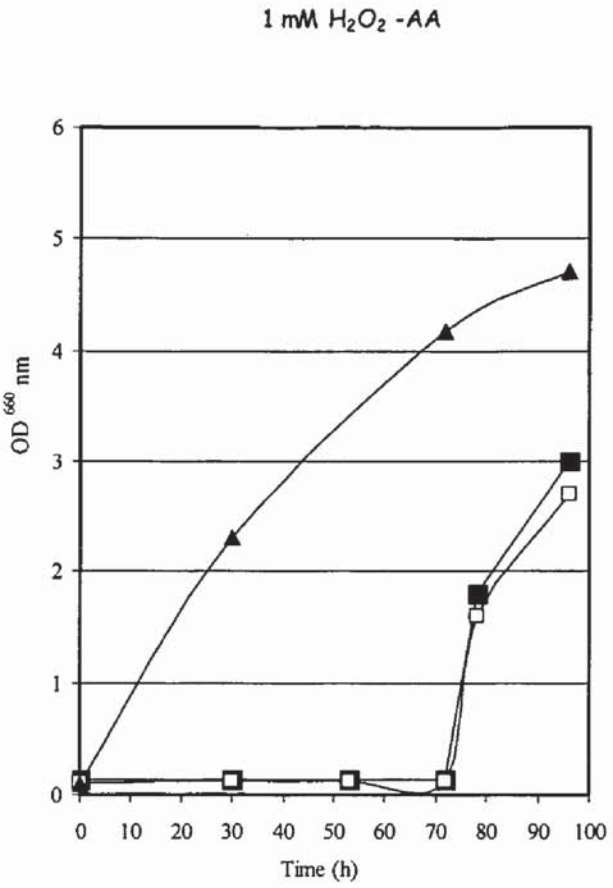

1 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 5A

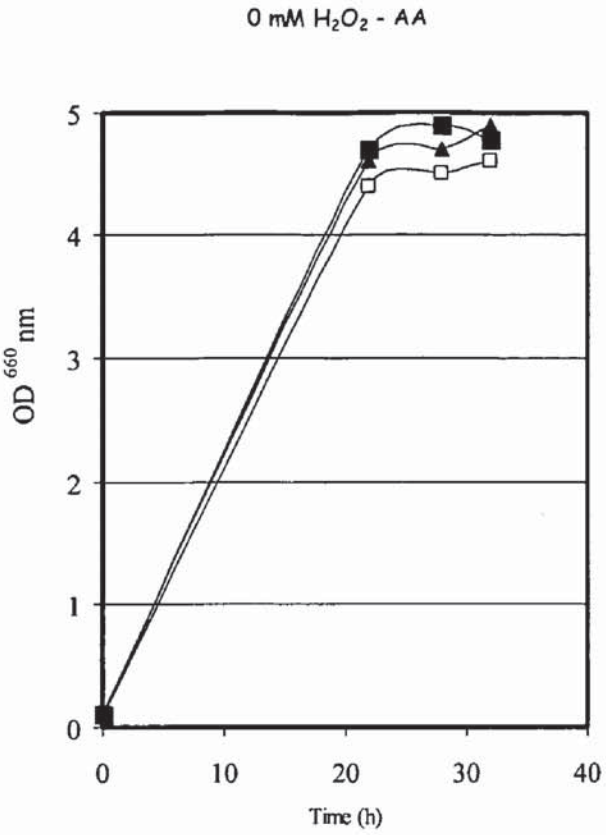


Figure 5b

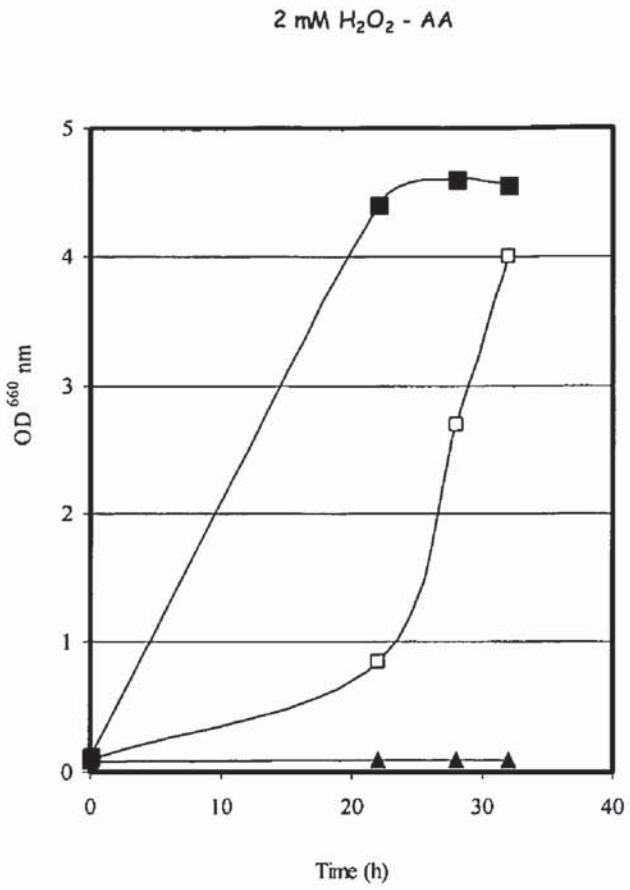


Figure 6

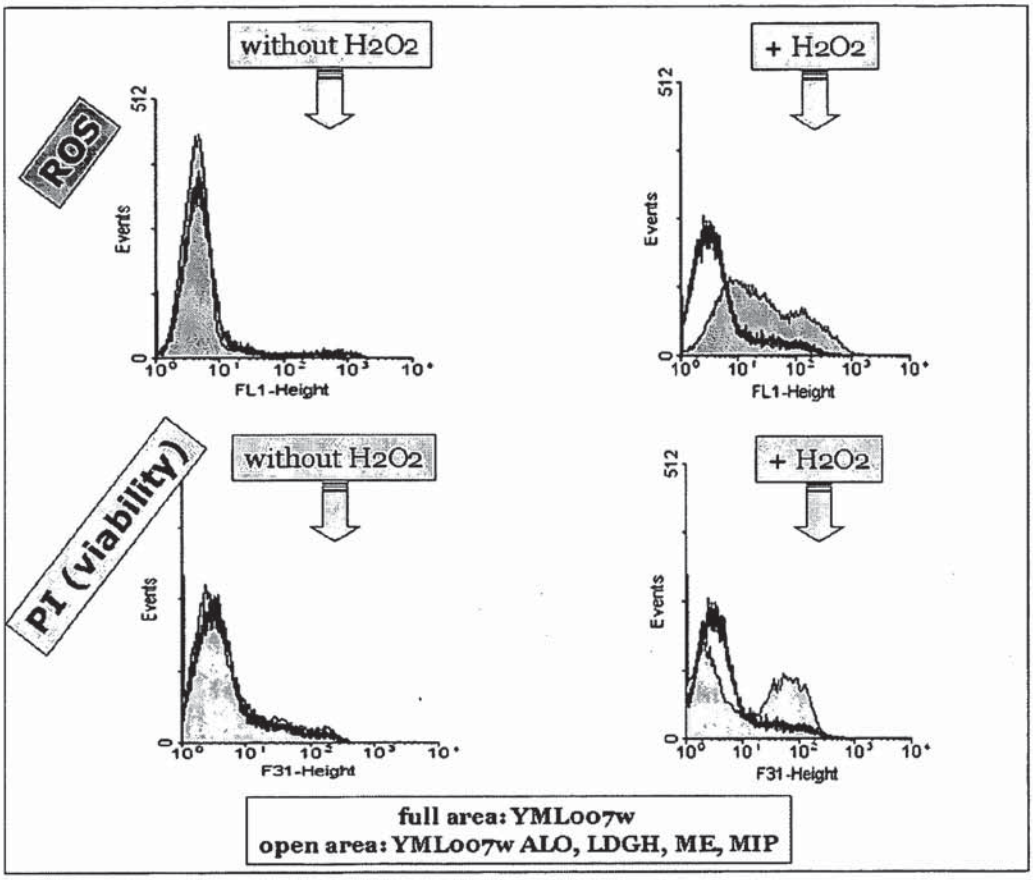
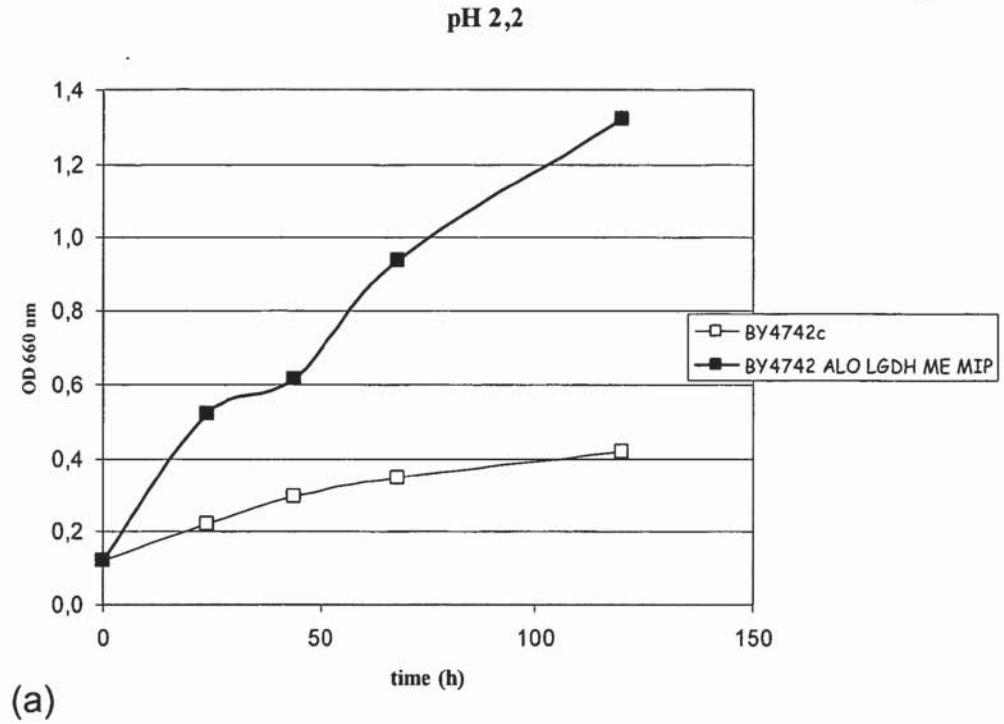
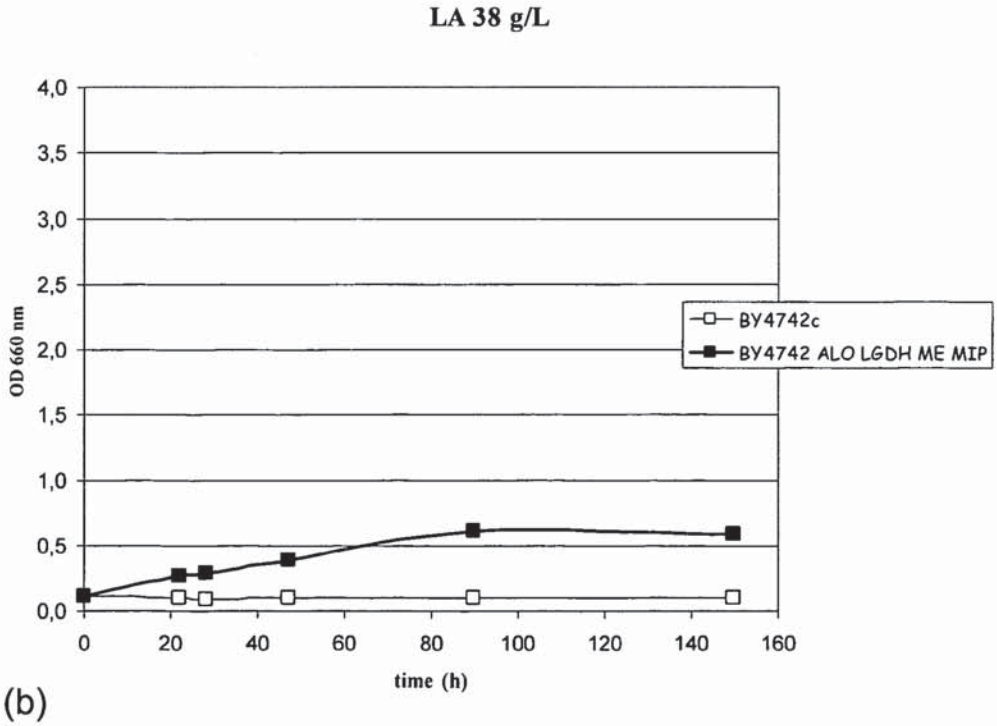

without H2O2
+ H2O2
ROS
PI (viability)
Events
512
0
FL1-Height
F31-Height
full area: YML007w
open area: YML007w ALO, LDGH, ME, MIP

Figure 7

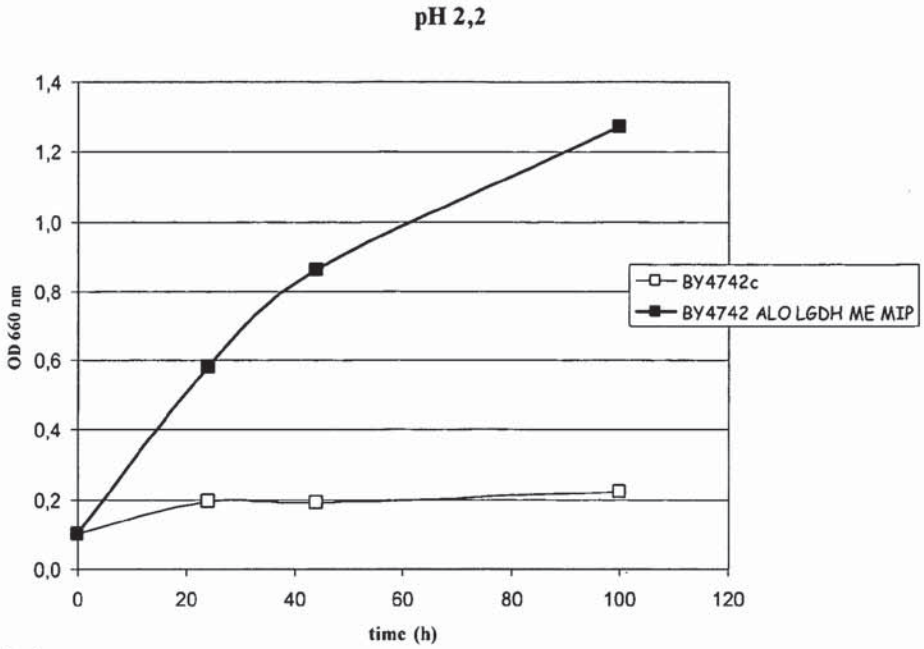

pH 2,2
OD 660 nm
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
50
100
150
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(a)

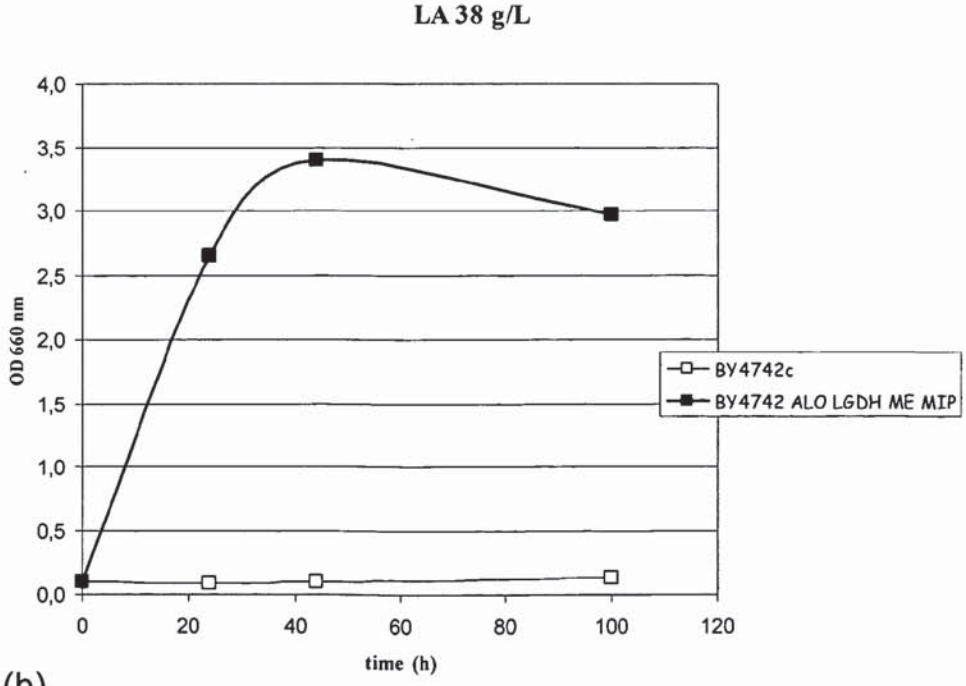

LA 38 g/L
OD 660 nm
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120
140
160
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(b)

Figure 8

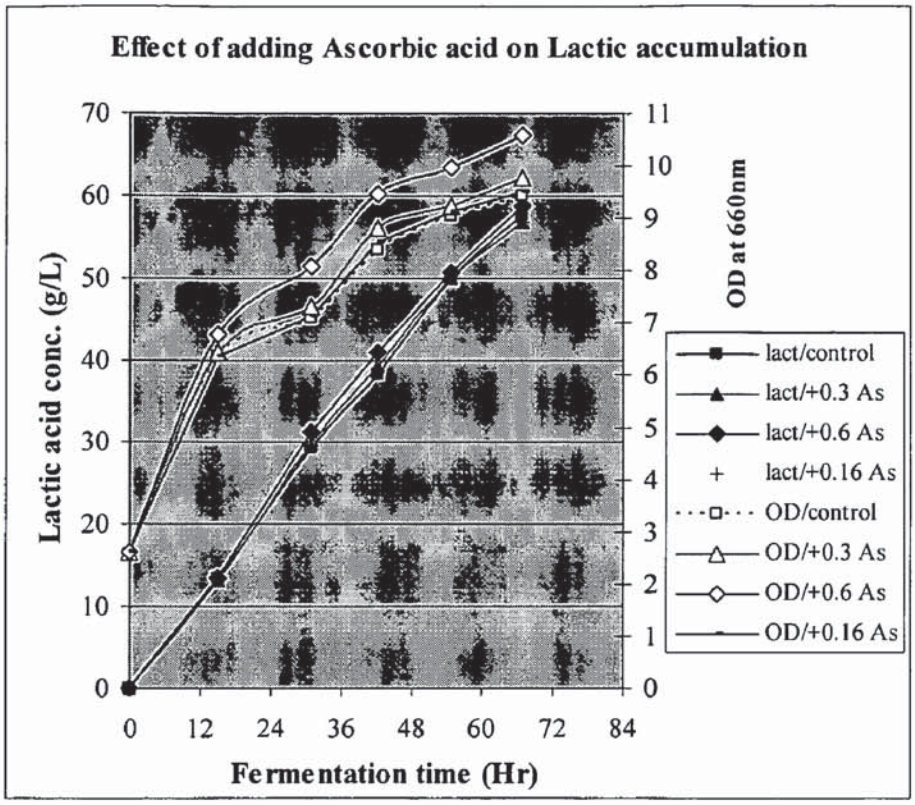

pH 2,2
OD 660 nm
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(a)


LA 38 g/L
OD 660 nm
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(b)

Figure 9


Effect of adding Ascorbic acid on Lactic accumulation
Lactic acid conc. (g/L)
OD at 660nm
Fermentation time (Hr)
0
12
24
36
48
60
72
84
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
9
10
11
lact/control
lact/+0.3 As
lact/+0.6 As
lact/+0.16 As
OD/control
OD/+0.3 As
OD/+0.6 As
OD/+0.16 As

INCREASE IN STRESS TOLERANCE WITH ASCORBIC ACID DURING FERMENTATION

RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Ser. No. 11/105,162, filed on Apr. 13, 2005, which is incorporated herein by reference. This application claims priority from U.S. Ser. No. 11/105,162 and from PCT/US06/012854, filed on Apr. 7, 2006, also incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of increasing stress tolerance in organisms used for industrial production. More particularly, it relates to a process for making L-ascorbic acid available to organisms during industrial production.

BACKGROUND

[0003] Microorganisms and cells can be easily grown on an industrial scale and are frequently employed in the commercial production of compounds such as organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides. Both prokaryotic and eukaryotic microorganisms are today easily and successfully used for the production of heterologous proteins as well as for the production of natural or engineered metabolites. Among prokaryotes, *Escherichia coli* and *Bacillus subtilis* are often used. Among eukaryotes, the yeasts, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, are often used. However, in an industrial process, wherein the organism is used as a means for production, stress on the organism typically leads to lower or zero production of the product, lower or zero productivity, a lower or zero yield of the product, or two or more thereof. Bacteria, yeast, other fungi, cultured animal cells, and cultured plant cells show similar responses to stress. (Close, D. C., et al., *Oxidative Stress, Exercise, and Aging*, H. M. Alessio, A. E. Hagerman, Eds. (2006), pp. 9-23; Sugiyama, K., et al., (2000), J Biol. Chem. 275, 15535-15540; Mongkolsuk, S. and Helmann, J. D. (2002), Molecular Microbiology 45, 9-15). Techniques for minimizing stress would therefore be useful for improving industrial production by these organisms.

[0004] Stresses may have cellular (internal or intracellular) origins, environmental (external or extracellular) origins, or both. Classical examples of the internally-originating stresses include protein and metabolite overproduction (in terms of weight/volume) and protein and metabolite overproductivity (in terms of weight/volume per unit time), among others. Examples of externally-originating stresses include high osmolarity, high salinity, oxidative stress, high or low temperature, non-optimal pH, presence of organic acids, presence of toxic compounds, and macro- and micronutrient starvation.

[0005] Stress is typically caused by stressors (or stimuli). Stressors are negative influences on a cell that require the cell to dedicate more effort to maintain equilibrium than is required in the absence of the stressor. This greater effort can lead to a higher or lower metabolic activity, lower growth rate, lower viability, or lower productivity, among other effects. Stressors are agents of a physical, chemical or biological nature that represent a change in the usual intracellular or extracellular conditions for any given life form. It follows that while a specific condition (e.g., a temperature of 65° C.) may be stressful (or even lethal) to a certain species that normally lives at 37° C., it may be optimal for a thermophilic organism.

[0006] At the cellular level, stress can damage DNA, lipids, proteins, membranes, and other molecules and macromolecules, induce apoptosis (programmed cell death), cell necrosis and cell lysis, and impair cell integrity and cell viability. These effects are often mediated by the generation of reactive oxygen species (ROS).

[0007] ROS can be generated through both intracellular and extracellular stimuli. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain. In addition, cytosolic enzyme systems, including NADPH oxidases and by-products of peroxisomal metabolism, are also endogenous sources of ROS. Generation of ROS also can occur through exposure to numerous exogenous agents and events including ionizing radiation, UV light, chemotherapeutic drugs, environmental toxins, and hyperthermia. Oxidative damage caused by intracellular ROS can result in DNA base modifications, single- and double-strand DNA breaks, and the formation of apurinic/apyrimidinic lesions, many of which are toxic and/or mutagenic. Therefore, the resulting DNA damage may also be a direct contributor to deleterious biological consequences (Tiffany, B. et al., (2004) Nucleic Acids Research 32, 3712-3723).

[0008] One example of an industrial process known to be hampered by stress responses is the production of lactic acid by bacteria or yeast. During a typical lactic acid fermentation, the accumulation of lactic acid in the medium also causes a drop in pH of the medium. The stress of low pH is amplified by the ability of the organic free acid to diffuse through the membrane and dissociate in the higher pH of the cytoplasm. The accumulation of lactic acid inhibits cell growth and metabolic activity. The toxicity of these stresses is mediated at least in part by reactive oxygen species. As a result, the extent of lactic acid production is greatly reduced by the accumulation of lactic acid in the medium.

[0009] The addition of $Ca(OH)_2$, $CaCO_3$, NaOH, or NH_4OH to the fermentation medium to neutralize the lactic acid and to thereby prevent the pH drop is a conventional operation in industrial processes to counteract the negative effects of free lactic acid accumulation. These processes allow the production of lactate(s) by maintaining the pH at a constant value in the range of about 5 to 7, which is well above the pKa of lactic acid (3.86).

[0010] However, this neutralization procedure has major disadvantages. Additional operations are required to regenerate free lactic acid from its salt and to dispose of or recycle the neutralizing cation, which adds expense to the process. The added operations and expense could be lessened if free lactic acid could be accumulated by organisms growing at low pH values. To this end, the use of recombinant yeast that are engineered for industrial production of free lactic acid, and, in particular, recombinant yeast from strains showing greater tolerance for extreme environmental conditions have been described. Engineered strains of recombinant yeast functionally transformed with a gene for lactate dehydrogenase (LDH) in the genera *Saccharomyes, Zygosaccharomyces, Torulaspora*, and *Kluveromyces* have been produced as

described in U.S. Pat. Nos. 6,429,006 and 7,049,108. While these recombinant strains show improved efficiency of lactic acid production at low pH, they are still adversely affected by stresses. In addition, it may be necessary to use organisms or strains that are less tolerant of extreme environmental conditions for the industrial production of specific compounds.

[0011] Ascorbic acid is a known antioxidant that is produced in all higher plants and many higher animals. Ascorbic acid has been shown to modulate the heat shock response in yeast through an effect on ROS(C. Moraitis and B. P. G. Curran. (2004), *Yeast* 21, 313-323), and to improve cell viability and reduce proteolysis of the end product of high cell-density fermentation (Xiao, A. et al. (2006), Appl. Microbiol. Biotechnol. 72, 837-844). These effects suggest that ascorbic acid could improve stress tolerance in general in organisms utilized for industrial production.

[0012] We have shown that recombinant yeast that are functionally transformed to produce L-ascorbic acid, the biologically active enantiomer, from D-glucose produce lower levels of ROS and exhibit improved growth and viability under conditions of low pH, oxidative stress, and in the presence of high concentrations of lactic acid. (Branduardi, P., et al., International Specialised Symposium on Yeast. ISSY25, Systems Biology of Yeast—From Models to Applications. "L-ascorbic acid production from D-glucose in metaboloic engineered *Saccharomyces cerevisiae* and its effect on strain robustness." Hanasaari, Espoo, Finland, Jun. 21, 2006).

[0013] Accordingly, it would be advantageous to industrial fermentation processes if the organisms and cells used for industrial production could endogenously produce L-ascorbic acid from D-glucose.

SUMMARY OF THE INVENTION

[0014] The present invention relates to a method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product. The method comprises making L-ascorbic acid available to the recombinant organism.

[0015] In one embodiment, ascorbic acid is made available by functionally transforming the recombinant organism with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a coding region encoding a D-arabinono-1,4-lactone oxidase (ALO). In a further embodiment, the functionally transformed, recombinant organism is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP).

[0016] In another embodiment, the L-ascorbic acid is made available by culturing the recombinant organism in culture medium containing an effective amount of L-ascorbic acid.

DESCRIPTION OF THE DRAWINGS

[0017] FIG. **1** shows the main plant pathway for the synthesis of L-ascorbic acid from D-glucose.

[0018] FIG. **2** shows the optical density at 660 nm of BY4742 (▲) and YML007w (yap1 mutant strain) (○) yeast in the absence (FIG. **2***a*) and presence (FIGS. **2***b*-**2***c*) of oxidative stress. Yap1 activates genes required for the response to oxidative stress; deletion of this gene leads to the observed phenotype.

[0019] FIG. **3** shows the impact of two stressors on yeast growth. FIGS. **3***a*-**3***b* show the optical density at 660 nm of BY4742 wt (▲) and YML007w (○) yeast in the presence of H_2O_2 in medium +/−ascorbic acid. FIG. **3***c* shows the optical density at 660 nm of wild type yeast GRFc, CEN.PK 113-5D, and BY4741 in the presence of 40 g/l lactic acid and zero, or increasing levels of ascorbic acid.

[0020] FIG. **4** shows the optical density at 660 nm of BY4742 wt (▲); YML007w expressing ALO, LDGH and ME (□); and YML007w expressing ALO, LDGH, ME and MIP (■) yeasts in the presence of oxidative stress (FIGS. **4***a*-**4***b*).

[0021] FIG. **5** shows the optical density at 660 nm of wild type GRFc (▲); GRF18U expressing ALO, LDGH and ME (□); and GRF18U expressing ALO, LDGH, ME and MIP (■) yeast strains in the absence (FIG. **5***a*) and presence (2 mM of H_2O_2) of oxidative stress. (FIG. **5***b*).

[0022] FIG. **6** shows ROS (upper panels) and viability (bottom panels) determination by flow cytometric analyses of *S. cerevisiae* cells producing (YML007w ALO, LDGH, ME, MIP, open area) or not producing (YML007w, full area) ascorbic acid when grown in minimal glucose medium in the presence (right) or absence (left) of hydrogen peroxide.

[0023] FIG. **7** shows growth curves of strains BY4742c (□) and BY4742 ALO, LDGH, ME, MIP (■) inoculated in minimal glucose medium at pH 2.2 (a), or in minimal glucose medium pH 3.0 containing 38 g/l of lactic acid (b).

[0024] FIG. **8** shows growth curves of strains BY4742c (□) and BY4742 ALO, LDGH, ME, MIP (■) that were first grown for 24 h in minimal glucose medium under nonlimiting conditions, and then transferred to minimal glucose medium at pH 2.2 (a), or to minimal glucose medium pH 3 containing 38 g/l of lactic acid (b).

[0025] FIG. **9** shows growth curves, as measured by OD660, and lactic acid production by *S. cerevisiae* strain NRRL Y-30696 grown in minimal glucose medium containing 2.78 g/L $CaCO_3$ and increasing concentrations of ascorbic acid (AA). 0 g/L AA (□), 0.16 g/L AA (+), 0.3 g/L AA (▲), or 0.6 g/L (♦)

DETAILED DESCRIPTION

[0026] The present invention relates to a method of increasing stress tolerance in recombinant cells or organisms that have been engineered for the industrial production of products such as organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides by increasing the available amount of ascorbic acid.

[0027] A "recombinant" cell or organism is one that contains a nucleic acid sequence that is not naturally occurring in that cell or organism, or one that contains an additional copy or copies of an endogenous nucleic acid sequence, wherein the nucleic acid sequence is introduced into the cell or organism or into an ancestor cell thereof by human action. Introduction of the gene into the cell or organism is known as "transformation" and the recipient organism or cell is said to be "transformed." Recombinant DNA techniques are

well-known to those of ordinary skill in the art, who will also understand how to choose appropriate vectors and promoters for the transformation of particular organisms or strains. (For example, see methods in Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual,* 3rd Edition, Cold Spring Harbor Laboratory Press, 2001). Very basically, a coding region of the homologous and/or heterologous gene is isolated from a "donor" organism that possesses the gene. The recombinant organism, as well as the donor, may be a prokaryote, such as a bacterium, or a eukaryote, such as a protozoan, alga, fungus, plant, or animal.

[0028] In one well-known technique, a coding region is isolated by first preparing a genomic DNA library or a cDNA library, and second, identifying the coding region in the genomic DNA library or cDNA library, such as by probing the library with a labeled nucleotide probe that is at least partially homologous with the coding region, determining whether expression of the coding region imparts a detectable phenotype to a library microorganism comprising the coding region, or amplifying the desired sequence by PCR. Other techniques for isolating the coding region may also be used.

[0029] Methods for preparing recombinant nucleotides and transferring them into a host organism are well-known to those of ordinary skill in the art. Briefly, the desired coding region is incorporated into the recipient organism in such a manner that the encoded protein is produced by the organism in functional form. That is, the coding region is inserted into an appropriate vector and operably linked to an appropriate promoter on the vector. If necessary, codons in the coding region may be altered, for example, to create compatibility with codon usage in the target organism, to change coding sequences that can impair transcription or translation of the coding region or stability of the transcripts, or to add or remove sequences encoding signal peptides that direct the generated protein to a specific location in or outside the cell, e.g., for secretion of the protein. Any type of vector, e.g., integrative, chromosomal, or episomal, may be used. The vector may be a plasmid, cosmid, yeast artificial chromosome, virus, or any other vector appropriate for the target organism. The vector may comprise other genetic elements, such as an origin of replication to allow the vector to be passed on to progeny cells of the host carrying the vector, sequences that facilitate integration into the host genome, restriction endonuclease sites, etc. Any promoter active in the selected organism, e.g., homologous, heterologous, constitutive, inducible, or repressible may be used. An "appropriate" vector or promoter is one that is compatible with the selected organism and will generate a functional protein in that organism. The recombinant organism thus transformed is referred to herein as being "functionally transformed."

[0030] The recombinant cells and organisms of the invention can be obtained by any method allowing a foreign DNA to be introduced into a cell, for example, transformation, electroporation, conjugation, fusion of protoplasts or any other known technique (Spencer J. F. et al. (1988), Journal of Basic Microbiology 28, 321-333). A number of protocols are known for transforming yeast, bacteria, and eukaryotic cells. Transformation can be carried out by treating the whole cells in the presence of lithium acetate and of polyethylene glycol according to Ito H. et al. ((1983), J. Bacteriol., 153:163), or in the presence of ethylene glycol and dimethyl sulphoxyde according to Durrens P. et al. ((1990) Curr. Genet., 18:7). An alternative protocol has also been described in EP 361991. Electroporation can be carried out according to Becker D. M. and Guarente L. ((1991) Methods in Enzymology, 194:18). The use of non-bacterial integrative vectors may be preferred when the yeast biomass is used at the end of the fermentation process as stock fodder or for other breeding, agricultural or alimentary purposes.

[0031] The transformed organism is propagated in an appropriate culture medium. Culturing techniques and specialized media are well known in the art. For industrial production, the organism is preferably cultured in an appropriate medium in a fermentation vessel.

[0032] Organisms frequently utilized for industrial production are yeast and bacteria. Yeast to be transformed can be selected from any known genus and species of yeast. Yeast species are described by N. J. W. Kreger-van Rij, ("The Yeasts," (1987) *Biology of Yeasts*, A. H. Rose and J. S. Harrison, Eds. London: Academic Press, Chapter 2) In one embodiment, the yeast genus is selected from the group consisting of *Saccharomyces, Zygosaccharomyces, Candida, Hansenula, Kluyveromyces, Debaromyces, Nadsonia, Lipomyces, Torulopsis, Kloeckera, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Cryptococcus, Trichosporon, Aureobasidium, Lipomyces, Phaffia, Rhodotorula, Yarrowia*, and *Schwanniomyces*. In another embodiment, the yeast is selected from *S. cerevisiae* strains, including GRF18U, W3031B, BY4742 (MATα; his3; leu2, lys2; ura3, EuroScarf Accession No. Y10000); *Z. bailii* ATCC 60483; *K. lactis* PM6-7A; BY4741 (MATα; his3; leu2; met15; ura3, Euroscarf Accession No. Y00000), CEN.PK 113-5D (MATα ura3-52; cir+), and yeast strains engineered to produce lactic acid, including NRRL Y-30696, NRRL Y-30698, NRRL Y-30742; *K. lactis* PM6-7/pEPL2, PMI/C1[pELP2]; *Zygosaccharomyces bailii* ATTC36947/pLAT-ADH, ATCC60483/pLAT-ADH.

[0033] Yeast have been widely utilized in the production of products. Yeast biomass is an important product as cultures for development of food products as well as a nutrient rich food and feed component. Genetic engineering has broadened the value of yeast production systems providing a route to organic acids (Porro, D. et al. (2002), U.S. Pat. No. 6,429,006); vitamins (Shiuan, D., US2003/0104584); polyols (Geertman, J. M, et al., (2006) Metabolic Engineering, June 30:(Epublication); biofuel (Ho, N. W. Y. and Tsao, G. T. (1998), U.S. Pat. No. 5,789,210); (Bosman, F., et al. (2006) U.S. Pat. No. 7,048,930); proteins (Gerard, G. F., et al. (2006). U.S. Pat. No. 7,115,406); and peptides (Lee, S. Y., et al., Lett. Appl. Microbiol (2003), 36, 121-128.).

[0034] Bacteria to be transformed can be selected from any known genus and species of the Eubacteria or the Archaea (also encompassed herein by the term, "bacteria"). Bacteria are cataloged at the NCBI Taxonomy website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy. In one embodiment the bacteria can be selected from the genera *Bacillus, Escherichia, Lactobacillus, Lactococcus, Pseudomonas*, or *Acetobacter.*

[0035] Bacteria have been widely utilized to produce industrial products. The natural range of available products has been extended by mutagenesis and screening and further by genetic engineering. Bacteria provide routes to organic acids (WO2006/083410); amino acids (WO2005/090589);

vitamins (Santos, et al., Abstracts of Papers, 232nd ACS National Meeting, San Francisco, Calif., United States, Sep. 10-14, 2006, BIOT-243); polyols (Dunn-Coleman, N. S., et al. (2006) U.S. Pat. No. 7,074,608); solvents (Harris, L. M., et al. (2001), Journal of Industrial Microbiology & Biotechnology 27, 322-328); biofuels (Ingram, L. O. and Zhou, S. WO2000/071729); therapeutics (Pizza, M., et al. (2006) U.S. Pat. No. 7,115,730); proteins (Gerard, G. F., et al. (2006) U.S. Pat. No. 7,115,406); and peptides (Knapp, S., et al. (1992) U.S. Pat. No. 5,159,062).

[0036] Filamentous fungi are widely utilized to produce organic acids (Bizukojc, M. and Ledakowicz, S., Process Biochemistry (2004), 39, 2261-2268.); and proteins (Wang, L., et al., (2003) Biotechnology Advances 23, 115-129). Filamentous fungi to be transformed can be selected from any known genus and species. Fungi are cataloged at the NCBI Taxonomy Website: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=4751.

[0037] In one embodiment the filamentous fungi can be selected from the genera *Rhizopus, Aspergillus*, or *Trichoderma.*

[0038] In one embodiment of the invention, the recombinant organism is functionally transformed with coding regions that encode a mannose epimerase (D-mannose:L-galactose epimerase; ME), L-galactose dehydrogenase (LGDH); and D-arabinono-1,4-lactone oxidase (ALO). These coding sequences enable the recombinant organism to produce enzymes necessary for the endogenous production of L-ascorbic acid from D-glucose. As a result of transformation with ME, LGDH, and ALO, and endogenous production of L-ascorbic acid, the organism shows increased tolerance to stress when compared with a strain of the same organism that cannot produce L-ascorbic acid.

[0039] An ME is any GDP-mannose-3,5-epimerase (5.1.3.18), that is any enzyme that catalyzes the conversion of GDP-mannose to GDP-L-galactose (FIG. **1**). An exemplary ME is encoded by the sequence listed as SEQ ID NO:1.

[0040] In one embodiment, the ME has at least about 95% identity with SEQ ID NO:1. "Identity" can be determined by a sequence alignment performed using the ClustalW program and its default values, namely: DNA Gap Open Penalty=15.0, DNA Gap Extension Penalty=6.66, DNA Matrix=Identity, Protein Gap Open Penalty=10.0, Protein Gap Extension Penalty=0.2, Protein matrix=Gonnet. Identity can be calculated according to the procedure described by the ClustalW documentation: "A pairwise score is calculated for every pair of sequences that are to be aligned. These scores are presented in a table in the results. Pairwise scores are calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). Both of these scores are initially calculated as percent identity scores and are converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site. We do not correct for multiple substitutions in these initial distances. As the pairwise score is calculated independently of the matrix and gaps chosen, it will always be the same value for a particular pair of sequences."

[0041] In another embodiment, the recombinant organism transformed with the coding sequences for ME, LGDH, and ALO is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP). An MIP is any myoinositol phosphatase (3.1.3.25), that also catalyzes the conversion of L-galactose-1P to L-galactose. L-galactose-1-phosphatase has been annotated as inositol/myo-inositol monophosphatase galactose-1-phosphatase and may be referred to as MIP/VTC4 (Conklin, P. L. et al. (2006) J. Biol. Chem. 281, 15662-70). In one embodiment, the MIP has at least about 95% identity with SEQ ID NO:2. Identity is determined as described above.

[0042] In another embodiment, the recombinant organism is further transformed with a coding region encoding an enzyme selected from L-galactono-1,4-lactone dehydrogenase (AGD), D-arabinose dehydrogenase (ARA) or L-gulono-1,4-lactone oxidase (GLO), as described, for example, in U.S. Pat. No. 6,630,330, which is incorporated herein by reference.

[0043] Although the pathway for the production of ascorbic acid in plants is shown in FIG. **1**, the present invention is not limited to the enzymes of the pathways known for the production of L-ascorbic acid intermediates or L-ascorbic acid in plants, yeast, or other organisms. (Examples of known L-ascorbic acid pathways in plants and animals are described in Conklin, P. L., et al. (2006), J. Biol. Chem. 281, 15662-15670; and in Valpuesta, V. and Botella, M. A. (2004) Trends in Plant Science 12, 573-577). One of ordinary skill in the art will understand that increasing flux through any pathway resulting in L-ascorbic acid biosynthesis will result in production of higher levels of L-ascorbic acid. This can be accomplished by increasing the levels of enzymes in the pathway that are limiting.

[0044] The coding regions for any of the desired enzymes may be isolated from any source or may be chemically synthesized. Following transformation with the coding regions for ME, LGDH, and ALO, (with or without the coding region for MIP), the recombinant organism is cultured in medium containing a carbon source that can be converted to L-ascorbic acid, such as D-glucose.

[0045] When the recombinant organism for industrial production is a eukaryotic organism, it is important to ensure that each of the enzymes used to produce ascorbic acid is appropriately compartmentalized in the eukaryotic cell. This is accomplished by including sequences encoding targeting labels in the recombinant vector. These types of sequences are disclosed, for example, in Alberts, B., et al., *Molecular Biology of the Cell,* 4th Edition, New York: Garland Science Publ., 2002, pages 659-710.

[0046] With respect to the invention, "production" means the process of making one or more products using a recombinant organism. Production can be quantified at any moment in time after commencement of the process by determining the weight of a product produced per weight or volume of the medium on which the recombinant organism's growth and survival is maintained, or weight or volume of the recombinant organism's biomass. "Productivity" means the amount of production, as quantified above, over a given period of time (e.g., a rate such as g/L per hour, mg/L per week, or g/g of biomass per hour). "Yield" is the amount of product produced per the amount of substrate converted into the product. This definition of "yield" also applies to endogenous production of L-ascorbic acid.

[0047] Stress tolerance, as used herein, may manifest as a decrease in the negative impact of stress on the organism,

such as a decline in the production of ROS or a positive effect on productivity, yield, or production. An increase in stress tolerance can be measured by a number of parameters, for example, as an increase in growth rate, an increase in cell density, a decrease in the inhibition of productivity, an increase in viability, an increase in metabolism, or an increase in yield, productivity, or production. An "effective amount" of L-ascorbic acid is an amount of L-ascorbic acid present in the culture medium that gives rise to an improvement in stress tolerance as measured by any of these parameters, when compared with stress tolerance of the organism grown in medium that does not contain L-ascorbic acid.

[0048] As shown in FIGS. **2-5**, yeast transformed with coding sequences for ME, LGDH, and ALO, or with this group of coding sequences plus a coding sequence for MIP, have greater stress tolerance than yeast that are not so transformed. FIG. **7** shows that endogenously produced L-ascorbic acid correlates with increased tolerance to low pH and oxidative stresses. This increased stress resistance can manifest as one or more of increased growth rate of the transformed organism, increased viability of the transformed organism, or increased production by the transformed organism.

[0049] We also show, in FIG. **3**, that the addition of L-ascorbic acid to the fermentation medium improves stress tolerance, in particular, tolerance to low pH and oxidative stress. Accordingly, in one embodiment of the invention, the available amount of ascorbic acid is increased by adding L-ascorbic acid to the fermentation medium. Exogenous L-ascorbic acid may be added to cultures that do or do not produce L-ascorbic acid endogenously.

[0050] Though not wishing to be bound by a single theory, we suggest that the increased stress tolerance results from an increase in antioxidant levels (specifically, L-ascorbic acid) and a reduction in the levels of endogenous reactive oxygen species (ROS) in the organism, imparting greater resistance to oxidative stress, as shown in FIG. **6**. The increased stress tolerance makes organisms that endogenously produce ascorbic acid particularly suitable for industrial production. Such organisms include plant and animal cells that produce ascorbic acid either naturally or through genetic engineering. (e.g., organisms described in Valpuesta, V. and Botella, M. A. (2004) Trends in Plant science 9, 573-577 and genetically engineered plant and animal cells.)

[0051] Organisms with increased stress tolerance that are to be used for industrial production may be created by any methods known to those of skill in the art for engineering recombinant organisms. The organism may be co-transformed with the necessary coding regions for production of L-ascorbic acid (i.e., ME, LGDH, ALO+/−MIP) and the coding sequences for the industrial product that the organism will produce. The organism may first be engineered to express the L-ascorbic acid coding sequences and then subsequently be transformed with coding regions for the industrial product. Alternatively, the organism may first be engineered to produce the industrial product and subsequently be transformed with the coding regions for production of L-ascorbic acid.

[0052] Endogenous production of L-ascorbic acid by the recombinant organism is particularly useful if the recombinant organism is cultivated under conditions of osmotic, pH, temperature, or oxidative stress. Osmotic stress is a condition in which the organism or cell encounters a difference in osmolarity from the optimal osmolarity defined for the respective microorganism. For example, in the yeast *S. cerevisiae*, an osmolarity greater than 500 mOsmol leads to a stress response.

[0053] A pH stress occurs if an organism or strain of organism encounters a difference in pH value from the optimal pH value for that strain of more than one to three pH units. For example, in the wild type strain of the yeast *S. cerevisiae*, the typical optimal pH for performance of bioprocesses is 5.0. A pH of less than 4.0 or more than 6.0 may cause a stress response in this strain that can affect the transcription of pH sensitive genes.

[0054] Temperature stress is a condition in which the organism encounters a cultivation temperature different the optimal temperature value for growth or production for a particular organism. In the yeast, *S. cerevisiae*, a temperature at or above 32° C. can cause stress responses. For the bacterium *E. coli*, a temperature at or above 38° C. can lead to stress responses.

[0055] Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, caused by the reactive oxygen species (ROS). This damage can affect a specific molecule or the entire organism. Reactive oxygen species, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants. Animal cells, as well as single-celled organisms, can be exposed to significant oxidative stress during standard cell culture conditions.

[0056] Endogenous production of L-ascorbic acid is also particularly useful in a cell or organism if it is subjected to stress due to overproduction of a metabolite or a protein. Such stresses may be indicated, for example, by the upregulation of genes related to the UPR (unfolded protein response), which is known in the art. (Foti, D. M., et al. (1999) J. Biol. Chem. 274, 30402-30409).

[0057] In one embodiment, the recombinant organism may be a yeast that has been engineered to produce and secrete lactic acid. The applications of lactic acid and its derivatives encompass many fields of industrial activities (e.g., chemistry, cosmetics, and pharmacy), as well as important aspects of food manufacture and use. Furthermore, today there is growing interest in the production of such an organic acid to be used directly for the synthesis of biodegradable polymer materials.

[0058] Lactic acid may be produced by chemical synthesis or by fermentation of carbohydrates using single-celled organisms. The latter method is now commercially preferred because organisms have been developed that produce exclusively one isomer, as opposed to the racemic mixture generated by chemical synthesis. The most important non-recombinant industrial organisms currently used to produce lactic acid, such as species of the genera *Lactobacillus, Bacillus*, and *Rhizopus*, produce L(+)-lactic acid. Production by fermentation of D(−)-lactic acid or mixtures of L(+)- and D(−)-lactic acid are also known.

[0059] During a typical lactic acid fermentation, the accumulation of lactic acid in the medium is detrimental to

metabolic activity. In addition, the accumulation of lactic acid lowers the pH of the medium, which also inhibits cell growth and metabolic activity. As a result, the extent of lactic acid production is reduced as the lactic acid product accumulates.

[0060] Methods for the construction of recombinant yeasts expressing at least one copy of a lactate dehydrogenase (LDH) gene, which shifts the glycolytic flux towards the production of lactic acid, have been described in U.S. Pat. Nos. 6,429,006 and 7,049,108, both of which are incorporated herein by reference. These references report that lactic acid can be produced by metabolically modified yeasts belonging to the genera of *Kluyveromyces, Saccharomyces, Torulaspora* and *Zygosaccharomyces*. While any yeast species could be used, these species are preferred because these strains can grow and/or metabolize at very low pH, especially in the range of pH 4.5 or less. In addition, genetic engineering methods for these strains are well-developed, and these strains are widely accepted for use in food-related applications.

[0061] The yield of lactic acid can be increased by increasing copy numbers of the LDH gene in each yeast. Higher yields (>80% g/g) of lactic acid may be obtained from these engineered yeast strains if both the ethanolic fermentation pathway and the use of pyruvate by mitochondria are replaced by lactic fermentation. The recombinant yeast can also be transformed to overexpress a lactate transporter, for example, the JEN1 gene encoding for the lactate transporter of *S. cerevisiae*, can to ensure secretion of the product.

[0062] The expression of a LDH gene in yeast strains allows the production of lactic acid at acid pH values so that the free acid is directly obtained and the cumbersome conversion and recovery of lactate salts are minimized. In this invention, the pH of the fermentation medium may initially be higher than 4.5, but will decrease to a pH of 4.5 or less, preferably to a pH of 3 or less at the termination of the fermentation.

[0063] The gene coding for LDH may be from any species (e.g., mammalian, such as bovine, or bacterial), and it may code for the L(+)-LDH or the D(−)-LDH. Alternatively, both types of LDH genes may be expressed simultaneously. In addition, any natural or synthetic variants of LDH DNA sequences, any DNA sequence with high identity to a wild-type LDH gene, any DNA sequence complementing the normal LDH activity may be used.

[0064] The co-expression of ascorbic acid in a lactic acid producing microorganism to improve the stress tolerance and robustness of that organism could be accomplished by introduction of ME, LGDH, ALO, and, optionally, MIP. The transformation of the yeast strains could be carried out by means of either integrative or replicative plasmid or linear vectors. In a particular embodiment of the invention, the recombinant DNA is part of an expression plasmid which can be of autonomous or integrative replication.

[0065] For the production of lactic acid, the recombinant yeast strains that endogenously produce ascorbic acid and produce and secrete lactic acid would be cultured in a medium containing a carbon source, D-glucose, and other essential nutrients. The lactic acid would be recovered at a pH of 7 or less, preferably at a pH of 4.5 or less, and even more preferably at a pH of 3 or less. Because the pH of the culture medium would be reduced, less neutralizing agent would be required. The formation of lactate salt would be correspondingly reduced and proportionally less regeneration of free acid would be necessary in order to recover lactic acid.

[0066] Because the recombinant yeast are more stress tolerant due to the endogenous production of L-ascorbic acid, the yeast cells separated from the lactic acid product could be utilized again as seed microorganisms for a fresh lactic acid fermentation. In addition, the yeast cells could be continuously separated and recovered during the lactic acid fermentation, and hence, the fermentation could be carried out continuously at low pH with less severe effects of pH and oxidative stress on yeast viability, production, productivity, and yield.

[0067] The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

[0068] "Ascorbic acid" as well as "ascorbate" as used herein, refers to L-ascorbic acid.

[0069] "Ascorbic acid precursor" is a compound that can be converted by an organism of the present invention, either directly or through one or more intermediates, into L-ascorbic acid.

[0070] "Amplification" refers to increasing the number of copies of a desired nucleic acid molecule or to increase the activity of an enzyme, by whatsoever means.

[0071] "Codon" refers to a sequence of three nucleotides that specify a particular amino acid.

[0072] "DNA ligase" refers to an enzyme that covalently joins two pieces of double-stranded DNA.

[0073] "Electroporation" refers to a method of introducing foreign DNA into cells that uses a brief, high voltage DC charge to permeabilize the host cells, causing them to take up extra-chromosomal DNA.

[0074] "Endonuclease" refers to an enzyme that hydrolyzes double stranded DNA at internal locations.

[0075] "Engineered for industrial production" refers to a recombinant organism that has been genetically modified to produce an industrial product.

[0076] Enzyme 1.1.3.37, D-arabinono-1,4-lactone oxidase, refers to a protein that catalyzes the conversion of D-arabinono-1,4-lactone+O_2 to D-erythroascorbate+H_2O_2. The same enzyme due to broadness of substrate range catalyses the conversion of L-galactono-1,4-lactone+O_2 to L-ascorbic acid+H_2O_2. Erroneously the same enzyme is referred to as L-galactono-1,4-lactone oxidase (enzyme 1.1.3.24) (Huh, W. K. et al. (1998), Mol. Microbiol. 30, 895-903)

[0077] Enzyme 1.3.2.3, L-galactono-1,4-lactone dehydrogenase, refers to a protein that catalyzes the conversion of L-galactono-1,4-lactone+2 ferricytochrome C to L-ascorbic acid+2 ferrocytochrome C.

[0078] Enzyme 1.1.3.8, L-gulono-1,4-lactone oxidase, refers to a protein that catalyzes the oxidation of L-gulono-1,4-lactone to L-xylo-hexulonolactone which spontaneously isomerizes to L-ascorbic acid.

[0079] Enzyme GDP-mannose-3,5-epimerase (5.1.3.18), refers to a protein that catalyzes the conversion of GDP-mannose to GDP-L-galactose.

[0080] Enzyme myoinositol phosphatase (3.1.3.23), refers to a protein that catalyzes the conversion of L-galactose-1P to L-galactose. L-galactose-1-phosphatase has been annotated as inositol/myo-inositol monophosphatase galactose-1-phosphatase and may be referred to as MIP/VTC4 (Conklin, P. L. (2006) J. Biol. Chem. 281, 15662-70).

[0081] Other enzymes of interest, and their classification numbers, are as follows:

GDP-Mannose 3,5-epimerase	5.1.3.18
L-Galactono-1,4-lactone dehydrogenase	1.3.2.3
UDP-Glucuronate 4-epimerase	5.1.3.6
L-Gulono-1,4-lactone oxidase	1.1.3.8
Myoinositol 1-P monophosphatase	3.1.3.25
UDP-Glucose 4-epimerase	5.1.3.2
D-arabinose 1-dehydrogenase (NAD)	1.1.1.116
D-arabinose 1-dehydrogenase (NADP)	1.1.1.117

[0082] The term "expression" refers to the transcription of a gene to produce the corresponding mRNA and translation of this mRNA to produce the corresponding gene product, i.e., a peptide, polypeptide, or protein.

[0083] The term "fermentation" refers to a process in which organisms growing in a liquid or solid medium produce an industrial product. As used herein, the term does not refer exclusively to non-oxidative metabolism.

[0084] The phrase "functionally linked" or "operably linked" refers to a promoter or promoter region and a coding or structural sequence in such an orientation and distance that transcription of the coding or structural sequence may be directed by the promoter or promoter region.

[0085] The phrase "functionally transformed" refers to an organism that has been transformed with an exogenous nucleic acid and is capable of producing a functional protein or peptide encoded by that amino acid.

[0086] The term "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression.

[0087] The term "genome" encompasses both the chromosomes and plasmids within a host cell. Encoding DNAs of the present invention introduced into host cells can therefore be either chromosomally integrated or plasmid-localized.

[0088] "Heterologous DNA" refers to DNA from a source different than that of the recipient cell.

[0089] "Homologous DNA" refers to DNA from the same source as that of the recipient cell.

[0090] "Hybridization" refers to the ability of a strand of nucleic acid to join with a complementary strand via base pairing. Hybridization occurs when complementary sequences in the two nucleic acid strands bind to one another.

[0091] The term "medium" refers to the chemical environment of the organism, comprising any component required for the growth of the organism and one or more precursors for the production of ascorbic acid. Components for growth and precursors for the production of ascorbic acid may or may be not identical.

[0092] "Open reading frame (ORF)" refers to a region of DNA or RNA encoding a peptide, polypeptide, or protein.

[0093] "Plasmid" refers to an extra chromosomal, replicatable piece of DNA.

[0094] "Polymerase chain reaction (PCR)" refers to an enzymatic technique to create multiple copies of one sequence of nucleic acid. Copies of DNA sequence are prepared by shuttling a DNA polymerase between two amplimers. The basis of this amplification method is multiple cycles of temperature changes to denature, then reanneal amplimers, followed by extension to synthesize new DNA strands in the region located between the flanking amplimers.

[0095] The term "promoter" or "promoter region" refers to a DNA sequence, usually found upstream (5') to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) or other functional RNAs, (e.g., tRNAs, rRNAs, sRNAs), by providing the recognition site for RNA polymerase and/or other factors necessary for start of transcription at the correct site.

[0096] A "recombinant cell" or "transformed cell" is a cell that contains a nucleic acid sequence not naturally occurring in the cell or an additional copy or copies of an endogenous nucleic acid sequence, wherein the nucleic acid sequence is introduced into the cell or an ancestor thereof by human action.

[0097] The term "recombinant vector" or "recombinant DNA or RNA construct" refers to any agent such as a plasmid, cosmid, virus, autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence, derived from any source, capable of genomic integration or autonomous replication, comprising a nucleic acid molecule in which one or more sequences have been linked in a functionally operative manner. Such recombinant constructs or vectors are capable of introducing a 5' regulatory sequence or promoter region and a DNA sequence for a selected gene product into a cell in such a manner that the DNA sequence is transcribed into a functional mRNA, which may or may not be translated and therefore expressed.

[0098] "Restriction enzyme" refers to an enzyme that recognizes a specific sequence of nucleotides in double stranded DNA and cleaves both strands; also called a restriction endonuclease. Cleavage typically occurs within the restriction site or close to it.

[0099] "Selectable marker" refers to a nucleic acid sequence whose expression confers a phenotype facilitating identification of cells containing the nucleic acid sequence. Selectable markers include those, which confer resistance to toxic chemicals (e.g. ampicillin, kanamycin) or complement a nutritional deficiency (e.g. uracil, histidine, leucine).

[0100] "Screenable marker" refers to a nucleic acid sequence whose expression imparts a visually distinguishing characteristic (e.g. color changes, fluorescence).

[0101] "Transcription" refers to the process of producing an RNA copy from a DNA template.

[0102] "Transformation" refers to a process of introducing an exogenous nucleic acid sequence (e.g., a vector, plasmid, or recombinant nucleic acid molecule) into a cell in which that exogenous nucleic acid is incorporated into a chromosome or is capable of autonomous replication. A cell that has undergone transformation, or a descendant of such a cell, is "transformed" or "recombinant."

[0103] "Translation" refers to the production of protein from messenger RNA.

[0104] "Unit" of enzyme refers to the enzymatic activity and indicates the amount of micromoles of substrate converted per mg of total cell proteins per minute.

[0105] "Vector" refers to a DNA or RNA molecule (such as a plasmid, cosmid, bacteriophage, yeast artificial chromosome, or virus, among others) that carries nucleic acid sequences into a host cell. The vector or a portion of it can be inserted into the genome of the host cell.

[0106] The term "yield" refers to the amount of industrial product or L-ascorbic acid produced by the recombinant organism, as (molar or weight/volume) divided by the amount of precursor consumed (molar or weight/volume) multiplied by 100.

[0107] List of Abbreviations:

Asc L-ascorbic acid (vitamin C)

AGD L-galactono-1,4-lactone dehydrogenase (without signaling peptide)

ALO D-arabinono-1,4-lactone oxidase

ARA D-arabinose dehydrogenase

Gal L-galactono-1,4-lactone

Gul L-gulono-1,4-lactone

LGDH L-galactose dehydrogenase

ME Mannose epimerase

MIP Myoinositol phosphatase

RGLO L-gulono-1,4-lactone oxidase

TCA trichloroacetic acid

TPI triosephosphateisomerase

EXAMPLES

[0108] The following examples are included to demonstrate particular embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials and Methods

[0109] 1. Determination of Ascorbic Acid

[0110] Ascorbic acid was determined spectrophotometrically following the method of Sullivan, M. X. et al. (1955), Assoc. Off. Agr. Chem., 38, 514-518). The sample (135 µl) was mixed in a cuvette with 40 µl of H_3PO_4 (85%). Then 675 µl of α,α'-Bipyridyl (0.5%) and 135 µl $FeCl_3$ (1%) were added. After 10 min the absorbance at 525 nm was measured. In some experiments, the identity of the ascorbic acid was confirmed by HPLC (Tracer Extrasil Column C8, 5 µM, 15×0.46 cm, Teknokroma, S. Coop. C. Ltda. # TR-016077; Eluent: 5 mM cetyltrimethylammonium bromide, 50 mM KH_2PO_4 in 95/5H_2O/Acetonitrile; Flow rate: 1 ml min^{-1}, Detection UV @ 254 nm) with pure L-ascorbic acid (Aldrich, A9,290-2) as standard.

[0111] 2. Amplification of Specific Gene Sequences

[0112] To amplify specific gene sequences, PfuTurbo DNA polymerase (Stratagene #600252) was used on a GeneAmp PCR System 9700 (PE Appl. Biosystems, Inc.). Standard conditions used were: 400 µM dNTP, 0.5 µM primers, 0.5 mM $MgCl_2$ (in addition to the buffer), and 3.75 U Pfu per 100 µl reaction.

[0113] The sequences of the genes used have been publicly reported via Genbank, as follows, except for MIP. The MIP sequence listed as SEQ ID NO:4 differed from the Genbank sequence, accession no. NM_111155, by two translationally silent point substitutions: at bp271, A (NM_111155) to T (SEQ ID NO:4); at bp 685, T (NM_11155) to G (SEQ ID NO:4).

Gene	Genbank accession no(s).	SEQ ID NO:
ME	AY116953	3
MIP	n.a.	4
ALO	U40390, AB009401	5, 6
LGDH		7

[0114] The following program was used for amplification of ALO:

94° C.	5 min		
94° C.	45 s	}	33 cycles
50° C.	30 s		
72° C.	1 min 40 s		
72° C.	7 min		
4° C.	To completion		

[0115] The following program was used for amplification of LGDH:

94° C.	5 min		
94° C.	45 s	}	33 cycles
56° C.	30 s		
72° C.	1 min 40 s		
72° C.	7 min		

-continued

4° C.	To completion	

[0116] The following program was used for amplification of ME:

94° C.	5 min	
94° C.	15 s	} 30 cycles
50° C.	30 s	
72° C.	1 min 30 s	
72° C.	7 min	
4° C.	To completion	

[0117] The following program was used for amplification of MIP:

94° C.	5 min	
94° C.	15 s	} 28 cycles
59.8° C.	30 s	
72° C.	45 s	
72° C.	7 min	
4° C.	To completion	

[0118] Template DNA for LGDH, ME, and MIP: 50 ng plasmid cDNA library pFL61 *Arabidopsis* (ATCC #77500 (Minet M. et al. (1992), Plant J. 2, 417-422)). Template DNA for ALO: 50 ng genomic DNA from *S. cerevisiae* GRF18U, extracted using a standard method. PCR products were blunt-end cloned into the EcoRV site of pSTBlue-1 using the perfectly blunt cloning kit from Novagen Inc. (#70191-4).

Oligonucleotides used		Gene amplified
SEQ ID NO:8:	tttcaccatatgtctactatcc	ALO
SEQ ID NO:9:	aaggatcctagtcggacaactc	(yeast)
SEQ ID NO:10:	atgacgaaaatagagcttcgagc	LGDH
SEQ ID NO:11:	ttagttctgatggattccacttgg	(plant)
SEQ ID NO:12:	gcgccatgggaactaccaatggaaca	ME
SEQ ID NO:13:	gcgctcgagtcactcttttccatca	(plant)
SEQ ID NO:14:	atccatggcggacaatgattctc	MIP
SEQ ID NO:15:	aatcatgcccctgtaagccgc	(plant)

[0119] 3. Plasmid Construction

[0120] The naming convention used herein is that pSTBlue-1 containing, for example, ALO in the sense direction regarding its multiple cloning site (MCS) was designated pSTB ALO-1. In a further example, pSTBlue-1 containing ALO in the antisense direction regarding its MCS was designated pSTB ALO-2, and so on.

[0121] Inserts were cloned using either the pYX series (R&D Systems, Inc.) or the centromeric expression plasmids pZ_3 and pZ_4 (P. Branduardi, et al. The Yeast *Zygosaccharomyces bailii*: a New Host for Heterologous Protein Production, Secretion and for Metabolic Engineering Applications, FEBS Yeast Research, FEMS Yeast Res. (2004) 4, 493-504). Standard procedures were employed for all cloning purposes, (Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual,* 3rd Edition, Cold Spring Harbor Laboratory Press, 2001).

pSTB LGDH-1	EcoRI	pYX022	pH LGDH	HIS 3 (marker)
pSTB ALO-1	EcoRI	pYX042	pL ALO	LEU 2 (marker)
pSTB ME-1	EcoRI	pZ_3	pZ_3 ME	Kan^r (marker)
pSTB ME-1	EcoRI	pZ_4	PZ_4 ME	Hph^r (marker)
pSTB MIP-1	EcoRI	pYX012	pU MIP	URA 3 (marker)

[0122] For all the work performed below, the yeast control strains were transformed with the corresponding empty vectors.

[0123] 4. Yeast Cultivation and Examination:

[0124] Yeast strains used were *S. cerevisiae* GRF18U (Brambilla, L. et al., 1999, FEMS Microb. Lett. 171, 133-140), *S. cerevisiae* GRFc (Brambilla et al. 1999 FEMS Microb. Lett. 171: 133-140), *S. cerevisiae* BY4742 (MATα; his3; leu2, lys2; ura3, EuroScarf Accession No. Y10000), *S. cerevisiae* YML007w (BY4742; MATα; his3; leu2, lys2; ura; YML007w::KanMX4 (the yap1 deleted strain) EuroScarf Accession No. Y10569); CEN.PK 113-5D (MATα ura3-52; cir+) (see, for example, VanDijken et al. (2000) Enzyme Microb. Technol. 26, 706-714); and BY4741 (MATα; his3; leu2; met 15; ura3, Euroscarf Accession No. Y00000), or strains derived from them through transformation with the different developed plasmids. All strains were cultivated in shake flasks in minimal medium (0.67% w/v YNB (Difco Laboratories, Detroit, Mich. #919-15), 2% w/v glucose or mannose, with addition of the appropriate amino acids or adenine or uracil, respectively, to 50 mL^{-1}) and/or the appropriate antibiotic (G418 or hygromicin to 500 mg/l and 400 mg/l, respectively) under standard conditions (shaking at 30° C.). The initial optical density at 660 nm was about 0.05 for ascorbic acid determination, and 0.1 for the kinetics of the recovery from oxidative stress.

[0125] Cells were recovered by centrifugation at 4000 rpm for 5 min at 4° C., washed once with cold distilled H_2O, and treated as follows: for determination of intracellular ascorbic acid, cells were resuspended in about 3 times the pellet volume of cold 10% TCA, vortexed vigorously, kept on ice for about 20 min, and then the supernatant was cleared from the cell debris by centrifugation.

[0126] 5. Yeast Transformation:

[0127] Transformation of yeast cells was performed by the standard LiAc/ss-DNA/PEG method (Gietz, R. D. and Schiestl, R. H. (1996), Transforming Yeast with DNA, Methods in Mol. and Cell. Biol.).

[0128] Experimental Results

[0129] 6. Expression of *Arabidopsis thaliana* ME, MIP, LDGH and *S. cerevisiae* ALO in GRF18U

[0130] The genes encoding *A. thaliana* ME, *S. cerevisiae* ALO, *A. thaliana* LGDH, and *A. thaliana* MIP were placed

under the control of the TPI (triosephosphateisomerase) promoter each on its own integrative plasmid, except ME, which was sub-cloned in a centromeric plasmid. Two or more of the genes were integrated into *S. cerevisiae* GRF18U and BY4742. Each gene was integrated at a unique locus.

[0131] FIG. **1** provides a schematic representation of the current understanding of the physiological biosynthetic pathway leading from D-glucose to L-ascorbic acid in plants. The following enzymes are involved: A, L-galactono-1,4-lactone dehydrogenase (1.3.2.3), B, L-galactose dehydrogenase, C, myoinositol phosphatase (3.1.3.23), D, pyrophosporylase, E, GDP-mannose-3,5-epimerase (5.1.3.18), F, mannose-1-phosphate guanylyltransferase (2.7.7.22), G, phosphomannomutase (5.4.2.8), H, mannose-6-phosphate isomerase (5.3.1.8), I, glucose-6-phosphate isomerase (5.3.1.9), J; hexokinase (2.7.1.1).

[0132] In the pathway shown in FIG. **1**, ALO catalyzes reaction A, LGDH catalyzes reaction B, ME catalyzes reaction E, and MIP catalyzes reaction C.

[0133] Wild-type yeast cells are known to produce GDP-mannose (reactions F-J in FIG. **1**) and to transport it to the endoplasmic reticulum.

[0134] The table below shows the conversion of D-Glucose and D-Mannose to ascorbic acid by *S. cerevisiae* GRFc (control), or *S. cerevisiae* GRF18U transformed with (i) ALO and LDGH; (ii) ALO, LDGH and ME; or (iii) ALO, LDGH, ME and MIP. Cells were grown on mineral medium (2% glucose or mannose, 0.67% YNB) starting from an OD^{660} of 0.05. After 24 hours of growth, ascorbic acid was determined. While both the wild-type GRFc and GRF18U cells transformed with ALO and LGDH did not accumulate ascorbic acid, cells transformed with ALO, LDGH and ME, or ALO, LDGH, ME and MIP, respectively unexpectedly accumulated considerable amounts (i.e. greater than background levels) of ascorbic acid.

[0135] Transformed yeast were batch grown on glucose- or mannose-based media:

Expressed gene	Total (ascorbate plus erythroascorbate) on glucose-containing media	Total (ascorbate plus erythroascorbate) on mannose-containing media
Wt (control)	0.0205	0.0220
ALO, LGDH (control)	0.0210	0.0221
ALO, LDGH, ME	0.0302	0.0332
ALO, LDGH, ME, MIP	0.0450	0.0296

(Total (ascorbate plus erythroascorbate) values are mg/OD^{660} of Biomass/L)

[0136] The values determined in the control strain indicate the production of erythroascorbate normally produced by wild type yeasts.

[0137] We conclude that the yeast endogenously possesses activities which can nonspecifically catalyze reactions from GDP-L-galactose to L-galactose (see FIG. **1**). Specifically, though not to be bound by theory, we conclude that GDP-L-galactose spontaneously hydrolyses to L-galactose-1-P and that a nonspecific phosphatase catalyzed the conversion of L-galactose-1-P to L-galactose, which was then converted to L-ascorbic acid by LGDH and ALO. MIP provided superior catalysis of L-galactose-1-P to L-galactose than did the putative nonspecific phosphatase (ALO, LGDH, ME, MIP vs. ALO, LGDH, ME).

[0138] We did not observe any ascorbic acid accumulation in the medium.

[0139] 7. Sensitivity to Oxidative Stress

[0140] FIG. **2** shows that YML007w yeast hosts are particularly sensitive to oxidative stress. Yap1p activates genes required for the response to oxidative stress; deletion of this gene leads to the observed phenotype (Rodrigues-Pousada C A, et al. (2004) FEBS Lett.

[0141] 567, 80-85)

[0142] The following yeast strains have been analyzed:

[0143] BY4742 (▲).

[0144] YML007w (○)

[0145] FIG. **2**A. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1.

[0146] FIG. **2**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in the presence of 0.8 mM of H_2O_2.

[0147] FIG. **2**C. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in the presence of 1.0 mM of H_2O_2.

[0148] The two strains grew in the absence of H_2O_2 (FIG. **2**A) while growth of the YML007w yeast host was strongly delayed in medium containing 0.8 mM of hydrogen peroxide (FIG. **2**B) and completely impaired in the medium containing 1 mM of hydrogen peroxide (FIG. **2**C).

[0149] 8. Effect of Ascorbic Acid in Media on Stress Tolerance

[0150] FIG. **3** shows that the growth sensitivity of YML007w yeast, as shown in FIG. **2**, can be rescued by adding ascorbic acid to the medium, and that the effect of ascorbic acid in the medium on robustness is concentration dependent and can be optimized for different yeast strains.

[0151] FIG. **3**A. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 0.8 mM of H_2O_2. Ascorbic acid was added at T=0 at a final concentration of 15 mg/L. BY4742 (▲); YML007w (○).

[0152] FIG. **3**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 1.0 mM of H_2O_2. Ascorbic acid was added at T=0 at a final concentration of 15 mg/L. BY4742 (▲); YML007w (○).

[0153] FIG. **3**C. Three yeast strains (GRFc, BY4741, and CEN.PK 113-5D) were grown in 2×YNB medium (2% glucose, 1.34% YNB), containing lactic acid at 40 g/l, pH3. Ascorbic acid was added to the medium at the concentrations shown. The data demonstrate that the negative effects of lactic acid on growth can be overcome by exogenous ascorbic acid, and that the effect of ascorbic acid is dose dependent.

[0154] 9. Effect of Endogenous Ascorbic Acid on Sensitivity to Oxidative Stress

[0155] FIG. **4** shows that the growth defects of the YML007w yeast hosts can be rescued following expression of ALO, LDGH, ME, and MIP.

[0156] The following yeast strains have been analyzed:

[0157] BY4742 (▲)

[0158] YML007w expressing ALO, LDGH and ME (□)

[0159] YML007w expressing ALO, LDGH, ME and MIP (■)

[0160] FIG. **4**A. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 0.8 mM of H_2O_2.

[0161] FIG. **4**B. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 1.0 mM of H_2O_2.

[0162] Endogenous production of ascorbic acid "rescued" the yeast from stress-induced growth inhibition in a manner similar to that obtained by adding ascorbic acid to the culture medium (see FIG. **3**).

[0163] 10. Effect of Endogenous Ascorbic Acid on Robustness of GRF Yeast Strains

[0164] FIG. **5** shows that the wild type GRF yeast strain is sensitive to fermentative stress conditions (stress condition induced by adding 2 mM of H_2O_2); surprisingly, the recombinant yeast strains producing ascorbic acid show a strong robustness, indicating an increased tolerance to stress. The following yeast strains were analyzed: GRFc (closed triangle); GRF18U expressing ALO, LDGH and ME (open square); and GRF18U expressing ALO, LDGH, ME and MIP (closed square).

[0165] FIG. **5**A. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD660 of 0.1.

[0166] FIG. **5**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD660 of 0.1 in presence of 2.0 mM of H_2O_2. The wild type strain does not consume glucose.

[0167] All the strains used in this experiment bear the same auxotrophic complementation and the same antibiotic resistance cassettes (that are necessary for the expression of the different heterologous genes), so that it was possible to use the same media for all of them, either the ones expressing 3 or 4 heterologous genes or the wild type strain.

[0168] For this experiment, as a classical example of stress, we challenged wild type yeast cells with H_2O_2. As expected, wild type cells grow well in the absence of H_2O_2 (FIG. **5**A), but the same yeast cells do not grow in the presence of the H_2O_2 (FIG. **5**B). It is generally accepted that this external stressor leads to damage to DNA, damage to lipids, damage to proteins, and damage to membranes, among other subcellular structures, and ultimately leads to a loss of cell viability and cell integrity. Therefore, it is not surprising that the presence of this stressor leads to zero production, zero productivity and zero yield of the product (in this case, wild type yeast biomass), as shown in FIG. **5**B.

[0169] By the transformation of wild type GRF yeast with (i) LGDH, ALO, and ME or (ii) LGDH, ALO, ME and MIP, the recombinant yeast produced ascorbic acid, as described above, whereas wild type yeasts do not naturally produce ascorbic acid. Surprisingly, the bioprocess based on these recombinant yeasts showed a high production, high productivity, and a high yield of the product, yeast biomass (FIG. **5**B). Values for production, productivity, and yield are greater than 0.00 in the recombinant yeast (values for the control strain).

[0170] This experiment shows the two recombinant GRF yeast strains are more tolerant to stress than wild type GRF yeast, and may therefore be more suitable for certain industrial processes. Though not to be bound by a single theory, we consider it likely the recombinant yeast are less sensitive to diverse stressors, possibly through both direct scavenging of reactive oxygen species (ROS) by ascorbic acid and interference by ascorbic acid with unwanted stress reactions, such as apoptosis, cell death, viability loss, and loss of cell integrity.

[0171] 11. Effect of Endogenous Ascorbic Acid on ROS and Viability

[0172] The *S. cerevisiae* strains YML007w and YML007w transformed to express ALO, LDGH, ME, and MIP were grown in minimal glucose medium with or without addition of H_2O_2. Each culture was then split into two, and one was stained with dehydrorodamine for the detection of reactive oxygen species (ROS), the other was stained with propidium iodide for viability determination. Samples were then analyzed with a flow cytometer and compared. FIG. **6** demonstrates a correlation between ascorbic acid production and reduction in ROS formation, as well as reduction of the fraction of nonviable cells.

[0173] 12. Effect of Endogenous Ascorbic Acid on Sensitivity to Low pH and Lactic Acid

[0174] The *S. cerevisiae* strains BY4742c and BY4742 transformed to express ALO, LDGH, ME, MIP were inoculated in minimal glucose medium, minimal glucose medium at low pH (2.2), or minimal glucose medium at pH 3.0 containing 38 g/l of lactic acid. FIG. **7** shows growth curves for BY4742c (open squares) and the same yeast background transformed to produce ascorbic acid (dark squares) in minimal glucose medium, pH 2.2 (FIG. **7***a*), and in minimal glucose medium containing 38 g/l lactic acid, pH 3.0 (FIG. **7***b*). In the transformed yeast strain producing ascorbic acid, peak levels of cells at low pH are approximately three-fold greater and peak levels of cells in medium containing lactic acid are approximately five-fold greater compared with the non-transformed strain.

[0175] The same experiment was conducted after the two yeast strains were grown for about 24 hours in minimal glucose medium and then inoculated in minimal glucose medium at low pH (2.2), or minimal glucose medium at pH 3.0 containing 38 g/l of lactic acid. The results are shown in FIG. **8**. At low pH, the transformed strain producing lactic acid showed more than a six-fold increase in peak cell numbers compared with the non-transformed strain (FIG. **8***a*). In medium containing lactic acid, the non-transformed strain showed no increase in growth, whereas the transformed yeast strain producing ascorbic acid showed exponentional growth with an approximately 3.5 fold increase at peak levels (FIG. **8***b*).

[0176] 13. Effect of Exogenous Ascorbic Acid on Growth of Lactic Acid Producing Yeast m850.

[0177] *S. cerevisiae* strain NRRL Y-30696 was inoculated in minimal glucose medium and 2.78 g/L $CaCO_3$ or minimal glucose medium with 2.78 g/L $CaCO_3$ and 0.16, 0.3, or 0.6 g/L ascorbic acid. OD660 (open symbols) and lactic acid (closed symbols) were monitored with time. The pH dropped in each case to 2.5 at 67 hours. FIG. **9** shows that growth, as measure by OD660, increased with increasing ascorbic acid, 0 g/L (O), 0.16 g/L (+), 0.3 g/L (▲), or 0.6 g/L (♦), while lactic acid production was equivalent at each level.

[0178] 14. Construction of a Yeast Strain Co-Producing Lactic Acid and Ascorbic Acid.

[0179] *S. cerevisiae* NRRL Y-30696 (Y-30696) has previously been engineered to produce lactic acid. The ability to co-produce a low level of endogenous ascorbic acid could be introduced by integrating the genes required for ascorbic acid production into Y-30696. As shown above, production of significant endogenous L-ascorbic acid can be achieved by the expression of sequences encoding ME, LGDH, and ALO+/−MIP. One or more of these genes, functionally coupled to an appropriate promoter, could be added to the L-LDH bearing plasmid of Y-30696, while additional genes, coupled to appropriate promoters, could be introduced at the sites of the deleted PDC genes. Methods for these steps are known in the art, and are found in Sauer, M., et al. (2004) Applied Environmental Microbiology 70, 6086-6091.

[0180] While the compositions and methods and yeast strains of this invention have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied without departing from the concept, spirit and scope of the invention.

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What is claimed is:

1. A method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product comprising functionally transforming the recombinant organism with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a coding region encoding a D-arabinono-1,4-lactone oxidase (ALO), whereby the recombinant organism is enabled to produce ascorbic acid endogenously.

2. The method of claim 1, wherein the recombinant organism is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP).

3. The method of claim 1, wherein the recombinant organism is further functionally transformed with a coding region encoding an enzyme selected from the group consisting of L-galactono-1,4-lactone dehydrogenase (AGD), D-arabinose dehydrogenase (ARA), and L-gulono-1,4-lactone oxidase (GLO).

4. The method of claim 1, wherein the recombinant organism produces lactic acid.

5. The method of claim 1, wherein the recombinant organism is an organism selected from the group consisting of bacteria, yeast, filamentous fungi, and animal cells.

6. The method of claim 1, wherein the recombinant organism is a yeast belonging to a genus selected from the group consisting of *Saccharomyces, Zygosaccharomyces, Candida, Hansenula, Kluyveromyces, Debaromyces, Nadsonia, Lipomyces, Torulopsis, Kloeckera, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Cryptococcus, Trichosporon, Aureobasidium, Lipomyces, Phaffia, Rhodotorula, Yarrowia*, and *Schwanniomyces*.

7. The method of claim 5, wherein the recombinant organism is a yeast selected from the group consisting of *S. cerevisiae* strain GRF18U; *S. cerevisiae* strains W3031B, BY4741, BY4742, CEN.PK 113-5D and YML007w; *K. lactis* strain CBS2359; *Z. bailii* strain ATCC 60483; *S. cerevisiae* strains NRRL Y-30696, NRRL Y-30698, NRRL Y-30742; *K. lactis* strains PM6-7/pEPL2, PMI/C1[pELP2]; *Z. bailii* strains ATTC36947/pLAT-ADH, ATCC60483/pLAT-ADH.

8. The method of claim 5, wherein the recombinant organism is a bacterium of a genus selected from the group consisting of *Bacillus, Escherichia, Lactobacillus, Lactococcus, Pseudomonas*, and *Acetobacter*.

9. The method of claim 5, wherein the recombinant organism is a bacterium selected from the group of bacterial strains producing lactic acid consisting of *Bacillus coagulans, Lactobacillus helveticus, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus pentosus*, and *Streptococcus thermophilus*.

10. The method of claim 5, wherein the recombinant organism is a filamentous fungus of a genus selected from the group consisting of *Aspergillis, Rhizopus*, and *Trichoderma*.

11. The method of claim 5, wherein the recombinant organism is a filamentous fungus selected from the group consisting of *Aspergillus kawachii, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Rhizopus arrhizus, Rhizopus microsporus, Rhizopus oryzae, Trichoderma harzianum, Trichoderma reesei*, and *Trichoderma viride*.

12. The method of claim 1, wherein the ME has at least about 95% identity with SEQ ID NO:1.

13. The method of claim 2, wherein the MIP has at least about 95% identity with SEQ ID NO:2.

14. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce at least one product selected from the group consisting of organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides.

15. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce organic acids.

16. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce lactic acid.

17. The method of claim 1, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce at least one product selected from the group consisting of organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides.

18. The method of claim 1, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce organic acids.

19. The method of claim 5, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce lactic acid.

20. The method of claim 1, wherein the recombinant organism is a filamentous fungus and wherein the filamentous fungus is engineered to produce at least one product selected from the group consisting of citric acid, lactic acid, and enzymes.

21. A method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product, comprising culturing the recombinant organism in a medium containing an effective amount of ascorbic acid.

22. The method of claim 21, wherein the effective amount of L-ascorbic acid is 0.005 to 2.0 grams/liter.

23. The method of claim 21 wherein the effective amount of L-ascorbic acid is 0.015 to 0.1 gram/liter.

24. The method of claim 21, wherein the recombinant organism is engineered for the industrial production of lactic acid.

25. The method of claim 21, wherein the recombinant organism is a bacterium, a yeast, a filamentous fungus, or an animal cell.

26. A method of increasing stress tolerance in an organism that produces lactic acid comprising culturing the organism in a medium containing 0.005 to 2.0 grams/liter of ascorbic acid.

* * * * *

EXHIBIT 9

US007393936B2

(12) **United States Patent**
Hertenberger et al.

(10) **Patent No.: US 7,393,936 B2**
(45) **Date of Patent: Jul. 1, 2008**

(54) **METHOD FOR THE RECOMBINANT PRODUCTION AND PURIFICATION OF PROTEIN KINASES**

(75) Inventors: **Hubert Hertenberger**, Weilheim (DE); **Konrad Honold**, Penzberg (DE); **Christian Klein**, Iffeldorf (DE); **Petra Rueger**, Penzberg (DE)

(73) Assignee: **Hoff Hoffmann-La Roche Inc.**, Nutley, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 262 days.

(21) Appl. No.: **11/000,867**

(22) Filed: **Dec. 1, 2004**

(65) **Prior Publication Data**

US 2005/0282250 A1 Dec. 22, 2005

(30) **Foreign Application Priority Data**

Dec. 2, 2003 (EP) 03027755

(51) **Int. Cl.**
C07K 1/02 (2006.01)
C07K 1/14 (2006.01)
C07K 1/20 (2006.01)
C12N 9/12 (2006.01)
C12N 15/70 (2006.01)

(52) **U.S. Cl.** **530/412**; 530/414; 435/69.1; 435/194; 435/320.1

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner—Nashaat T. Nashed
Assistant Examiner—William W. Moore
(74) *Attorney, Agent, or Firm*—George W. Johnston; Patricia S. Rocha-Tramaloni

(57) **ABSTRACT**

A method for the recombinant production and purification of a protein kinase selected from the group consisting of tyrosine protein kinases and serine/threonine kinases by expressing a nucleic acid encoding said kinase in a microbial host cell, forming inclusion bodies containing said kinase, isolating, solubilizing, naturing, and purifying said kinase wherein said purification is performed by hydrophobic interaction with an hydrophobic adsorbent under conditions whereby at least 70% of said protein kinase are not bound to said adsorbent and the protein kinase not bound to said adsorbent is recovered.

12 Claims, 3 Drawing Sheets

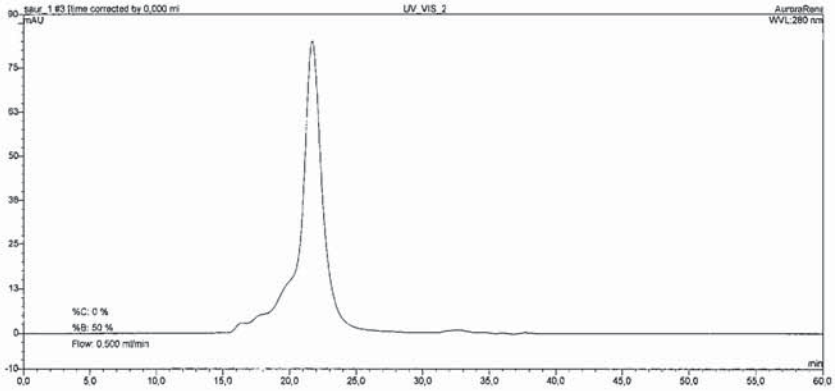

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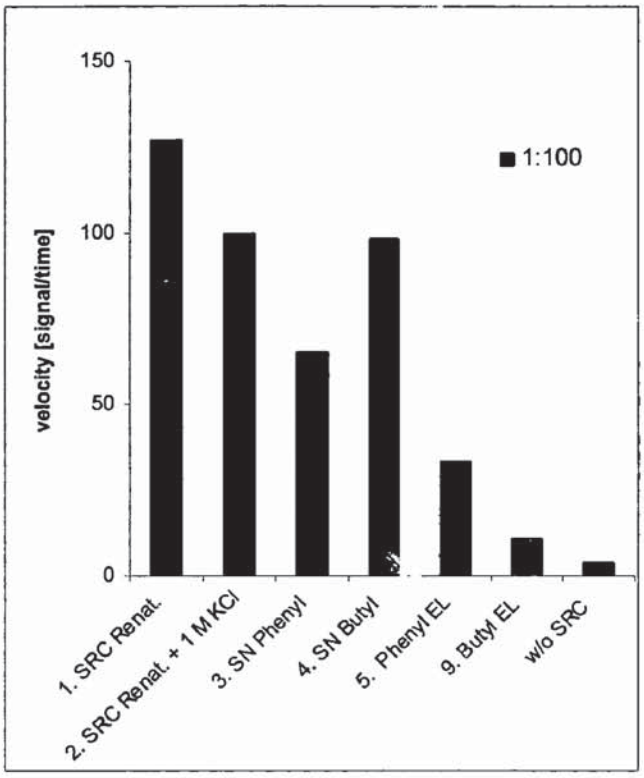
Fig. 1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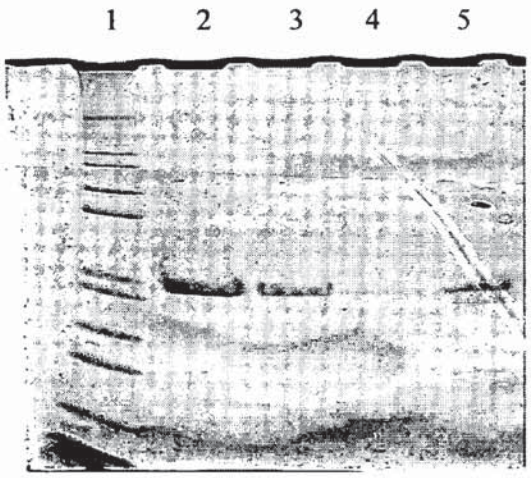
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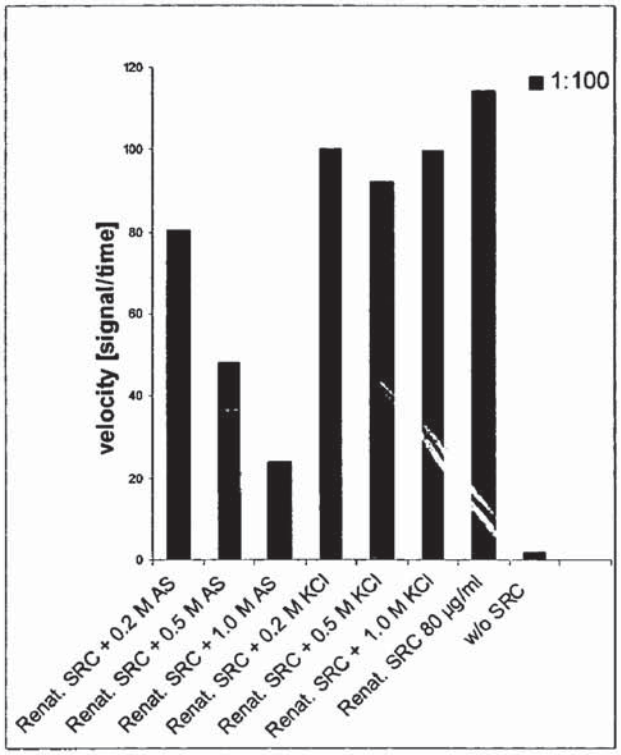
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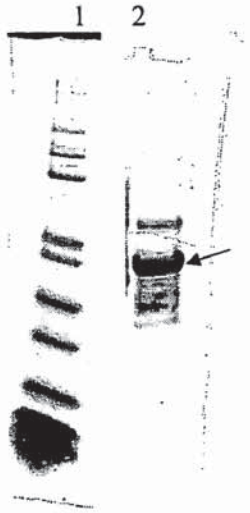
Fig. 4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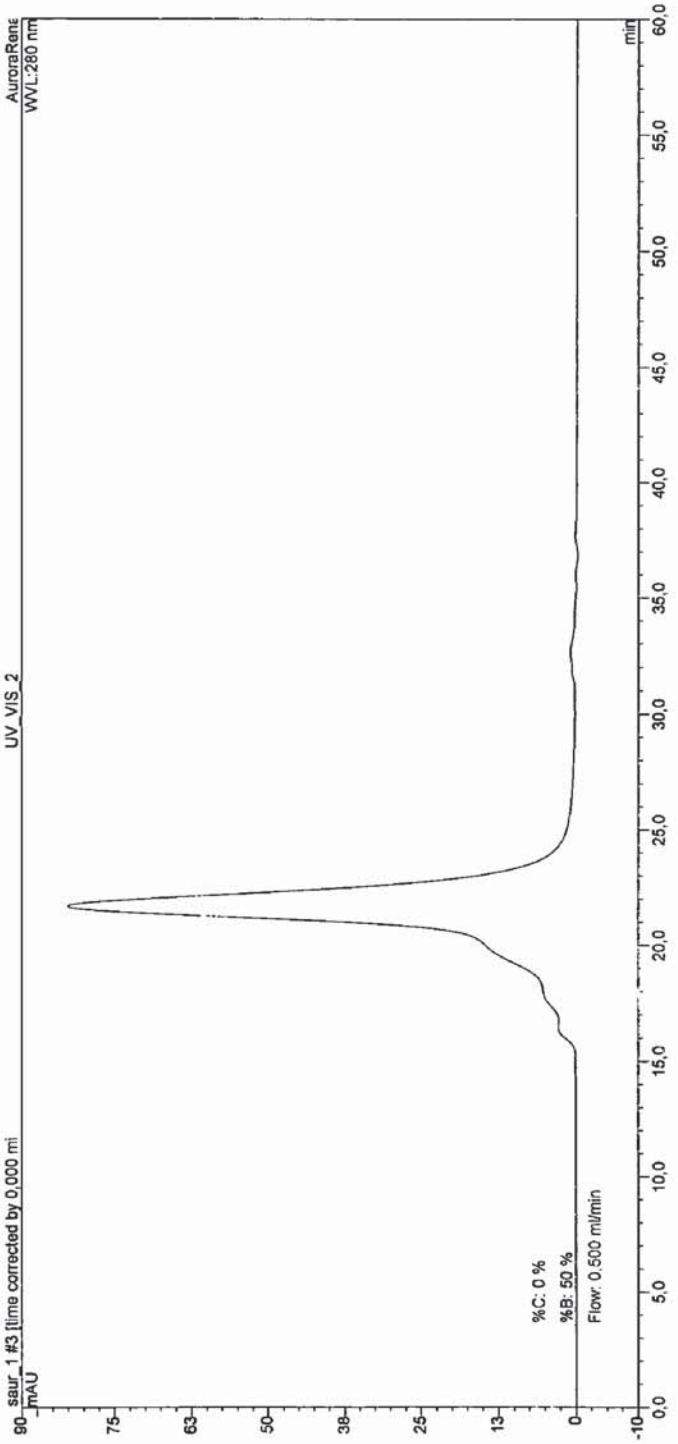
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METHOD FOR THE RECOMBINANT PRODUCTION AND PURIFICATION OF PROTEIN KINASES

BACKGROUND OF THE INVENTION

Protein kinases regulate many different cell proliferation, differentiation, and signaling processes by adding phosphate groups to proteins (Hunter, T., Cell 50 (1987) 823-829). Protein kinases are usually named after their substrate, their regulatory molecules, or some aspect of a mutant phenotype. With regard to substrates, the protein kinases may be divided into two groups; those that phosphorylate tyrosine residues (protein tyrosine kinases, PTK) and those that phosphorylate serine or threonine residues (serine/threonine kinases, STK). Almost all kinases contain a similar 250-300 amino acid catalytic domain. The N-terminal domain binds and orients the ATP (or GTP) donor molecule. The larger C terminal part binds the protein substrate and carries out the transfer of phosphate from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue.

The kinases may be categorized into families by the different amino acid sequences (generally between 5 and 100 residues) located on either side of, or inserted into loops of, the kinase domain. These added amino acid sequences allow the regulation of each kinase as it recognizes and interacts with its target protein. The primary structure of the kinase domains is conserved and can be further subdivided into 11 subdomains. Each of the 11 subdomains contain specific residues and motifs or patterns of amino acids that are characteristic of that subdomain and are highly conserved (Hardie, G., and Hanks, S., The Protein Kinase Facts Books I, Academic Press, San Diego, Calif., 1995, pp. 7-20).

The second messenger dependent protein kinases primarily mediate the effects of second messengers such as cyclic AMP (cAMP) cyclic GMP, inositol triphosphate, phosphatidylinositol, 3,4,5-triphosphate, cyclic ADP ribose, arachidonic acid and diacylglycerol. Cyclic -AMP dependent protein kinases (PKA) and mitogen-activated protein kinases (MAPK) are e.g. members of the STK family. Cyclic -AMP is an intracellular mediator of hormone action in all procaryotic and animal cells that have been studied. Such hormone-induced cellular responses include thyroid hormone secretion, cortisol secretion, progesterone secretion, glycogen breakdown, bone resorption, and regulation of heart rate and force of heart muscle contraction. PKA is found in all animal cells and is thought to account for the effects of cyclic -AMP in most of these cells. Altered PKA expression is implicated in a variety of disorders and diseases including cancer, thyroid disorders, diabetes, atherosclerosis, and cardiovascular disease.

MAP kinases like p38 also regulate intracellular signaling pathways. They mediate signal transduction from the cell surface to the nucleus via phosphorylation cascades. Several subgroups have been identified, and each manifests different substrate specificities and responds to distinct extracellular stimuli (Egan, S. E., and Weinberg, R. A., Nature 365 (1993) 781-783).

Protein kinase B (PKB/Akt) is a component of an intracellular signalling pathway of fundamental importance that functions to exert the effects of growth and survival factors, and which mediates the response to insulin and inflammatory signals (Datta, S. R., et al., Genes Dev. 13 (1999) 2905-2927; Brazil, D. P., and Hemmings, B. A., Trends Biochem. Sci. 11 (2001) 657-664). The recombinant production and purification of PKB is described in WO 2003/016516 using Phenyl

TSK hydrophobic interaction chromatography. PKB was adsorbed to the column and eluted after washing PKB using a linear gradient.

Src kinases are implicated in cancer, immune system dysfunction and bone remodeling diseases. For general reviews, see Thomas, S. M., and Brugge, J. S., Annu. Rev. Cell Dev. Biol. 13 (1997) 513-609. Members of the Src family are e.g. Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, and Blk. These are nonreceptor protein kinases that range in molecular mass from 52 to 62 kD. All are characterized by a common structural organization that is comprised of six distinct functional domains: Src homology domain 4 (SH4), a unique domain, SH3 domain, SH2 domain, a catalytic domain (SH1), and a C-terminal 15 regulatory region.

In prokaryotic organisms, the protein synthesis, also referred to as translation, takes place on the ribosomes in the cytoplasm. In expressing recombinant DNA in prokaryotic host organisms, such as, e.g., *E. coli* the resultant recombinant gene product/protein often precipitates in the cytoplasm in the form of insoluble inclusion bodies. After completion of fermentation and lysis of the cells, the inclusion bodies are isolated and optionally purified and the recombinant protein contained therein is solubilized by adding denaturants such as urea or guanidinium hydrochloride and naturation of said protein is accomplished by reducing the denaturing conditions. Such methods are well-known and have long been used successfully also for the industrial manufacture of recombinant proteins (cf., e.g., Lee, S. Y., Trends Biotechnol. 14 (1996) 98-105; Panda, A. K., et al., J. Biotechnol. 75 (1999) 161-172; Mattes, R., Semin. Thromb. Hemost. 27 (2001) 325-336; Clark, E. D., Curr. Opin. Biotechnol. 12 (2001) 202-207; Misawa, S., and Kumagai, I., Biopolymers 51 (1999) 297-307; and Lilie, H., Current Opinion Biotechnol. 9 (1998) 497-501).

However, expression of mammalian proteins in microbial host cells like *E. coli* is often a challenging task due to poor solubility, improper folding, lack of stability and other problems. Past attempts to produce such protein kinases by recombinant expression in microbial host cells pursuant to known methods in the art generally result in general only low amounts of active soluble kinases but with large amounts of undesired and inactive dimers and higher aggregates.

Now it was surprisingly found, that using the method of the invention kinases can be recovered after recombinant production in microbial host cells in a correctly folded form in large amounts.

SUMMARY OF THE INVENTION

The invention relates to an improved method for the recombinant production and purification of protein kinases in prokaryotes via inclusion bodies.

The object of the invention therefore is a method for the recombinant production and purification of a protein kinase selected from the group consisting of tyrosine protein kinases and serine/threonine kinases comprising a) expressing a nucleic acid encoding said kinase in a microbial host cell, b) forming inclusion bodies containing said protein kinase, and c) isolating, solubilizing, naturing, and purifying said protein kinase wherein said purification is performed by hydrophobic interaction with an hydrophobic adsorbent under conditions whereby at least 70% of said correctly folded protein kinase are not bound to said adsorbent and the protein kinase not bound to said adsorbent is recovered and unfolded protein is bound to the adsorbent.

Preferably said protein kinase is Src, PKB, c-Met, Llck, Aurora or p38 MAPK.

The adsorbent according to the invention comprises a solid or gel material, perferably a solid or gel material comprising cellulose, cross-linked dextran, cross-linked agarose or the like modified with hydrophobic residues like phenyl-, butyl-, or octylresidues (HIC adsorbent, hydrophobic interaction chromatography adsorbent).

Preferably the kinase is treated with the hydrophobic adsorbent in an aqueous solution comprising at least 0.1 M, more preferably at least 1M of a salt wherein the salt preferably comprises a cation, selected from the group consisting of sodium, potassium and ammonium, and an anion selected from the group consisting of chloride, sulfate (except not with ammonium) and phosphate, more preferably NaCl or KCl. Higher salt concentrations are possible as long as the protein kinase does not bind in an undesired large amount exceeding 70% of the total amount of protein kinase protein material, wherein the total amount of protein kinase material includes the correctly folded protein kinase, the not correctly folded protein kinase aggregates, and other undesired protein kinase material. In addition high salt concentrations (e.g., above 5M) may destabilze and/or denature protein kinases.

In addition the above hydrophobic interaction treatment is performed preferably in the presence of at least about 0.5 M arginine, guanidine or a compound comprising the general formula I

$$R_2—CO—NRR_1 \quad (I),$$

or combination thereof,

wherein

R and R_1 are hydrogen or a saturated or unsaturated branched or unbranched C_1-C_4 alkyl chain and

R_2 is hydrogen, NHR_1 or a saturated or unsaturated branched or unbranched C_1-C_3 alkyl chain.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**: Src activity after renaturation and hydrophobic chromatography as described. Activity is found in supernatant (SN) (EL=eluate).

FIG. **2**: SDS page of renaturation and butyl chromatography fractions, Coomassie stained. While retaining activity in butyl supernatant (SN) high amounts of inactive Src protein are cut off.

1: Molecular weight protein standard, 2: Renaturation, 3: Renaturation+KCl, 4: Butyl SN, 5: Ultrafiltration/concentration.

FIG. **3**: Activity of Src after renaturation and addition of KCl or ammonium sulfate. Addition of ammonium sulfate that is common in hydrophobic chromatography results in loss of active-Src in supernatant. KCl up to 1M keeps active Src in solution and allows separation of inactive Src on Butyl sepharose.

FIG. **4**: SDS page, Coomassie Blue staining. Lane 1 standard proteins, lane 2 Aurora kinase preparation after refolding and Butyl Sepharose. Arrow: Aurora kinase.

FIG. **5**: Size exclusion chromatography of Aurora kinase after refolding and Butylsepharose batch. Superdex 75 (Pharmacia) 10/30. Buffer: 50 mM TRIS pH7.5, 500 mM NaCl, 10% Glycerol, 3 mM Chaps. Flow rate: 0.5 ml/min.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an improved method for the recombinant production and purification of protein kinases in prokaryotes via inclusion bodies.

The object of the invention therefore is a method for the recombinant production and purification of a protein kinase selected from the group consisting of tyrosine protein kinases and serine/threonine kinases comprising a) expressing a nucleic acid encoding said kinase in a microbial host cell, b) forming inclusion bodies containing said protein kinase, and c) isolating, solubilizing, naturing, and purifying said protein kinase wherein said purification is performed by hydrophobic interaction with an hydrophobic adsorbent under conditions whereby at least 70% of said correctly folded protein kinase are not bound to said adsorbent and the protein kinase not bound to said adsorbent is recovered and unfolded protein is bound to the adsorbent.

Preferably said protein kinase is Src, PKB, c-Met, Lck, Aurora or p38 MAPK.

The adsorbent according to the invention comprises a solid or gel material, perferably a solid or gel material comprising cellulose, cross-linked dextran, cross-linked agarose or the like modified with hydrophobic residues like phenyl-, butyl-, or octylresidues (HIC adsorbent, hydrophobic interaction chromatography adsorbent).

Preferably the kinase is treated with the hydrophobic adsorbent in an aqueous solution comprising at least 0.1 M, more preferred 1M of a salt wherein the salt preferably comprises a cation, selected from the group consisting of sodium, potassium and ammonium, and an anion selected from the group consisting of chloride, sulfate (except not with ammonium) and phosphate, more preferably NaCl or KCl. Higher salt concentrations are possible as long as the protein kinase does not bind in an undesired large amount exceeding 70% of the total amount of protein kinase protein material, wherein the total amount of protein kinase material includes the correctly folded protein kinase, the not correctly folded protein kinase aggregates, and other undesired protein kinase material. In addition high salt concentrations (e.g., above 5M) may destabilze and/or denature protein kinases.

In addition the above hydrophobic interaction treatment is performed preferably in the presence of at least about 0.5 M arginine, guanidine or a compound comprising the general formula I

$$R_2—CO—NRR_1 \quad (I),$$

or combinations thereof,

wherein

R and R1 are hydrogen or a saturated or unsaturated branched or unbranched C_1-C_4 alkyl chain and

R_2 is hydrogen, NHR_1 or a saturated or unsaturated branched or unbranched C_1-C_3 alkyl chain.

The protein kinases which can be produced and purified according to the invention have been defined above. Preferably the method according to the invention is useful for the production and purification of Src kinases and cyclic -AMP dependent protein kinases (PKA). Members of the Src family are e.g. Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, and Blk. These are nonreceptor protein kinases that range in molecular mass from 52 to 62 kD. All are characterized by a common structural organization that is comprised of six distinct functional domains: Src homology domain 4 (SH4), a unique domain, SH3 domain, SH2 domain, a catalytic domain (SH1), and a C-terminal 15 regulatory region. Cyclic -AMP dependent protein kinases (PKA) are members of the STK family. Cyclic -AMP is an intracellular mediator of hormone action in all procaryotic and animal cells that have been studied. Such hormone-induced cellular responses include thyroid hormone secretion, cortisol secretion, progesterone secretion, glycogen breakdown, bone resorption, and regulation of

heart rate and force of heart muscle contraction. PKA is found in all animal cells and is thought to account for the effects of cyclic -AMP in most of these cells.

The above described protein kinases that can be produced according to the current invention all play an important role in biochemical processes. For further elucidation of metabolic correlations not only the natural protein kinases are of interest but also mutants of these naturally occuring protein kinases.

An adsorbent (HIC or hydrophobic adsorbent) useful according to the invention comprises preferably a gel matrix substituted with hydrophobic ligands. The degree of substitution is usually in the range of 10-50 μmol/ml gel. Preferred ligands are e.g. C_2-C_8 alkyl residues or simple aryl (like phenyl) residues. Usually hydrophobic interaction is increased by adding salt. If HIC adsorbents are used for chromatography the kinase in the correctly folded form is not bound to a considerable extent and therefore recovered in the flow-through.

Especially preferred is the use of a cross-linked agarose substituted with phenyl-, butyl- or octylgroups. Such adsorbent is for example phenyl-, butyl- or octyl-Sepharose (e.g. cross-linked agarose, 4% spherical, mean particle size 90 μm, particle size range 45-165 μm, degree of substitution approx. 50 μmol butyl groups/ml gel), available from Amersham Biosciences (General Electric Healthcare).

The treatment with the hydrophobic adsorbent can be performed according to methods known to one of ordinary skill in the art, e.g. treatment of the solution containing the kinase with a suspension of the adsorbent or as a chromatography (HIC). For example, the hydrophobic adsorbent is equilabraited in the renaturation buffer and added to the renaturation solution of the protein. The mixture is agitated, preferably in a cold storage room, for about one hour. The adsorbent is separated from the solution by filtration, such as for example plaited paper filter. Other chromatographic steps, such as for example ion exchange and size exclusion chromatography, optionally may be performed to achieve further purification.

The term "at least 70% of said protein kinase are not bound" means that from the protein recovered after recombinant production and naturation, which contains said protein kinase in a correctly folded form, not correctly folded form (e.g. multimers and the like) and other protein impurities from the host cell present after naturation in the aqueous solution containing the kinase, at least 70% of the correctly folded form are not bound to the adsorbent and found either in the supernant after treatment of the kinase solution with the adsorbent (the chromatographic material) or if the adsorbent (the chromatographic material) is used within a chromatographic HIC purification found in the flow-through.

The term "chromatographic material" as used herein refers to the material that is used for a purification process and more specifically comprises the adsorbent in the solution of the protein kinase. Preferably, the chromatogrpahic material comprises the use of the cross-linked agarose substituted with phenyl, butyl, or octyl groups, such as butyl-sepharose, in the solution of the protein kinase.

The term "correctly folded" means that the protein after naturation adopts the natural three dimensional structure. This is independent of catalytical activity and comprises also catalytically inactive mutants. Preferably active protein kinases are produced according to the current invention.

Binding of a correctly folded kinase to the adsorbent with a rate below 30% can be reached by treating an aqueous solution of such a kinase after naturation with at least 0.1 M of a salt solution, more preferably at least 1M of a salt, wherein the salt is selected from one or more of the group comprising of a cation selected from the group consisting of sodium, potassium, and ammonium, and an anion seelected from the group consisting of chloride, sulfate, and phosphate, except that where the cation is ammonium the anion is not sulfate. More preferably, the salt is KCl or NaCl Higher preferred salt concentrations are possible as long as the protein kinase do not bind in a undesired large amount exceeding 70% of the total amount of protein kinase protein material. In addition very high salt concentrations may destabilze protein kinases.

In a preferred embodiment of the invention, wherein the protein kinase is applied to a chromatogrpahic material in an aqueous solution containing at least 0.1 M of a salt, and more preferably, at least 1M of a salt, the aqueous solution contains (comprises) in addition at least 0.5 M of arginine, guanidine or a compound having the general formula I or combinations thereof. The aqueous solution containing in addition at least 0.5M arginine, guanidine or a compound having the general formula I or combinations thereof, preferably comprise one or more compounds selected from the group consisting of guanidine, arginine, general formula I compounds, and pharmaceutical salts of guanidine, arginine and general formula I compounds.

The compounds having the general formula I preferably comprise one or more compounds selected from the group consisting of formamide, acetamide, urea or urea derivatives, such as ethyl urea or methyl urea, and pharmaceutically acceptable salts of such compounds. The term guanidine comprises and includes the base guanidine as well as pharmaceutrically acceptable salts of guanidine. The term arginine comprises and includes the base arginine as well as pharmaceutically acceptable salts of arginine. For example, arginine can be used as a hydrochloride or as another titrated form of the base arginine. Preferably however L-arginine, more preferably the hydrochloride form of L-arginine, is employed.

Insoluble inclusion bodies are formed during recombinant expression of polypeptides in microbial host cells. Inclusion bodies are refractile aggregates of protease-resistant misfolded desired protein that occur upon over-expression of the encoding gene (Misawa, S., and Kumagai, I., Biopolymers 51 (1999) 297-307).

Suitable prokaryotic host cells for recombinant gene expression are, for example, gram-negative or gram-positive organisms, such as, e.g., *E. coli* and *Bacillus subtilis*. Suitable *E. coli* strains are, for instance, *E. coli* strains such as BL20, BL21, UT5600, AB101, XL1, K12, X1776 and W3110. However, other enterobacteriaceae as well as microorganisms such as *Klebsiella, Salmonella* or *Bacillus subtilis, Pseudomonas* or *Streptomyces* are also suitable as host cells. Also suitable as host cells are yeast strains, such as, e.g., *Saccharomyces, Pichia, Hansenula, Kluyveromyces* and *Schizosaccharomyces.*

The nucleic acid coding for the polypeptide is usually inserted in an expression vector. Suitable vectors are well-known to one skilled in the art and are, for example, plasmids or phages. See, e.g., "Sambook, J., Russel, D. W., Molecular Cloning, 3rd Edition, Chapter 15", relevant parts thereof are which hereby incorporated by reference.

The fermentation of the host cells is also accomplished according to methods known to one skilled in the art. After a predetermined number of cells has been reached (measured via the optical density of the fermentation broth/cell suspension), the expression of the recombinant polypeptide is induced and cultivation is performed until the stationary phase is reached (in the case of batch cultures). After completion of cell growth, the cells are harvested and the inclusion bodies are isolated and processed by solubilization and matu-

ration according to known methods. Recovery of the correctly folded protein kinases from the solution, and not bound to the adsorbent, is according to methods known to those of ordinary skill in the art, depending upon the purity of the IB and intended use of the kinase. For example, as a first step the adsorbent is removed from the solution (e.g. via filtration, ultrafiltration, centrifugation etc). If further treatment steps are required the steps are varying. For example, for a change of the buffer salts dialysis may be employed. The protein kinase recovered afterwards is still in solution and can be further processed. If further purification steps follow, that don't require a concentrated protein solution, the recovered solution is directly applied, e.g. to a Ni-affinity-column. From this column the protein can be recovered as concentrated solution. If a step follows that needs a concentrated solution, e.g., a size-exclusion-chromatography, a concentration step, e.g, ultrafiltration, is inserted.

In general after the purification is finished the correctly folded protein kinase is recovered from the solution. Depending upon the ultimate product desired for the kinase, the solution may be even freeze dried, potentially after demineralization by e.g., dialysis or ion exchange, (for e.g., a desired protein powder) or a concentration step with ultrafiltration is applied (for e.g., a desired liquid formulation).

The invention provides therefore an improved method for the recombinant production and purification of a protein kinase selected from the group consisting of tyrosine protein kinases and serine/threonine kinases comprising: a) expressing a nucleic acid encoding said kinase in a microbial host cell, b) forming inclusion bodies containing said kinase, and c) isolating, solubilizing, naturing, and purifying said kinase, wherein said purification is performed by hydrophobic interaction with an hydrophobic adsorbent under conditions whereby at least 70% of said correctly folded protein kinase are not bound to said adsorbent and the protein kinase not bound to said adsorbent is recovered. With the method of the invention substantial amounts of correctly folded protein kinases can be recovered and purified up to a purity of about >90-95% and more.

The following examples, figures and references are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

The following exmaples are provided for illustrative purposes and are not intended to limit the scope of applicants invention.

EXAMPLE 1

Recombinant Production of Src

a) Cloning

Src kinase domain was cloned BamHI/HindIII into pQE32 (QIAGEN GmbH) with an N-terminal thrombin cleavage site introduced by PCR Constructs for the bacterial expression of mouse full length wild type Src kinase and derivatives for application in kinase assays and for structural purposes were cloned according to the following procedure.

The ORF coding for mouse full length Src kinase was amplified from cDNA plasmid pUSE Src wt (Upstate) via standard PCR using gene-specific oligonucleotide primers and Tgo/Tag DNA Polymeerases (Roche Diagnostics GmbH), and subsequently sub cloned via BamHI and HindIII restriction, sites into bacterial expression vector pQE32 (Qiagen GmbH). An N-terminal thrombin cleavage site was introduced by PCR. This vector served as a template for the generation of truncated and mutated versions of Src kinase domain and full lenth via site-directed mutagenesis. All inserts under control of the T5 promotor were confirmed by sequencing.

b) Fermentation

Vectors plus co-vector pUBS520 (Brinkmann, U., Mattes, R. E., Buckel, P., Gene 85 (1989) 109 114) were subsequently transformed into *E. coli* BL21 strain (Stratagene) for protein expression by large scale fermentation as inclusion bodies.

To prepare the inoculum 1 ml of a glycerol stock of the appropriate *E. coli* strain, harboring the expression plasmid to produce recombinant kinase protein, is added to 100 ml LB media and incubated for 8 to 10 hours on a rotary shaker at 37° C. This pre-culture is transferred to the sterilized fermenter vessel containing further LB media and glucose. The temperature of the main culture is maintained at 37° C. when insoluble expression of the kinase protein to inclusion bodies is desired. For example, proteins were expressed in *E. coli* BL21 grown at 37° C. in LB medium supplemented with ampicillin (100 μg/ml) to an absorbance of 0.5-0.8 before overnight induction at 37° C. with 0.5 mM IPTG (isopropl-D-thiogalactopyranoside). The dissolved oxygen concentration of the media throughout the fermentation is kept above 20% saturation by increasing the agitation speed. Additional feeding of the culture is performed with the addition of glucose at rising pH values and the continuous dosing of a yeast-tryptone solution (2 ml/min). The fed batch fermentation ends when no more increase of the optical density is measurable. The culture broth is harvested by centrifugation after incubation, resuspended and inclusion bodies are then prepared (Example 1, part c) according to the given procedure.

REFERENCES

Brinkmann, U., Mattes, R. E., Buckel, P., Gene 85 (1989) 109-114: High level expression of recombinant genes in *Escherichia coli* is dependent on the availability of the dnaY gene product.

c) IB Preparation

The biomass is suspended in a buffer containing Tris and $MgSO_4$. Lysozym and DNase (e.g. Benzonase from Merck) are added if required before disintegration of the cells of the biomass (bacteria cells). The bacteria cells are disrupted by homogenization to release the inclusion bodies from inside. Additional DNase keeps the suspension liquid what is essential for further handling. After an incubation period at room temperature a second buffer solution containing NaCl, EDTA and Brij solution is added to the suspension. The total incubation time is about one hour. The covered inclusion bodies are separated from the supernatant by centrifugation after an additional incubation time at room temperature. Then the pellet is suspended with a third buffer solution containing Tris and EDTA to wash the IBs (endotoxin release) and incubated under stirring at room temperature and centrifuged.

d) Naturation of Src

1.3 g inclusion bodies were suspended in 100 ml 0.1M TRIS pH 8.0, 8M Guanidin HCl, 10 mM EDTA, 10 mM DTT at room temperature. This Src solubilisate is added dropwise under stirring into 101 refolding buffer containing 1M TRIS pH 7.0, 0.5M Arginine, 10 mM DTT, 10 tablets of Complete. The refolding process is continued 3-5 days at 8° C. without stirring.

Dialysis against different buffers commonly used in the prior art (acetate, phosphate, MES, TRIS pH 5-9 with various additives) at this stage was not successful due to high aggregation of inactive not corerctly folded Src and then co-immu-

noprecipitation of the active fraction. It was necessary to remove the inactive, not correctly folded Src solubilised in renaturation buffer by hydrophobic chromatography before dialysis.

e) Hydrophobic Batch Chromatography

The refolded protein solution was set to 1M KCl. Subsequently 40 g Butylsepharose 4 Fast Flow (Amersham) were added and binding allowed for 1 h at 8° C. After removal of Butylsepharose by filtration the supernatant contains the correctly folded active Src protein. Under these conditions not correctly folded, non-active protein is bound to Butylsepharose. This method allows separation of active and inactive Src (FIGS. **1** and **2**). Further purification can be achieved if necessary by additional chromatography steps i.e. Ni-chelate chromatography and size exclusion chromatography or other methods known to one of ordinary skill in the art, depending upon the subsequent intended application of the protein.

Addition of KCl (0.2M, 0.5M and 1.0M) proved to be superior versus ammonium sulfate as can be seen in FIG. **3**. Similar concentrations of ammonium sulfate already result in precipitation of active Src. Using conditions that lead to binding of the active Src on hydrophobic materials were not suitable because of high loss of activity on the column. Eluates show only low Src protein and activity recovery.

f) Src Assay

Phosphorylation of Src substrate peptide YA133 labeled by Src kinase is measured by using a phosphotyrosine antibody Eu labeled (PT66 Lance Eu-W1024 (Wallac)) and detection of time resolved fluorescence signal.

EXAMPLE 2

Recombinant Production of Aurora

a) Cloning

Vector constructs for the bacterial expression of human full-length wildtype Aurora A kinase and derivatives for application in kinase assays (e.g. Elisa, HTRF, FP) and for biostructural purposes were designed and cloned according to the following procedure.

The ORF coding for human full-length Aurora A kinase (residues 1-403) was amplified from a human HeLa cDNA library (Clontech) via standard PCR using gene-specific oligonucleotide primers and Pwo DNA polymerase (Roche Diagnostics GmbH), and subsequently subcloned into bacterial expression vectors. These vectors served as a template for the generation of truncated versions of Aurora A via PCR using gene-specific oligonucleotide primers and of Aurora A muteins via site-directed mutagenesis. For the final expression constructs wildtype and mutant Aurora A kinase domains (residues 114-403) were amplified from the corresponding Aurora A basic vectors via PCR using gene-specific primers and Pwo DNA polymerase. PCR products were subcloned via NdeI and XhoI restriction sites into modified pQE40 expression vectors under control of a T5 promoter with and without an N-terminal RGS-$(His)_6$-tag. All vector insert sequences were confirmed by sequencing. Vectors were subsequently transformed into *E. coli* BL21, 20 and UT5600 strains co-transformed with the pUBS520 co-repressor plasmid (Brinkmann, U., Mattes, R. E., Buckel, P., Gene. 85 (1989) 109-114). Aurora protein was subsequently expressed by large scale fermentation as inclusion bodies. Proteins were expressed in *E. coli* strains BL21 and UT5600 grown at 37° C. in LB medium supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml) to an absorbance of 0.5-0.8 before overnight induction at 37° C. with 1 mM isopropyl-D-thiogalactopyranoside. After induction, cells were harvested by centrifugation, resuspended and inclusion bodies were prepared according to the given procedure.

b) Fermentation

To prepare the inoculum 1 ml of a glycerol stock of the appropriate *E. coli* strain, harboring the expression plasmid to produce recombinant kinase protein, is added to 100 ml LB media and incubated for 8 to 10 hours on a rotary shaker at 37° C. This pre-culture is transferred to the sterilized fermenter vessel containing further LB media and glucose. The temperature of the main culture is maintained at 37° C. Additional feeding of the culture is performed with the addition of glucose at rising pH values and the continuous dosing of a yeast-tryptone solution (2 ml/min). The fed batch fermentation ends when no more increase of the optical density is measurable. The culture broth is harvested by centrifugation.

c) IB Preparation

The biomass is suspended in a buffer containing Tris and $MgSO_4$. Lysozym and DNase (e.g. Benzonase from Merck) are added if required. The bacteria cells are disrupted by homogenization to release the inclusion bodies from inside. After an incubation period at room temperature a second buffer solution containing NaCl, EDTA and Brij solution is added to the suspension. The covered inclusion bodies are separated from the supernatant by centrifugation after an additional incubation time at room temperature. Then the pellet is suspended with a third buffer solution containing Tris and EDTA to wash the IBs and incubated under stirring at room temperature and centrifuged.

d) Naturation of Aurora

160 mg inclusion bodies were suspended in 10 ml 0.1M TRIS pH 8.0, 8M Guanidin HCl, 10 mM EDTA, 10 mM DTT at room temperature. This aurora solubilisate is added dropwise under stirring into 1 l refolding buffer containing 1M TRIS pH 7.0, 0.5M Arginine, 10 mM DTT. The refolding process is continued 1 day at 8° C. without stirring.

e) Hydrophobic Batch Chromatography

The refolded protein solution was set to 1M KCl. After 30 min 5 g Butylsepharose 4 Fast Flow (Amersham Biosciences) were added and binding allowed for 1 h at 8° C. After removal of Butylsepharose by filtration the supernatant contains mainly the correctly folded active aurora protein. Under these conditions not correctly folded protein is bound to Butylsepharose. Further purification can be achieved if necessary by additional chromatography steps, e.g., ion exchange, size exclusion chromatography, etc.

f) Analysis of Refolded Protein

After refolding the supernatant of the Butyl Sepharose batch contains >90% monomeric Aurora kinase as is seen by SDS-Page and size exclusion chromatography.

Various embodiments of the invention have been described. The descriptions and examples are intended to be illustrative of the invention rather than limiting. Indeed, it will be apparent to those of skill in the art that modifications may be made to the various embodiments of the invention described without departing from the spirit of the invention or scope of the appended claims set forth below.

Each reference cited herein is hereby incorporated by reference in its entirety.

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The invention claimed is:

1. A method for the recombinant production and purification of a correctly folded protein kinase selected from the group consisting of tyrosine protein kinases and serine/threonine kinases comprising:

a) expressing a nucleic acid encoding said protein kinase in a microbial host cell, wherein inclusion bodies containing said protein kinase are formed in said host cell,

b) isolating said protein kinase from the cell, solubilizing said protein kinase, and then renaturing said protein kinase to provide a renatured protein kinase, and

c) purifying said renatured protein kinase by hydrophobic interaction chromatography with a hydrophobic adsorbent wherein said renatured protein kinase is applied directly to said hydrophobic adsorbent in an aqueous solution comprising at least a 1 M salt solution wherein the cation is selected from the group consisting of sodium, potassium and ammonium, and an anion selected from the group consisting of chloride, phosphate and sulfate, except that where the cation is ammonium, the anion is not sulfate,

whereby at least 70% of said protein kinase recovered is correctly folded and is not bound to said hydrophobic adsorbent.

2. The method according to claim **1**, wherein the hydrophobic adsorbent comprises a gel matrix substituted with hydrophobic ligands in the range of 10-50 μmol/ml gel, wherein the hydrophobic ligands are selected from the group C_2-C_8 alkyl residues or simple aryl(phenyl) residues.

3. The method according to claim **2**, wherein the hydrophobic adsorbent comprises a cross-linked agarose substituted with phenyl-, butyl- or octyl groups.

4. The method according to claim **3**, wherein the hydrophobic adsorbent is selected from the group consisting of phenyl-, octyl- or butyl-sepharose.

5. The method of claim **1**, wherein the salt is KCl or NaCl.

6. The method according to claim **1**, wherein the aqueous solution comprises in addition at least 0.5 M arginine, guanidine or a compound having the general formula I

$$R_2—CO—NRR_1 \quad (I),$$

or combinations thereof,
wherein

R and R_1 are hydrogen or a saturated or unsaturated branched or unbranched C_1-C_4 alkyl chain and

R_2 is hydrogen, NHR_1 or a saturated or unsaturated branched or unbranched C_1-C_3 alkyl chain.

7. A method for the recombinant production and purification a correctly folded protein kinase selected from the group consisting of a Src, a PKB, a c-Met, a Lck, an Aurora or a p38 MAPK protein kinase comprising:

a) expressing a nucleic acid encoding said protein kinase in a microbial host cell, wherein inclusion bodies containing said protein kinase are formed in said host cell,

b) isolating said protein kinase from the cell, solubilizing said protein kinase, and then renaturing said protein kinase to provide a renatured protein kinase, and

c) purifying said renatured protein kinase by hydrophobic interaction chromatography with a hydrophobic adsorbent wherein said renatured protein kinase is applied directly to said hydrophobic adsorbent in an aqueous solution comprising at least a 1 M salt solution wherein the cation is selected from the group consisting of sodium, potassium and ammonium, and an anion selected from the group consisting of chloride, phosphate and sulfate, except that where the cation is ammonium, the anion is not sulfate,

whereby at least 70% of said protein kinase recovered is correctly folded and is not bound to said hydrophobic adsorbent.

8. The method according to claim **7**, wherein the hydrophobic adsorbent comprises a gel matrix substituted with hydrophobic ligands in the range of 10-50 μmol/ml gel, wherein the hydrophobic ligands are selected from the group comprising C_2-C_8 alkyl residues or simple aryl(phenyl) residues.

9. The method according to claim **8**, wherein the hydrophobic adsorbent comprises a cross-linked agarose substituted with phenyl-, butyl- or octyl groups.

10. The method according to claim **9**, wherein the hydrophobic adsorbent is selected from the group consisting of phenyl-, octyl- or butyl-sepharose.

11. The method of claim **7**, wherein the salt is KCl or NaCl.

12. The method according to claim **7**, wherein the aqueous solution comprises in addition at least 0.5 M arginine, guanidine or a compound having the general formula I

$$R_2—CO—NRR_1 \quad (I),$$

or combinations thereof,
wherein

R and R_1 are hydrogen or a saturated or unsaturated branched or unbranched C_1-C_4 alkyl chain and

R_2 is hydrogen, NHR_1 or a saturated or unsaturated branched or unbranched C_1-C_3 alkyl chain.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,393,936 B2
APPLICATION NO. : 11/000867
DATED : July 1, 2008
INVENTOR(S) : Hertenberger et al.

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

Title Page, Item (73) Assignee - Delete "Hoff"

Signed and Sealed this

First Day of December, 2009

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office

EXHIBIT 10

US006258560B1

(12) **United States Patent**
Leung et al.

(10) **Patent No.: US 6,258,560 B1**
(45) **Date of Patent: Jul. 10, 2001**

(54) **PROCESS FOR BACTERIAL PRODUCTION OF POLYPEPTIDES**

(75) Inventors: **Woon-Lam Susan Leung**, San Mateo; **James R. Swartz**, Menlo Park, both of CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/607,756**

(22) Filed: **Jun. 29, 2000**

Related U.S. Application Data

(62) Division of application No. 09/422,712, filed on Oct. 21, 1999, now Pat. No. 6,180,367.
(60) Provisional application No. 60/106,052, filed on Oct. 28, 1998.

(51) **Int. Cl.**[7] **C12P 21/00**; C12N 15/09; C12N 1/20

(52) **U.S. Cl.** **435/69.1**; 435/69.7; 435/252.1; 435/252.3; 435/320.1

(58) **Field of Search** 435/69.1, 69.7, 435/320.1, 252.1, 252.3, 252.33; 536/23.1

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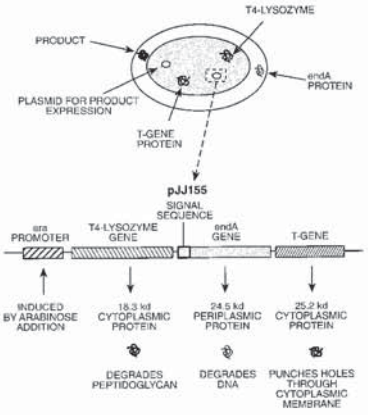
Primary Examiner—David Guzo
Assistant Examiner—Gerald G. Leffers, Jr.
(74) *Attorney, Agent, or Firm*—Janet E. Hasak

(57) **ABSTRACT**

Processes are described for recovering heterologous polypeptide from bacterial cells, including the periplasm and cytoplasm. One process involves culturing the bacterial cells, which cells comprise nucleic acid encoding phage lysozyme and nucleic acid encoding a protein that displays DNA-digesting activity, wherein these nucleic acids are linked to a first promoter, and nucleic acid encoding the heterologous polypeptide, which nucleic acid is linked to a second promoter, under certain conditions to produce a broth lysate; and recovering accumulated heterologous polypeptide from the broth lysate. Another process entails culturing bacterial cells that comprise nucleic acid encoding phage lysozyme, gene t, and nucleic acid encoding a protein that displays DNA-digesting activity under the control of a signal sequence for secretion of said DNA-digesting protein, wherein said nucleic acids are linked to one or more promoters, and nucleic acid encoding the heterologous polypeptide and a signal sequence for secretion of the heterologous polypeptide, which nucleic acid encoding the heterologous polypeptide is linked to a another promoter that is inducible, under certain conditions to produce a broth lysate; and recovering accumulated heterologous polypeptide from the broth lysate.

20 Claims, 13 Drawing Sheets



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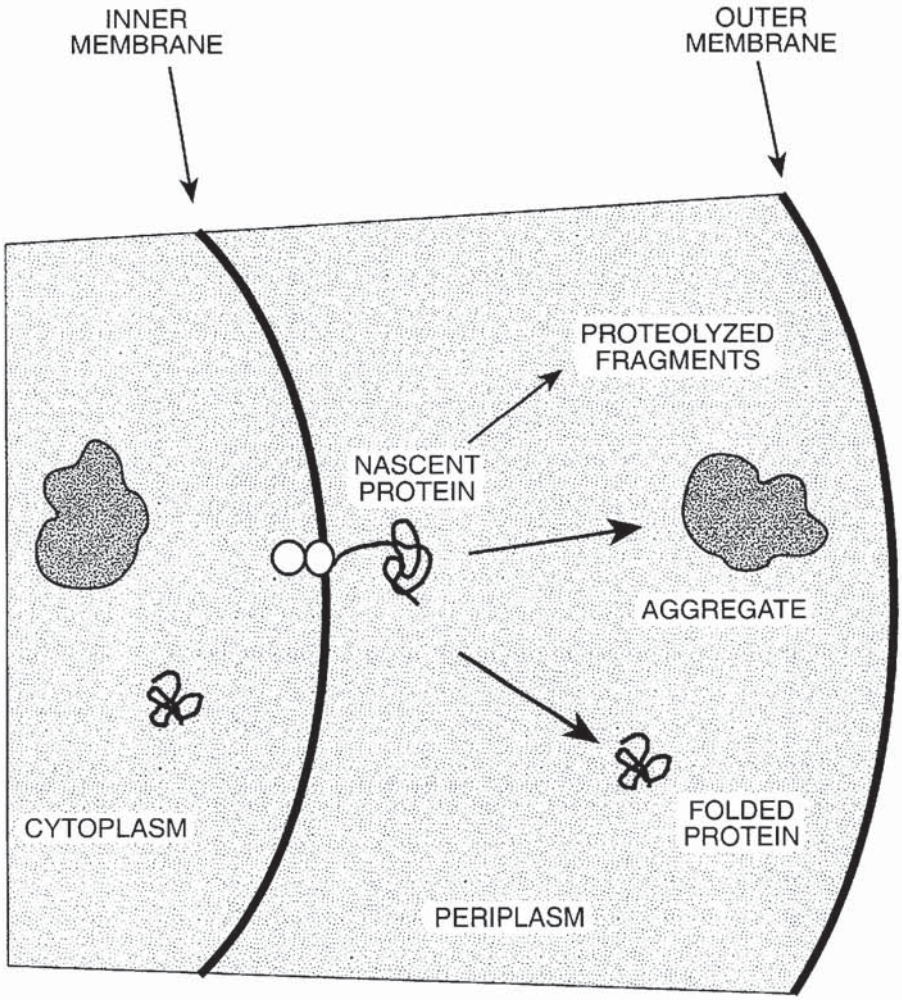

INNER MEMBRANE
OUTER MEMBRANE
PROTEOLYZED FRAGMENTS
NASCENT PROTEIN
AGGREGATE
FOLDED PROTEIN
CYTOPLASM
PERIPLASM

FIG._1

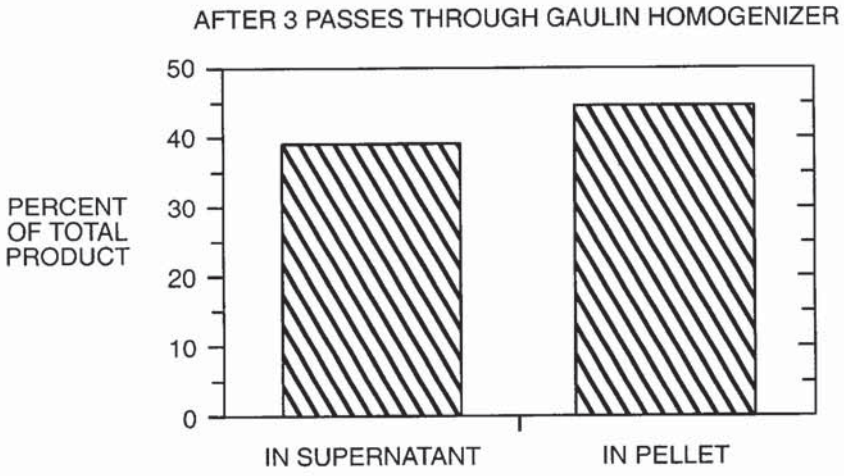

AFTER 3 PASSES THROUGH GAULIN HOMOGENIZER
PERCENT OF TOTAL PRODUCT
50
40
30
20
10
0
IN SUPERNATANT
IN PELLET

FIG._2

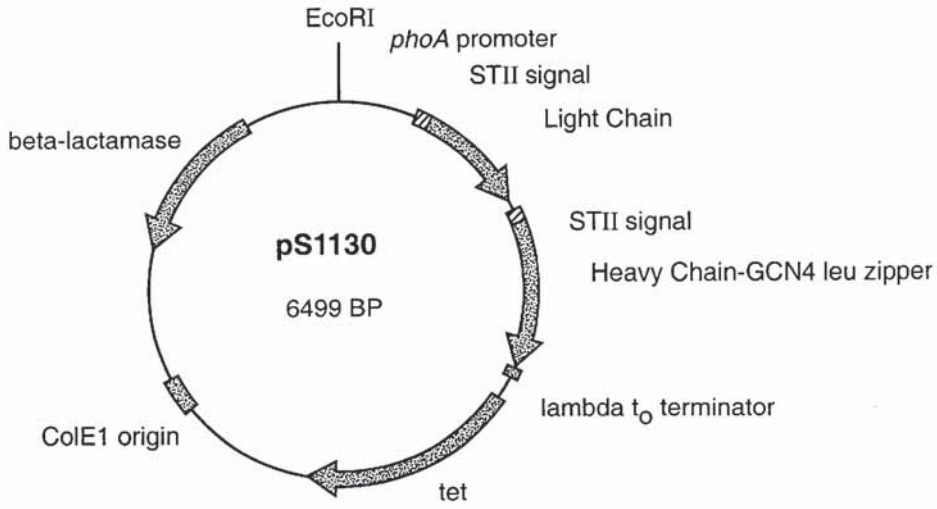

EcoRI
phoA promoter
STII signal
Light Chain
STII signal
Heavy Chain-GCN4 leu zipper
lambda t0 terminator
tet
ColE1 origin
beta-lactamase
pS1130
6499 BP

FIG._3

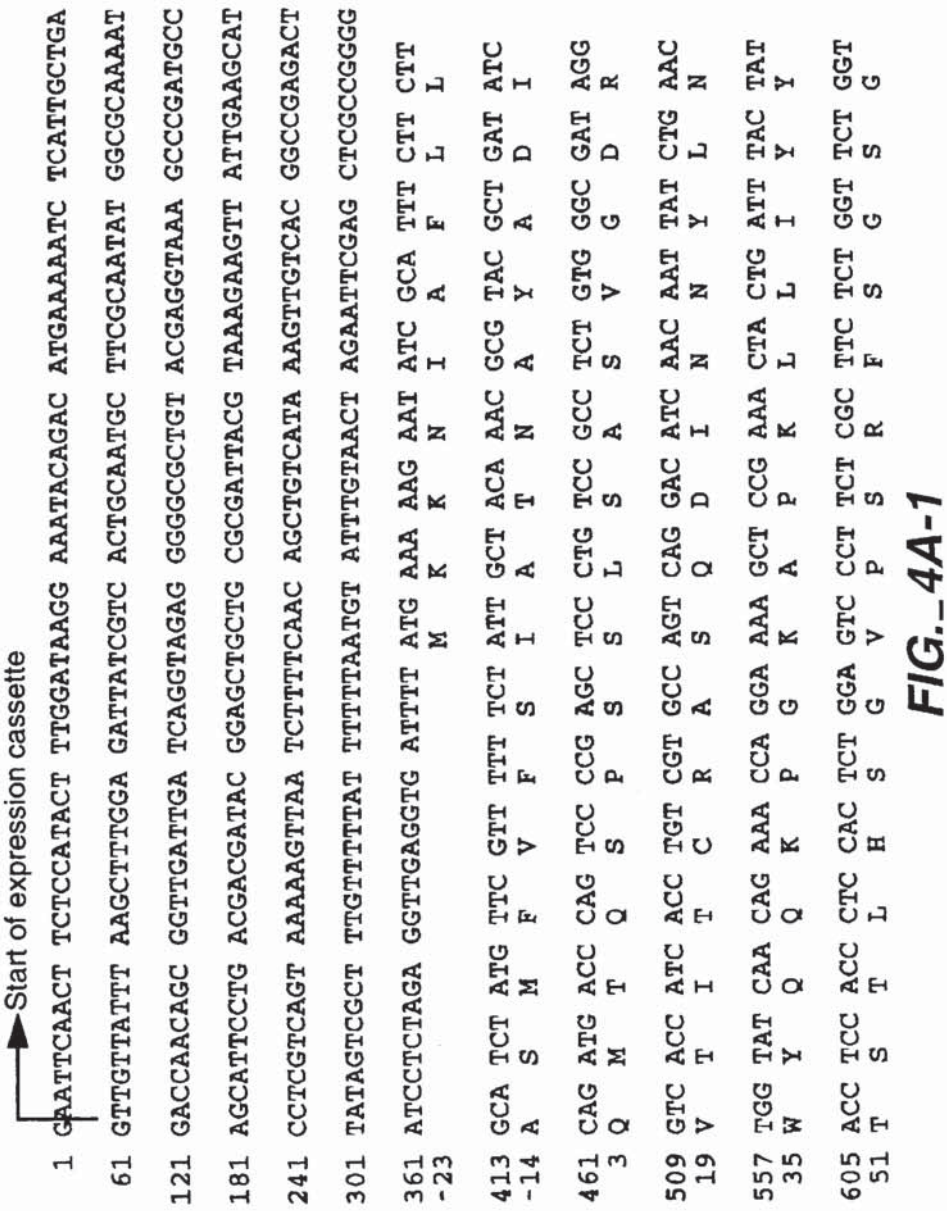

Start of expression cassette
1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA
61 GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT
121 GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC
181 AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT
241 CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT
301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGCCGGGG
361 ATCCTCTAGA GGTTGAGGTG ATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT
M K K N I A F L L
-23
413 GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAC GCT GAT ATC
-14 A S M F V F S I A T N A Y A D I
461 CAG ATG ACC CAG TCC CCG AGC TCC CTG TCC GCC TCT GTG GGC GAT AGG
3 Q M T Q S P S S L S A S V G D R
509 GTC ACC ATC ACC TGT CGT GCC AGT CAG GAC ATC AAC AAT TAT CTG AAC
19 V T I T C R A S Q D I N N Y L N
557 TGG TAT CAA CAG AAA CCA GGA AAA GCT CCG AAA CTA CTG ATT TAC TAT
35 W Y Q Q K P G K A P K L L I Y Y
605 ACC TCC ACC CTC CAC TCT GGA GTC CCT TCT CGC TTC TCT GGT TCT GGT
51 T S T L H S G V P S R F S G S G

FIG._4A-1

```
653 TCT GGG ACG GAT TAC ACT CTG ACC ATC AGC AGT CTG CAA CCG GAG GAC
 67 S   G   T   D   Y   T   L   T   I   S   S   L   Q   P   E   D

701 TTC GCA ACT TAT TAC TGT CAG CAA GGT AAT ACT CTG CCG CCG ACG TTC
 83 F   A   T   Y   Y   C   Q   Q   G   N   T   L   P   P   T   F

749 GGA CAG GGC ACG AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT
 99 G   Q   G   T   K   V   E   I   K   R   T   V   A   A   P   S

797 GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC
115 V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A

845 TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA
131 S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K   V

893 CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT
147 Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q   E   S

941 GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC
163 V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T

989 CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC
179 L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A   C

1037 GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC
195 E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N

1085 AGG GGA GAG TGT TAA  G CTGATCCTCT ACGCCGGACG CATCGTGGCG
211 R   G   E   C
```

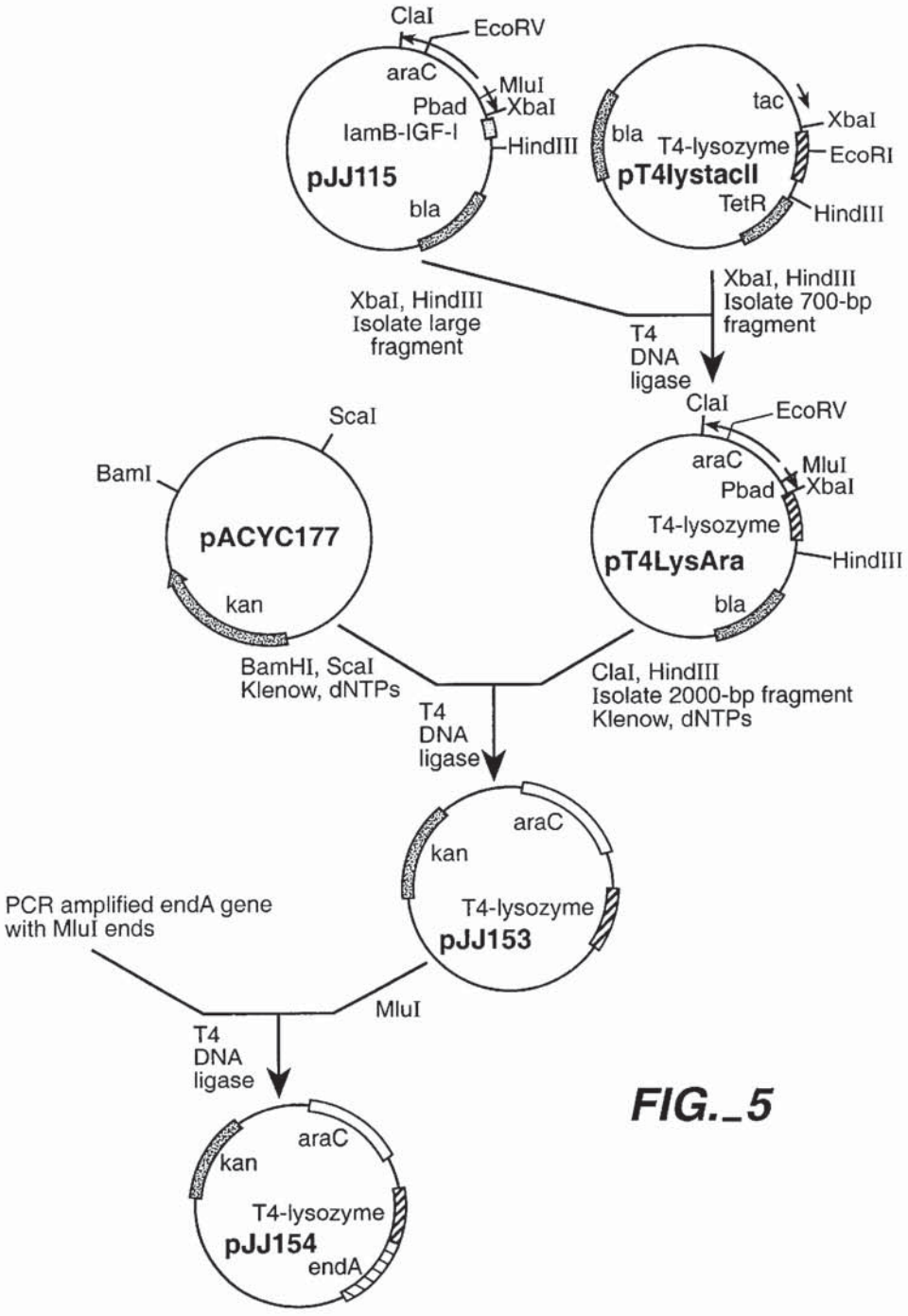
FIG._4A-2

```
1131 CTAGTACGCA AGTTCACGTA AAAACGGTAT CTAGAGGTTG AGGTGATTTT   ATG AAA
  -23                                                        M   K

1187 AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
 -21 K   N   I   A   F   L   L   A   S   M   F   V   F   S   I   A

1235 ACA AAC GCG TAC GCT GAG GTT CAG CTG GTG GAG TCT GGC GGT GGC CTG
  -5 T   N   A   Y   A   E   V   Q   L   V   E   S   G   G   G   L

1283 GTG CAG CCA GGG GGC TCA CTC CGT TTG TCC TGT GCA ACT TCT GGC TAC
  12 V   Q   P   G   G   S   L   R   L   S   C   A   T   S   G   Y

1331 ACC TTT ACC GAA TAC ACT ATG CAC TGG ATG CGT CAG GCC CCG GGT AAG
  28 T   F   T   E   Y   T   M   H   W   M   R   Q   A   P   G   K

1379 GGC CTG GAA TGG GTT GCA GGG ATT AAT CCT AAA AAC GGT GGT ACC AGC
  44 G   L   E   W   V   A   G   I   N   P   K   N   G   G   T   S

1427 CAC AAC CAG AGG TTC ATG GAC CGT TTC ACT ATA AGC GTA GAT AAA TCC
  60 H   N   Q   R   F   M   D   R   F   T   I   S   V   D   K   S

1475 ACC AGT ACA GCC TAC ATG CAA ATG AAC AGC CTG CGT GCT GAG GAC ACT
  76 T   S   T   A   Y   M   Q   M   N   S   L   R   A   E   D   T

1523 GCC GTC TAT TAT TGT GCT AGA TGG CGA GGC CTG AAC TAC GGC TTT GAC
  92 A   V   Y   Y   C   A   R   W   R   G   L   N   Y   G   F   D

1571 GTC CGT TAT TTT GAC GTC TGG GGT CAA GGA ACC CTG GTC ACC GTC TCC
 108 V   R   Y   F   D   V   W   G   Q   G   T   L   V   T   V   S

1619 TCG GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC
 124 S   A   S   T   K   G   P   S   V   F   P   L   A   P   S   S
```

FIG._4B-1

```
1667 AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC
  140 K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D

1715 TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC
  156 Y   F   P   E   P   V   T   V   S   W   N   S   G   A   L   T

1763 AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC TAC
  172 S   G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y

1811 TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC AGC TTG GGC ACC CAG
  188 S   L   S   S   V   V   T   V   P   S   S   S   S   L   G   T   Q

1859 ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTC GAC
  204 T   Y   I   C   N   V   N   H   K   P   S   N   T   K   V   D

1907 AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCG CCG
  220 K   K   V   E   P   K   S   C   D   K   T   H   T   C   P   P

1955 TGC CCA GCA CCA GAA CTG CTG GGC GGC CGC ATG AAA CAG CTA GAG GAC
  236 C   P   A   P   E   L   L   G   G   R   M   K   Q   L   E   D

2003 AAG GTC GAA GAG CTA CTC TCC AAG AAC TAC CAC CTA GAG AAT GAA GTG
  252 K   V   E   E   L   L   S   K   N   Y   H   L   E   N   E   V

2051 GCA AGA CTC AAA AAG CTT GTC GGG GAG CGC TAA  GCATGCG ACGGCCCTAG
  268 A   R   L   K   K   L   V   G   E   R

2101 AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA
```

FIG._4B-2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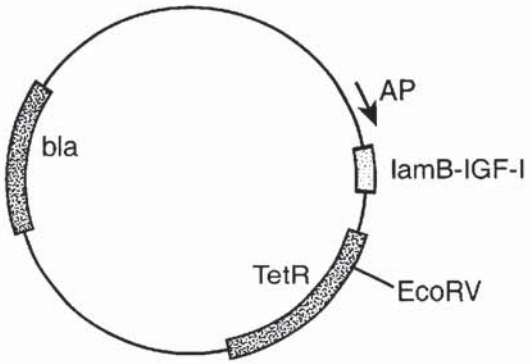
EcoRV
araC
MluI
Pbad
XbaI
lamB-IGF-I
HindIII
pJJ115
bla
tac
XbaI
bla
T4-lysozyme
EcoRI
pT4lystacII
TetR
HindIII
XbaI, HindIII
Isolate large
fragment
XbaI, HindIII
Isolate 700-bp
fragment
T4
DNA
ligase
ScaI
BamI
pACYC177
kan
ClaI
EcoRV
araC
MluI
Pbad
XbaI
T4-lysozyme
pT4LysAra
HindIII
bla
BamHI, ScaI
Klenow, dNTPs
ClaI, HindIII
Isolate 2000-bp fragment
Klenow, dNTPs
T4
DNA
ligase
araC
kan
T4-lysozyme
pJJ153
PCR amplified endA gene
with MluI ends
MluI
T4
DNA
ligase
araC
kan
T4-lysozyme
pJJ154
endA

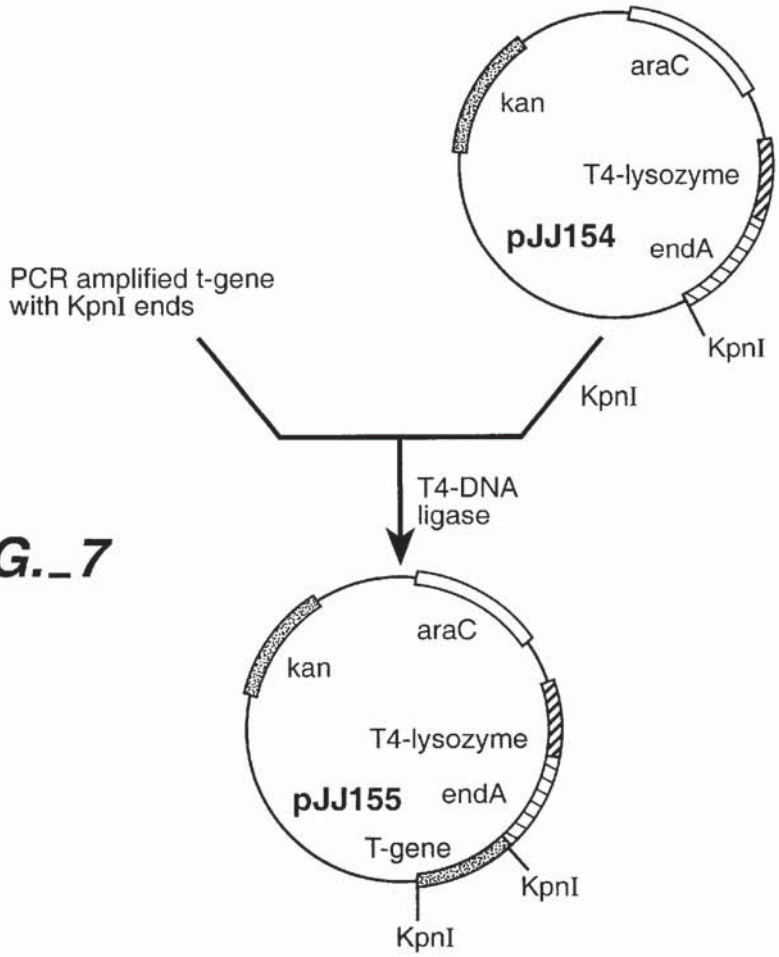
FIG._5

FIG._6



pLBIGF57

FIG._7



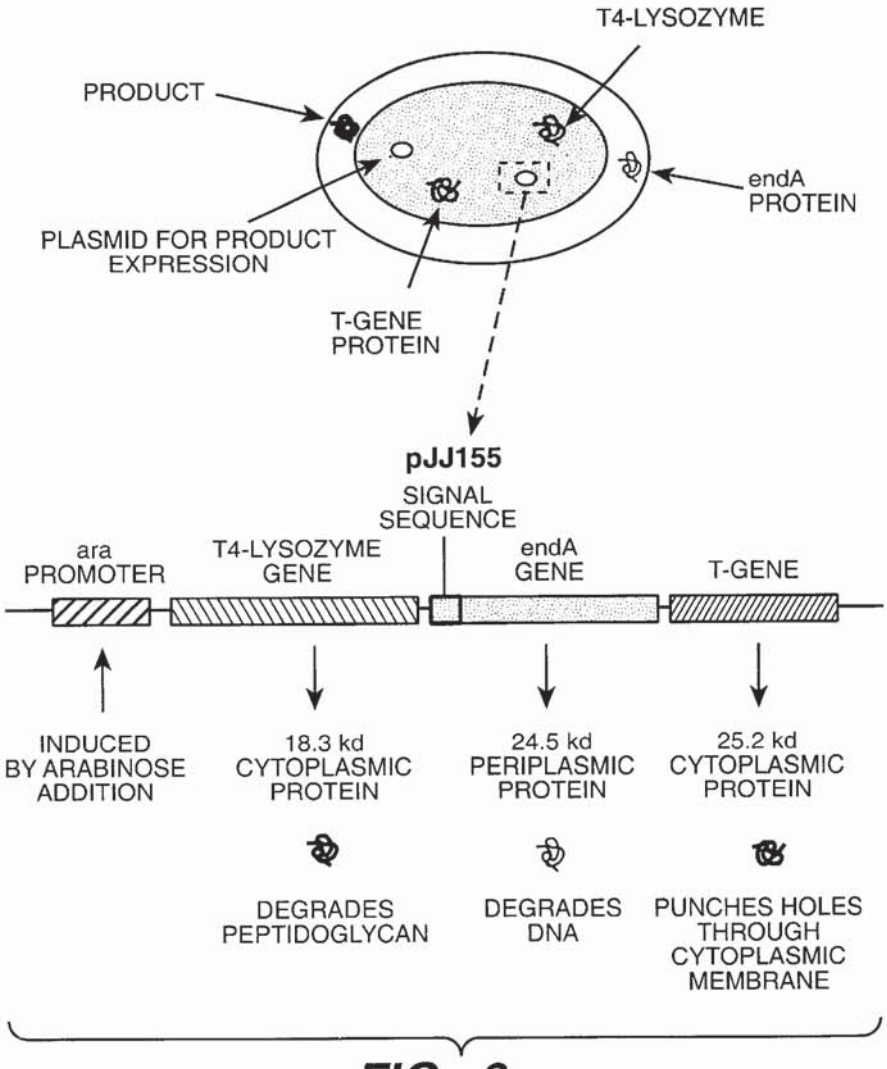

T4-LYSOZYME
PRODUCT
endA
PROTEIN
PLASMID FOR PRODUCT
EXPRESSION
T-GENE
PROTEIN
pJJ155
SIGNAL
SEQUENCE
ara
PROMOTER
T4-LYSOZYME
GENE
endA
GENE
T-GENE
INDUCED
BY ARABINOSE
ADDITION
18.3 kd
CYTOPLASMIC
PROTEIN
24.5 kd
PERIPLASMIC
PROTEIN
25.2 kd
CYTOPLASMIC
PROTEIN
DEGRADES
PEPTIDOGLYCAN
DEGRADES
DNA
PUNCHES HOLES
THROUGH
CYTOPLASMIC
MEMBRANE

FIG._8

CONTROL BROTH BEFORE EDTA ADDITION
RESUSPENDED PELLET

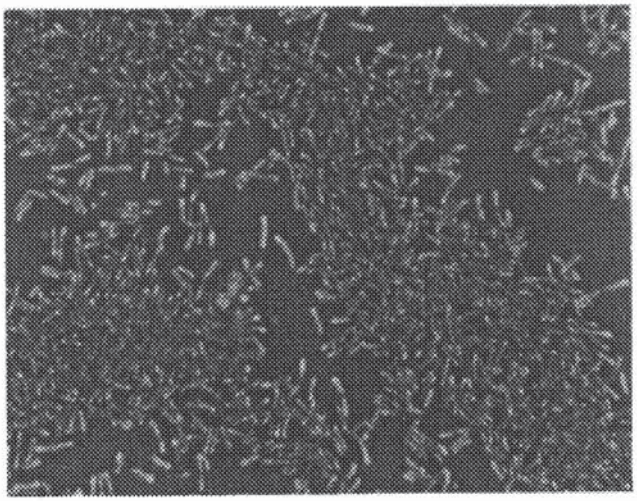


FIG._9A

CONTROL BROTH AFTER EDTA ADDITION
RESUSPENDED PELLET

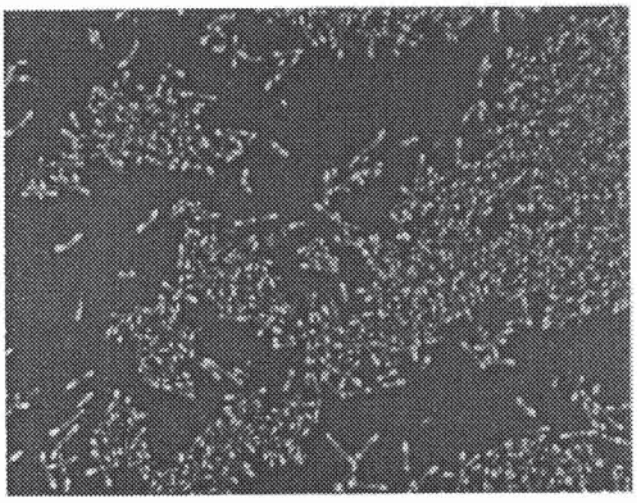


FIG._9B

BROTH WITH T4-LYSOZYME +endA +t-GENE CO-EXPRESSION FERMENTATION HARVEST WHOLE BROTH - UNDILUTED WHOLE BROTH

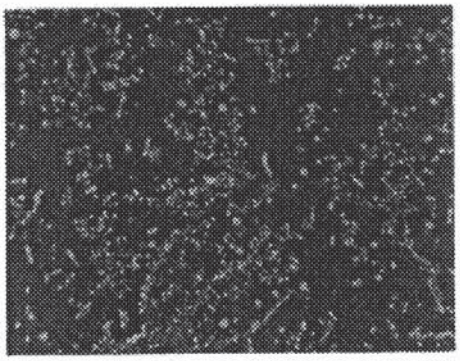


FIG._9C

BROTH WITH T4-LYSOZYME +endA +t-GENE CO-EXPRESSION BEFORE EDTA ADDITION - RESUSPENDED PELLET

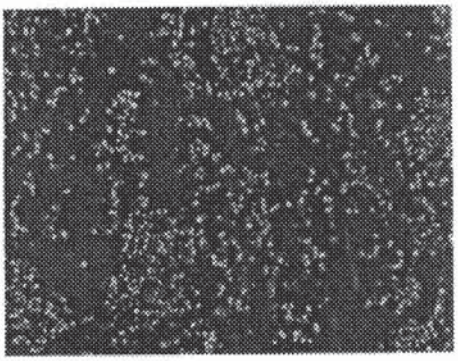


FIG._9D

BROTH WITH T4-LYSOZYME +endA +t-GENE CO-EXPRESSION AFTER EDTA ADDITION - RESUSPENDED PELLET

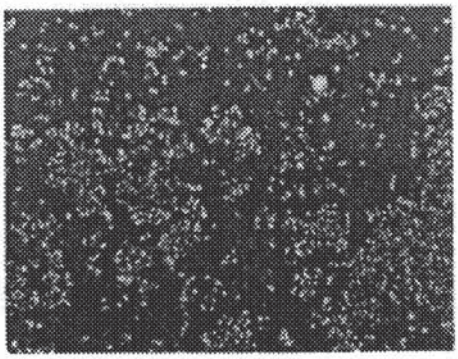


FIG._9E

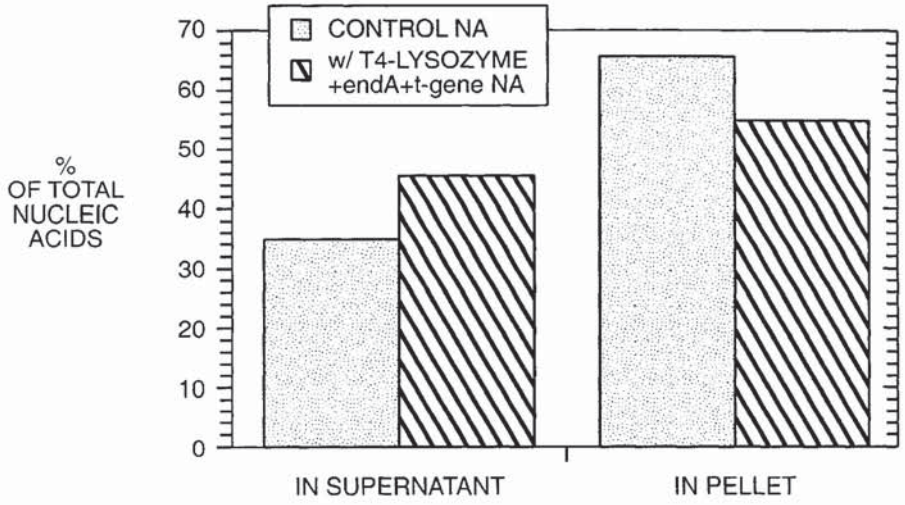


FIG._10A

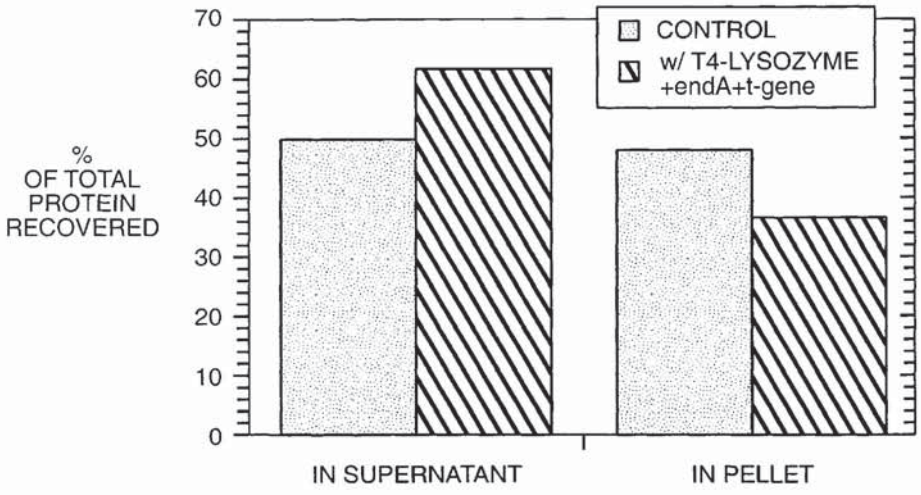


FIG._10B

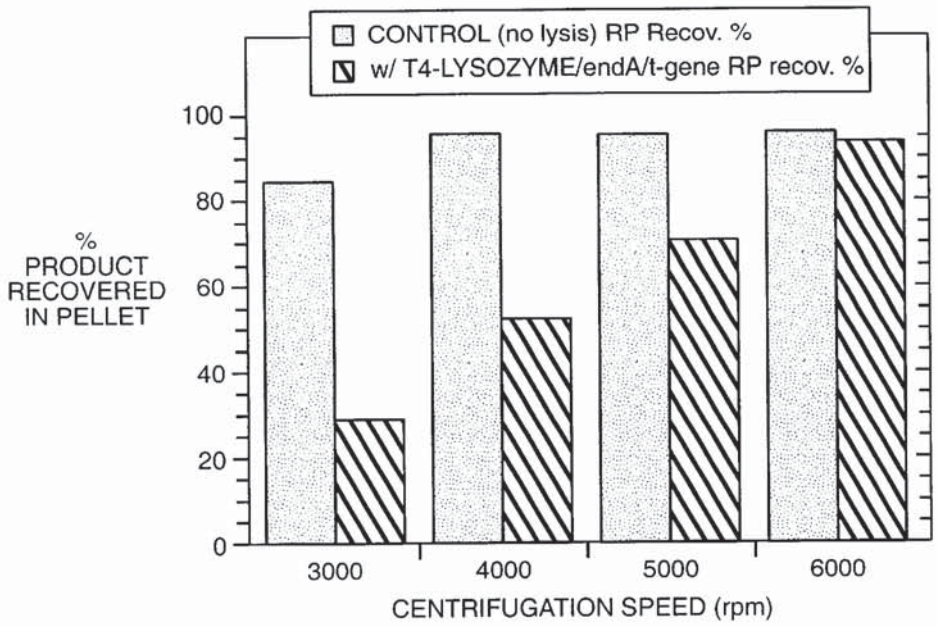

CONTROL (no lysis) RP Recov. %
w/ T4-LYSOZYME/endA/t-gene RP recov. %
% PRODUCT RECOVERED IN PELLET
100
80
60
40
20
0
3000
4000
5000
6000
CENTRIFUGATION SPEED (rpm)

FIG._11

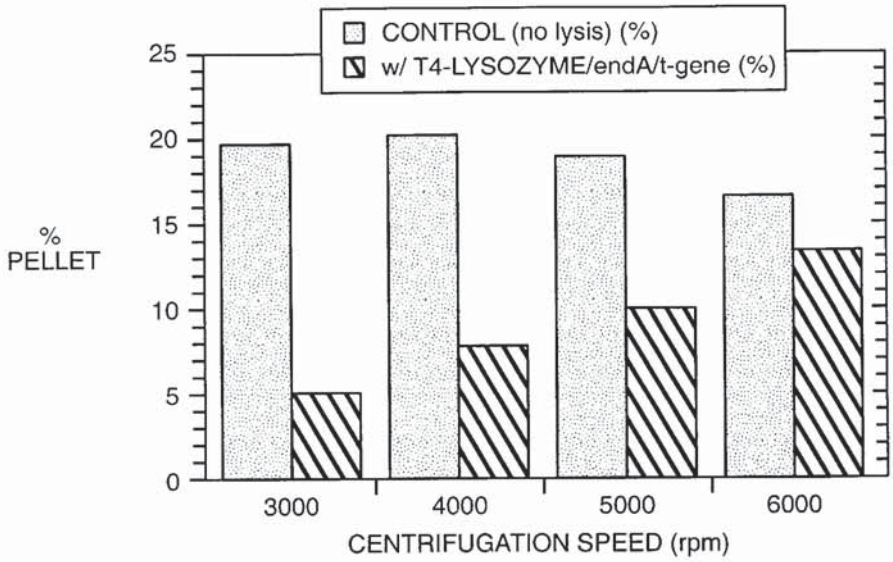

CONTROL (no lysis) (%)
w/ T4-LYSOZYME/endA/t-gene (%)
% PELLET
25
20
15
10
5
0
3000
4000
5000
6000
CENTRIFUGATION SPEED (rpm)

FIG._12

PROCESS FOR BACTERIAL PRODUCTION OF POLYPEPTIDES

RELATED APPLICATIONS

This is a divisional application claiming priority to application Ser. No. 09/422,712, filed Oct. 21, 1999, now U.S. Pat. No. 6,180,367; which application claims priority to provisional application No. 60/106,052, filed Oct. 28, 1998, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a process for producing and recovering heterologous polypeptides from bacterial cells. More particularly, this invention relates to a process wherein recovery of soluble or aggregated recombinant heterologous polypeptides from bacterial cytoplasm and periplasm is facilitated or increased.

2. Description of Related Disclosures

Escherichia coli has been widely used for the production of heterologous proteins in the laboratory and industry. *E. coli* does not generally excrete proteins to the extracellular medium apart from colicins and hemolysin (Pugsley and Schwartz, *Microbiology,* 32: 3–38 (1985)). Heterologous proteins expressed by *E. coli* may accumulate as soluble product or insoluble aggregates. See FIG. **1** herein. They may be found intracellularly in the cytoplasm or be secreted into the periplasm if preceded by a signal sequence. How one proceeds initially in the recovery of the products greatly depends upon how and where the product accumulates. Generally, to isolate the proteins, the cells may be subjected to treatments for periplasmic extraction or be disintegrated to release trapped products that are otherwise inaccessible.

The conventional isolation of heterologous polypeptide from gram-negative bacteria poses problems owing to the tough, rigid cell walls that surround these cells. The bacterial cell wall maintains the shape of the cell and protects the cytoplasm from osmotic pressures that may cause cell lysis; it performs these functions as a result of a highly cross-linked peptidoglycan (also known as murein) backbone that gives the wall its characteristic rigidity. A recent model described the space between the cytoplasmic and outer membranes as a continuous phase filled with an inner periplasmic polysaccharide gel that extends into an outer highly cross-linked peptidoglycan gel (Hobot et al., *J. Bact.,* 160: 143 (1984)). This peptidoglycan sacculus constitutes a barrier to the recovery of any heterologous polypeptide not excreted by the bacterium into the medium.

To release recombinant proteins from the *E. coli* periplasm, treatments involving chemicals such as chloroform (Ames et al., *J. Bacteriol.,* 160: 1181–1183 (1984)), guanidine-HCl, and Triton X-100 (Naglak and Wang, *Enzyme Microb. Technol.,* 12: 603–611 (1990)) have been used. However, these chemicals are not inert and may have detrimental effects on many recombinant protein products or subsequent purification procedures. Glycine treatment of *E. coli* cells, causing permeabilization of the outer membrane, has also been reported to release the periplasmic contents (Ariga et al., *J. Ferm. Bioeng.,* 68: 243–246 (1989)). These small-scale periplasmic release methods have been designed for specific systems. They do not translate easily and efficiently and are generally unsuitable as large-scale methods.

The most widely used methods of periplasmic release of recombinant protein are osmotic shock (Nosal and Heppel, *J. Biol. Chem.,* 241: 3055–3062 (1966); Neu and Heppel, *J. Biol. Chem.,* 240: 3685–3692 (1965)), hen eggwhite (HEW)-lysozyme/ethylenediamine tetraacetic acid (EDTA) treatment (Neu and Heppel, *J. Biol. Chem.,* 239: 3893–3900 (1964); Witholt et al., *Biochim. Biophys. Acta,* 443: 534–544 (1976); Pierce et al., *ICheme Research. Event,* 2: 995–997 (1995)), and combined HEW-lysozyme/osmotic shock treatment (French et al., *Enzyme and Microb. Tech.,* 19: 332–338 (1996)). Typically, these procedures include an initial disruption in osmotically-stabilizing medium followed by selective release in non-stabilizing medium. The composition of these media (pH, protective agent) and the disruption methods used (chloroform, HEW-lysozyme, EDTA, sonication) vary among specific procedures reported. A variation on the HEW-lysozyme/EDTA treatment using a dipolar ionic detergent in place of EDTA is discussed by Stabel et al., *Veterinary Microbiol.,* 38: 307–314 (1994). For a general review of use of intracellular lytic enzyme systems to disrupt *E. coli*, see Dabora and Cooney in *Advances in Biochemical Engineering/Biotechnology,* Vol. 43, A. Fiechter, ed. (Springer-Verlag: Berlin, 1990), pp. 11–30.

Conventional methods for the recovery of recombinant protein from the cytoplasm, as soluble protein or refractile particles, involved disintegration of the bacterial cell by mechanical breakage. Mechanical disruption typically involves the generation of local cavitation in a liquid suspension, rapid agitation with rigid beads, sonication, or grinding of cell suspension (*Bacterial Cell Surface Techniques,* Hancock and Poxton (John Wiley & Sons Ltd, 1988), Chapter 3, p. 55). These processes require significant capital investment and constitute long processing time.

HEW-lysozyme acts biochemically to hydrolyze the peptidoglycan backbone of the cell wall. The method was first developed by Zinder and Arndt, *Proc. Natl. Acad. Sci. USA,* 42: 586–590 (1956), who treated *E. coli* with egg albumin (which contains HEW-lysozyme) to produce rounded cellular spheres later known as spheroplasts. These structures retained some cell-wall components but had large surface areas in which the cytoplasmic membrane was exposed.

U.S. Pat. No. 5,169,772 discloses a method for purifying heparinase from bacteria comprising disrupting the envelope of the bacteria in an osmotically-stabilized medium, e.g., 20% sucrose solution using, e.g., EDTA, lysozyme, or an organic compound, releasing the non-heparinase-like proteins from the periplasmic space of the disrupted bacteria by exposing the bacteria to a low-ionic-strength buffer, and releasing the heparinase-like proteins by exposing the low-ionic-strength-washed bacteria to a buffered salt solution.

There are several disadvantages to the use of the HEW-lysozyme addition for isolating periplasmic proteins. The cells must be treated with EDTA, detergent, or high pH, all of which aid in weakening the cells. Also, the method is not suitable for lysis of large amounts of cells because the lysozyme addition is inefficient and there is difficulty in dispersing the enzyme throughout a large pellet of cells.

Many different modifications of these methods have been used on a wide range of expression systems with varying degrees of success (Joseph-Liazun et al., *Gene,* 86: 291–295 (1990); Carter et al., *Bio/Technology,* 10: 163–167 (1992)). Although these methods have worked on a laboratory scale, they involve too many steps for an efficient large-scale recovery process.

Efforts to induce recombinant cell culture to produce lysozyme have been reported. EP 155,189 discloses a means for inducing a recombinant cell culture to produce lysozymes, which would ordinarily be expected to kill such host cells by means of destroying or lysing the cell wall

structure. Russian Pat. Nos. 2043415, 2071503, and 2071501 disclose plasmids and corresponding strains for producing recombinant proteins and purifying water-insoluble protein agglomerates involving the lysozyme gene. Specifically, the use of an operon consisting of the lysozyme gene and a gene that codes for recombinant protein enables concurrent synthesis of the recombinant protein and a lysozyme that breaks the polysaccharide membrane of *E. coli*.

U.S. Pat. No. 4,595,658 discloses a method for facilitating externalization of proteins transported to the periplasmic space of *E. coli*. This method allows selective isolation of proteins that locate in the periplasm without the need for lysozyme treatment, mechanical grinding, or osmotic shock treatment of cells. U.S. Pat. No. 4,637,980 discloses producing a bacterial product by transforming a temperature-sensitive lysogen with a DNA molecule that codes, directly or indirectly, for the product, culturing the transformant under permissive conditions to express the gene product intracellularly, and externalizing the product by raising the temperature to induce phage-encoded functions. JP 61–257931 published Nov. 15, 1986 discloses a method for recovering IL-2 using HEW-lysozyme. Asami et al., *J. Ferment. and Bioeng.,* 83: 511–516 (1997) discloses synchronized disruption of *E. coli* cells by T4 phage infection, and Tanji et al., *J. Ferment. and Bioeng.,* 85: 74–78 (1998) discloses controlled expression of lysis genes encoded in T4 phage for the gentle disruption of *E. coli* cells.

The development of an enzymatic release method to recover recombinant periplasmic proteins suitable for large-scale use is reported by French et al., *Enzyme and Microbial Technology,* 19: 332–338 (1996). This method involves resuspension of the cells in a fractionation buffer followed by recovery of the periplasmic fraction, where osmotic shock immediately follows lysozyme treatment. The effects of overexpression of the recombinant protein, *S. thermoviolaceus* α-amylase, and the growth phase of the host organism on the recovery are also discussed.

In a 10-kiloliter-scale process for recovery of IGF-I polypeptide (Hart et al., *Bio/Technology,* 12: 1113 (1994)), the authors attempted the typical isolation procedure involving a mechanical cell breakage step followed by a centrifugation step to recover the solids. The results were disappointing in that almost 40% of the total product was lost to the supernatant after three passes through the Gaulin homogenizer. Hart et al., *Bio/Technology* 12: 1113 (1994). See FIG. **2** herein. Product recovery was not significantly improved even when the classical techniques of EDTA and HEW-lysozyme additions were employed.

While HEW-lysozyme is the only practical commercial lysozyme for large-scale processes, lysozyme is expressed by bacteriophages upon infection of host cells. Lysis of *E. coli,* a natural host for bacteriophages, for example the T4 phages, requires the action of two gene products: e and t. Gene e encodes a lysozyme (called T4-lysozyme for the T4 phage) that has been identified as a muramidase (Tsugita and Inouye, *J. Biol. Chem.,* 243: 391 (1968)), while gene t seems to be required for lysis, but does not appear to have lysozyme activity. Gene t is required for the cessation of cellular metabolism that occurs during lysis (Mukai et al., *Vir.,* 33: 398 (1967)) and is believed to degrade or alter the cytoplasmic membrane, thus allowing gene product e to reach the periplasm and gain access to the cell wall (Josslin, *Vir.,* 40: 719 (1970)). Phage are formed by gene t- mutants, but lysis of the *E. coli* host does not occur except by addition of chloroform (Josslin, supra). Wild-type T4-lysozyme activity is first detected about eight minutes after T4 infection at 37° C., and it increases through the rest of the infection, even if lysis inhibition is induced. In the absence of secondary adsorption, cells infected by gene e mutants shut down progeny production and metabolism at the normal time, but do not lyse (*Molecular Genetics of Bacteriophage T4*, J. D. Karam, ed. in chief (American Society for Microbiology, Washington DC, ASM Press, 1994), p. 398).

Recovery of insoluble IGF-I using T4-lysozyme was disclosed on Oct. 28, 1997 at the "Separation Technology VII meeting entitled 'Separations for Clean Production'" in Davos, Switzerland, sponsored by the Engineering Foundation.

Upon cell lysis, genomic DNA leaks out of the cytoplasm into the medium and results in significant increase in fluid viscosity that can impede the sedimentation of solids in a centrifugal field. In the absence of shear forces such as those exerted during mechanical disruption to break down the DNA polymers, the slower sedimentation rate of solids through viscous fluid results in poor separation of solids and liquid during centrifugation. Other than mechanical shear force, there exist nucleolytic enzymes that degrade DNA polymer. In *E. coli,* the endogenous gene endA encodes for an endonuclease (molecular weight of the mature protein is approx. 24.5 kD) that is normally secreted to the periplasm and cleaves DNA into oligodeoxyribonucleotides in an endonucleolytic manner. It has been suggested that endA is relatively weakly expressed by *E. coli* (Wackernagel et al., *Gene,* 154: 55–59 (1995)).

For controlling cost of goods and minimizing process time, there is a continuing need for increasing the total recovery of heterologous polypeptide from cells. At large scale, there is a significant incentive to avoid mechanical cell breakage to release the soluble or aggregated recombinant polypeptide from the cytoplasmic and periplasmic compartments and to condition the lysate for efficient product recovery in the subsequent step.

SUMMARY OF THE INVENTION

Accordingly, this invention provides processes using biochemical disruption to recover both soluble and insoluble heterologous product from bacterial cells.

In one aspect the present invention provides a process for recovering a heterologous polypeptide from bacterial cells comprising:

(a) culturing bacterial cells, which cells comprise nucleic acid encoding phage lysozyme and nucleic acid encoding a protein that displays DNA-digesting activity under the control of a signal sequence for secretion of said DNA-digesting protein, wherein said nucleic acids are linked to a first promoter, and nucleic acid encoding the heterologous polypeptide and a signal sequence for secretion of the heterologous polypeptide, which nucleic acid encoding the heterologous polypeptide is linked to a second promoter, wherein the second promoter is inducible and the first promoter is either a promoter with low basal expression or an inducible promoter, the culturing being under conditions whereby when an inducer is added, expression of the nucleic acid encoding the phage lysozyme and DNA-digesting protein is induced after about 50% or more of the heterologous polypeptide has accumulated, whereby the phage lysozyme accumulates in a cytoplasmic compartment, whereby the DNA-digesting protein is secreted to the periplasm, and whereby the cells are lysed to produce a broth lysate; and

(b) recovering accumulated heterologous polypeptide from the broth lysate.

In yet another aspect, the invention supplies a process for recovering a heterologous polypeptide from bacterial cells in which it is produced comprising:

(a) culturing the bacterial cells, which cells comprise nucleic acid encoding phage lysozyme, gene t, and nucleic acid encoding a protein that displays DNA-digesting activity, wherein these nucleic acids are linked to a first promoter, and nucleic acid encoding the heterologous polypeptide, which nucleic acid is linked to a second promoter, wherein the second promoter is inducible and the first promoter is either a promoter with low basal expression or an inducible promoter, the culturing being under conditions whereby when an inducer is added, expression of the nucleic acids encoding the phage lysozyme, gene t, and DNA-digesting protein is induced after about 50% or more of the heterologous polypeptide has accumulated, and under conditions whereby the phage lysozyme accumulates in a cytoplasmic compartment, whereby the DNA-digesting protein is secreted into the periplasm, and is whereby the cells are lysed to produce a broth lysate; and

(b) recovering accumulated heterologous polypeptide from the broth lysate.

In a third aspect, the invention provides a process for recovering a heterologous polypeptide from bacterial cells in which it is produced comprising:

(a) culturing the bacterial cells, which cells comprise nucleic acid encoding phage lysozyme, gene t, and nucleic acid encoding a protein that displays DNA-digesting activity, wherein the nucleic acid encoding the phage lysozyme and DNA-digesting protein is linked to a first promoter that is inducible or with low basal expression, the gene t is linked to a second inducible promoter, and the nucleic acid encoding the heterologous polypeptide is linked to a third inducible promoter, under conditions whereby when an inducer is added and all three promoters are inducible, expression of the nucleic acids encoding the phage lysozyme, gene t, and DNA-digesting protein is induced after about 50% or more of the heterologous polypeptide has accumulated, with the third promoter being induced before the first promoter and the second promoter induced after the first promoter, and whereby if the phage lysozyme and DNA-digesting protein are linked to a promoter with low basal expression, expression of the gene t is induced after about 50% or more of the heterologous polypeptide has accumulated, and under conditions whereby the phage lysozyme accumulates in a cytoplasmic compartment, whereby the DNA-digesting protein is secreted into the periplasm, and whereby the cells are lysed to produce a broth lysate; and

(b) recovering accumulated heterologous polypeptide from the broth lysate.

Biochemical lysis or biochemically-assisted mechanical lysis is superior to mechanical disruption for recovering heterologous polypeptide from bacterial cells. Coordinated expression of nucleic acid encoding phage lysozyme with gene t and DNA-digesting protein, and nucleic acid encoding the heterologous polypeptide of interest provides a highly effective method for releasing insoluble or soluble polypeptide from the entanglement with the peptidoglycan layer, as well as releasing product trapped in the cytoplasm. When the phage lysozyme gene is cloned behind a tightly-controlled promoter, for example, the pBAD promoter (also referred to as the ara promoter), cytoplasmic accumulation of phage lysozyme may be induced by the addition of an inducer (such as arabinose) at an appropriate time near the end of fermentation. By placing the nucleic acid expression of heterologous polypeptide and lytic enzymes under separate promoter control, one can independently regulate their production during fermentation. Without a signal sequence, the accumulated phage lysozyme is tightly locked up in the cytoplasmic compartment, and gene t functions to release the phage lysozyme to degrade the peptidoglycan layer. Furthermore, the optimal pH for T4-phage-lysozyme activity, which is a preferred embodiment, is about 7.3, which is about the neutral pH of most typical harvest broths.

The induction of the genes encoding the bacteriophage lysozyme, DNA-digesting protein, and gene t after expression of the nucleic acid encoding the heterologous polypeptide results in a significant amount of insoluble or soluble heterologous polypeptide recovered from the cytoplasm or periplasm of bacteria. Besides product yield, the success of a recovery process is judged by the ease of operation, the process flow, the turn-around time, as well as the operation cost. The present invention alleviates several if not all these bottlenecks encountered in the large-scale recovery process.

The processes herein also allow use of biochemical cell lysis at high cell density and increased scale. At high density, excessive expression of T4-lysozyme, gene t, and endA could have disastrous results, such as premature cell lysis and reduction in heterologous polypeptide production. Further, it would not be expected that induction at the end of a long fermentation process and after substantial product accumulation would produce enough of the lytic enzymes to be effective. The present processes do not pose problems at high cell densities such as increased viscosity and excessive foaming during the fermentation process. It is expected that the processes herein will enable the attainment of high cell density, effective induction and action of the system, and the processing of broth lysates derived from high-density cultures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts a schematic diagram of how a polypeptide product is disposed in the cytoplasm and in the periplasm, that is, it forms an aggregate, proteolyzed fragment, or folded soluble polypeptide.

FIG. **2** depicts IGF-I aggregate recovery from the supernatant and pellet by the typical isolation procedure involving mechanical cell disruption followed by centrifugation, after three passes through the Gaulin homogenizer.

FIG. **3** depicts a plasmid map of pS1130, an expression plasmid for rhuMAb CD18 $F(ab')_2$-leucine zipper precursor (herein also referred to as anti-CD18 antibody fragment).

FIGS. **4A-1**, **4A-2**, **4B-1**, and **4B-2** show the sequence of the expression cassette of pS1130 (SEQ ID NOS:1 and 2).

FIG. **5** shows plasmid construction of pJJ154 used to co-express T4-lysozyme and endA (*E. coli* DNase).

FIG. **6** shows a plasmid map of pLBIGF57 used to express IGF-I.

FIG. **7** shows plasmid construction of pJJ155 used to express T4-lysozyme, endA, and gene t, which construction is from pJJ154.

FIG. **8** depicts a schematic of the two-plasmid system for co-expression of T4-lysozyme, a preferred phage lysozyme, endA, a preferred DNA-digesting protein, and gene t (pJJ155) with IGF-I-encoding nucleic acid in accordance with an example of this invention.

FIGS. **9A–9E** disclose photographs from phase-contrast microscopy of the harvest broth and resuspended pellets from centrifugation of fermentation broth with and without co-expression of T4-lysozyme and endA and t-gene before and after EDTA addition. Specifically, the photographs show the resuspended pellet from centrifugation of control broth with no lytic enzyme co-expression before and after EDTA

addition respectively (FIGS. **9**A and **9**B), the fermentation harvest undiluted whole broth resulting from co-expression of IGF-I with the three lytic enzymes, T4-lysozyme, endA, and t-gene (FIG. **9**C), the resuspended pellet from centrifugation of harvest broth with co-expression of IGF-I with the three lytic enzymes and no EDTA addition (FIG. **9**D), and the resuspended pellet from centrifugation of harvest broth with the co-expression of IGF-I with the three lytic enzymes and EDTA addition (FIG. **9**E).

FIGS. **10**A and **10**B show, respectively, nucleic acid quantitation in the supernatant and pellet by OD260 determination for IGF-I-expressing *E. coli* with co-expression of the three lytic enzymes versus control, and total protein in the supernatant and pellet from IGF-I-expressing *E. coli* with co-expression of the three lytic enzymes versus no co-expression control.

FIG. **11** shows IGF-I product recovery by centrifugation using three lytic enzymes versus no-lysis control broth for various centrifugation speeds.

FIG. **12** shows solids recovery during centrifugation using three lytic enzymes co-expressed with IGF-I versus no-lysis control broth for various centrifugation speeds.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Definitions

As used herein, "phage lysozyme" refers to a cytoplasmic enzyme that facilitates lysis of phage-infected bacterial cells, thereby releasing replicated phage particles. The lysozyme may be from any bacteriophage source, including T7, T4, lambda, and mu bacteriophages. The preferred such lysozyme herein is T4-lysozyme.

As used herein, "T4-lysozyme" or "E protein" refers to a cytoplasmic muramidase that facilitates lysis of T4 phage-infected bacterial cells, thereby releasing replicated phage particles (Tsugita and Inouye, *J. Mol. Biol.,* 37: 201–12 (1968); Tsugita and Inouye, *J. Biol. Chem.,* 243: 391–97 (1968)). It is encoded by gene e of T4 bacteriophage and hydrolyzes bonds between N-acetylglucosamine and N-acetylmuramic acid residues in the rigid peptidoglycan layer of the *E. coli* cell envelope. The enzyme is a single polypeptide chain of a molecular weight of 18.3 kd. It is approximately 250-fold more active than HEW-lysozyme against bacterial peptidoglycan (Matthews et al., *J. Mol. Biol.,* 147: 545–558 (1981)). The optimal pH for T4-lysozyme enzyme activity is 7.3, versus 9 for HEW-lysozyme (*The Worthington Manual;* pp 219–221).

As used herein, "gene t" or "t gene" or "holin" refers to a lytic gene of bacteriophage T4 that is required for lysis but does not appear to have lysozyme activity. See also *Molecular Genetics of Bacteriophage T4*, supra, p. 398–399.

The term "protein that displays DNA-digesting activity" or "DNA-digesting protein" refers to a protein that will digest DNA such as, for example, mammalian or bacterial DNase. Preferably, the DNA-digesting protein is human DNase or bacterial endA.

As used herein, the phrase "lytic enzymes" refers collectively to at least phage lysozyme and DNA-digesting protein; where applicable it also refers to a phage gene t gene product or equivalent in combination with phage lysozyme and DNA-digesting protein.

As used herein, the phrase "agent that disrupts the outer cell wall" of bacteria refers to a molecule that increases permeability of the outer cell wall of bacteria, such as chelating agents, e.g., EDTA, and zwitterions.

As used herein, the term "bacteria" refers to any bacterium that produces proteins that are transported to the periplasmic space. Generally, the bacteria, whether gram positive or gram negative, has phage lysozyme and nuclease expression under control so that they are only expressed near the end of the fermentation, a preferred embodiment, or expressed at a low level during fermentation. The nuclease is generally relatively stable when secreted to the periplasm or medium. The term "non-temperature-sensitive bacteria" refers to any bacterium that is not significantly sensitive to temperature changes. One preferred embodiment herein is bacteria that are not temperature sensitive. The most preferred bacteria herein are gram-negative bacteria.

As used herein, "a time sufficient to release the polypeptide contained in the cytoplasm or periplasm" refers to an amount of time sufficient to allow the lysozyme to digest the peptidoglycan to a sufficient degree to release the cytoplasmic or periplasmic aggregate or soluble heterologous polypeptide.

As used herein, "signal sequence" or "signal polypeptide" refers to a peptide that can be used to secrete the heterologous polypeptide or protein that displays DNA-digesting activity into the periplasm of the bacteria. The signals for the heterologous polypeptide or DNA-digesting protein may be homologous to the bacteria, or they may be heterologous, including signals native to the heterologous polypeptide or DNA-digesting protein being produced in the bacteria.

The promoters of this invention may be "inducible" promoters, i.e., promoters that direct transcription at an increased or decreased rate upon binding of a transcription factor.

As used herein, a promoter "with low basal expression" or a "low-basal-expression promoter" is a promoter that is slightly leaky, i.e., it provides a sufficiently low basal expression level so as not to affect cell growth or product accumulation and provides a sufficiently low level of promotion not to result in premature cell lysis.

"Transcription factors" as used herein include any factors that can bind to a regulatory or control region of a promoter and thereby effect transcription. The synthesis or the promoter-binding ability of a transcription factor within the host cell can be controlled by exposing the host to an "inducer" or removing a "repressor" from the host cell medium. Accordingly, to regulate expression of an inducible promoter, an inducer is added or a repressor removed from the growth medium of the host cell.

As used herein, the phrase "induce expression" means to increase the amount of transcription from specific genes by exposure of the cells containing such genes to an effector or inducer.

An "inducer" is a chemical or physical agent which, when given to a population of cells, will increase the amount of transcription from specific genes. These are usually small molecules whose effects are specific to particular operons or groups of genes, and can include sugars, alcohol, metal ions, hormones, heat, cold, and the like. For example, isopropylthio-β-galactoside (IPTG) and lactose are inducers of the tacII promoter, and L-arabinose is a suitable inducer of the arabinose promoter.

A "repressor" is a factor that directly or indirectly leads to cessation of promoter action or decreases promoter action. One example of a repressor is phosphate As the repressor phosphate is depleted from the medium, the alkaline phosphatase (AP) promoter is induced.

As used herein, "polypeptide" or "polypeptide of interest" refers generally to peptides and proteins having more than about ten amino acids. The polypeptides are "heterologous," i.e., foreign to the host cell being utilized, such as a human protein produced by *E. coli*. The polypeptide may be pro-

duced as an insoluble aggregate or as a soluble polypeptide in the periplasmic space or cytoplasm.

Examples of mammalian polypeptides include molecules such as, e.g., renin, a growth hormone, including human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; thrombopoietin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial naturietic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; cardiotrophins (cardiac hypertrophy factor) such as cardiotrophin-1 (CT-1); platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1–3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; anti-HER-2 antibody; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressing; regulatory proteins; antibodies; and fragments of any of the above-listed polypeptides.

The preferred exogenous polypeptides of interest are mammalian polypeptides, most preferably human polypeptides. Examples of such mammalian polypeptides include t-PA, VEGF, gp120, anti-HER-2, anti-CD11a, anti-CD18, DNase, IGF-I, IGF-II, brain IGF-I, growth hormone, relaxin chains, growth hormone releasing factor, insulin chains or pro-insulin, urokinase, immunotoxins, neurotrophins, and antigens. Particularly preferred mammalian polypeptides include, e.g., IGF-I, DNase, or VEGF, most preferably IGF-I, if the polypeptide is produced as an insoluble aggregate in the periplasm, and anti-CD18 antibodies or fragments thereof such as anti-recombinant human CD18 Fab, Fab' and $(Fab')_2$ fragments, if the polypeptide is produced in a soluble form in the periplasm.

As used herein, "IGF-I" refers to insulin-like growth factor from any species, including bovine, ovine, porcine, equine, and preferably human, in native-sequence or in variant form and recombinantly produced. In a preferred method, the IGF-I is cloned and its DNA expressed, e.g., by the process described in EP 128,733 published Dec. 19, 1984.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably-linked coding sequence in a particular host organism. The control sequences that are suitable for bacteria include a promoter such as the alkaline phosphatase promoter, optionally an operator sequence, and a ribosome-binding site.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a heterologous polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading frame. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

B. Modes for Carrying Out the Invention

In one embodiment, the invention provides a process for recovering a heterologous polypeptide, soluble or insoluble, from bacterial cells in which it is produced. This process involves, in a first step, culturing the bacterial cells, which cells comprise nucleic acid encoding the lytic enzymes, wherein these nucleic acids are linked to a first promoter, and nucleic acid encoding the heterologous polypeptide, which nucleic acid is linked to a second inducible promoter. In an alternative embodiment, the phage lysozyme and DNA-digesting protein are linked to a first inducible promoter or a promoter with low basal expression, the t-gene is linked to a second inducible promoter, and the heterologous polypeptide is linked to a third inducible promoter.

The culturing takes place under conditions whereby expression of the nucleic acids encoding the lytic enzymes, when induced, commences after about 50% or more of the heterologous polypeptide has accumulated, and under conditions whereby the phage lysozyme accumulates in a cytoplasmic compartment and the DNA-digesting protein is secreted into the periplasmic compartment.

In the processes herein, induction of the promoters is preferred; however, the processes also contemplate the use of a promoter for the phage lysozyme and DNA-digesting protein that is a promoter with low basal expression (slightly leaky), wherein no induction is carried out. This type of promoter has a leakiness that is low enough not to result in premature cell lysis and results in a sufficiently low basal expression level so as not to affect cell growth or product accumulation.

In a second step, the accumulated heterologous polypeptide is recovered from the bacterial cells. An agent that increases permeability of the outer cell wall of the bacterial cells may be added, as described in detail below, before the recovery step is carried out. The need to disrupt cells mechanically to release the phage lysozyme is either

reduced or is completely avoided. In a preferred embodiment, after lytic enzyme expression the cells are incubated for a time sufficient to release the heterologous polypeptide contained in the cytoplasm or periplasm.

While the processes can apply to the recovery of insoluble aggregates such as IGF-I, VEGF, and DNase by sedimentation of product, they are also applicable to heterologous polypeptides that are soluble in the cytoplasm or periplasm, such as, for example, anti-CD18 antibody fragment. Advantages for recovery of soluble heterologous polypeptides by biochemical cell lysis include avoiding or reducing the need for mechanical lysis, thereby avoiding loss of heat-labile proteins, and obtaining a low and consistent fluid viscosity compatible with downstream recovery processes such as expanded bed absorption technology and centrifugation.

Expanded bed absorption (EBA) chromatography, described, for example, in "Expanded Bed Adsorption: Principles and Methods", *Pharmacia Biotech,* ISBN 91-630-5519-8), is useful for the initial recovery of target proteins from crude feed-stock or cell culture. The process steps of clarification, concentration, and initial purification can be combined into one unit operation, providing increased process economy due to a decreased number of process steps, increased yield, shorter overall process time, reduced labor cost, and reduced cost. In EBA chromatography an adsorbent is expanded and equilibrated by applying an upward liquid flow to the column. A stable fluidized bed is formed when the adsorbent particles are suspended in equilibrium due to the balance between particle sedimentation velocity and upward liquid flow velocity. Crude cell mixture or broth lysate is applied to the expanded bed with an upward flow. Target proteins are bound to the adsorbent while cell debris and other contaminants pass through unhindered. Weakly bound material is washed from the expanded bed using upward flow of a wash buffer. Flow is then stopped and the adsorbent is allowed to settle in the column. The column adaptor is then lowered to the surface of the sedimented bed. Flow is reversed and the captured proteins are eluted from the sedimented bed using an appropriate buffer. The eluate contains the target protein in a reduced elution pool volume, partially purified in preparation for packed bed chromatography (*Pharmacia Biotech,* supra). EBA, wherein the whole cell lysate containing soluble product is pumped up through the column and the protein is absorbed onto a resin (fluidized bed) and the cell debris flows through, utilizes only one chromatography step, thereby saving a step.

In another embodiment, the invention provides a process for recovering soluble heterologous polypeptide from the cytoplasm or periplasm of bacterial cells. This process involves culturing bacterial cells, which cells comprise nucleic acid encoding phage lysozyme and nucleic acid encoding a DNA-digesting protein that displays DNA-digesting activity under the control of a signal sequence for secretion of said DNA-digesting protein. In this process, the nucleic acids are linked to a first promoter, and nucleic acid encoding the heterologous polypeptide and a signal sequence for secretion of the heterologous polypeptide, which nucleic acid encoding the heterologous polypeptide is linked to a second inducible promoter. This culturing takes place under conditions whereby over-expression of the nucleic acid encoding the phage lysozyme and DNA-digesting protein is weakly and constitutively promoted or, if induced, commences after about 50% or more of the heterologous polypeptide has accumulated, and under conditions whereby the heterologous polypeptide and DNA-digesting protein are secreted into the periplasm of the bacteria and the phage lysozyme accumulates in a cytoplasmic compartment.

In a second step, the resulting heterologous polypeptide is recovered from the broth lysate.

In a third embodiment, the invention provides a process for recovering a heterologous polypeptide from bacterial cells in which three different promoters are used. Specifically, in the first step, the bacterial cells are cultured, where the cells comprise nucleic acid encoding phage lysozyme, gene t, and nucleic acid encoding a protein that displays DNA-digesting activity, as well as nucleic acid encoding the heterologous polypeptide. The nucleic acid encoding the lysozyme and DNA-digesting protein is linked to a first promoter that is inducible or with low basal expression, the gene t is linked to a second inducible promoter, and the nucleic acid encoding the heterologous polypeptide is linked to a third inducible promoter.

The culturing is carried out under conditions whereby when an inducer is added and all three promoters are inducible, expression of the nucleic acids encoding the phage lysozyme, gene t, and DNA-digesting protein is induced after about 50% or more of the heterologous polypeptide has accumulated, with the third promoter being induced before the first promoter, and the second promoter induced last, and whereby if the phage lysozyme and DNA-digesting protein are linked to a promoter with low basal expression, expression of the gene t is induced after about 50% or more of the heterologous polypeptide has accumulated. Culturing is also carried out under conditions whereby the phage lysozyme accumulates in a cytoplasmic compartment, whereby the DNA-digesting protein is secreted to and accumulates in the periplasm, and whereby the cells are lysed to produce a broth lysate.

In a second step, accumulated heterologous polypeptide is recovered from the broth lysate.

In the above processes, while the signal sequence for the DNA-digesting protein may be any sequence, preferably, it is a native sequence of the DNA-digesting protein. Also, in a preferred embodiment, the DNA-digesting protein is DNase or bacterial, e.g., *E. coli* endA product.

In a preferred embodiment, the culturing step takes place under conditions of high cell density, that is, generally at a cell density of about 15 to 150 g dry weight/liter, preferably at least about 40, more preferably about 40–150, most preferably about 40 to 100. In optical density, 120 OD550 (A_{550}) is about 50 g dry wt./liter. In addition, the culturing can be accomplished using any scale, even very large scales of 100,000 liters. Preferably, the scale is about 100 liters or greater, more preferably at least about 500 liters, and most preferably from about 500 liters to 100,000 liters.

The nucleic acids encoding the lytic enzymes are linked to one promoter, i.e., put in tandem, as by placing a linker between the nucleic acids. The promoter for the heterologous polypeptide expression is different from that used for the lytic enzymes, such that one is induced before the other. While the promoters may be any suitable promoters for this purpose, preferably, the promoters for the lytic enzymes with or without gene t and heterologous polypeptide are, respectively, arabinose promoter and alkaline phosphatase promoter.

The promoters for the heterologous polypeptide and for the lytic enzymes for all three processes herein must be different, such that the nucleic-acid-encoded heterologous polypeptide expression is induced before expression of nucleic-acid-encoded lytic enzymes or at a much higher level, when the promoters are inducible. While the promoters may be any suitable promoters for this purpose,

preferably, the promoters for the phage lysozyme and heterologous polypeptide are, respectively, arabinose promoter and alkaline phosphatase promoter. Alternatively, the compartmentalization of the phage lysozyme and DNA-digesting protein may allow for the use of a promoter with low basal expression for expression of the nucleic acid encoding phage lysozyme and DNA-digesting protein. If a promoter with low basal expression is employed, such as arabinose as opposed to tac or trp promoter, then an active step of induction is not required.

The induction of expression of the nucleic acid encoding the lytic enzymes is preferably carried out by adding an inducer to the culture medium. While, in this respect, the inducers for the promoters may be added in any amount, preferably if the inducer is arabinose, it is added in an amount of about 0–1% by weight, and if inducer is added, 0.1–1% by weight.

In the processes described above, typically the expression elements are introduced into the cells by transformation therein, but they may also be integrated into the genome or chromosome of the cells along with their promoter regions. This applies to any of the lytic enzymes or the heterologous polypeptide gene. The bacterial cells may be transformed with one or more expression vectors containing the nucleic acid encoding the lytic enzymes, and the nucleic acid encoding the heterologous polypeptide. In one such embodiment, the bacterial cells are transformed with two vectors respectively containing the nucleic acid encoding the lytic enzymes and the nucleic acid encoding the heterologous polypeptide. In another embodiment, the nucleic acid encoding the lytic enzymes and the nucleic acid encoding the heterologous polypeptide are contained on one vector with which the bacterial cells are transformed. In another specific embodiment that may be preferred, the phage lysozyme and DNA-digesting enzyme are carried on a plasmid to ensure high copy number and the gene t is integrated into the host chromosome to down-regulate expression and prevent premature cell lysis to avoid leakiness.

In the first step of the above processes, the heterologous nucleic acid (e.g., cDNA or genomic DNA) is suitably inserted into a replicable vector for expression in the bacterium under the control of a suitable promoter for bacteria. Many vectors are available for this purpose, and selection of the appropriate vector will depend mainly on the size of the nucleic acid to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the particular host cell with which it is compatible. The vector components for bacterial transformation may include a signal sequence for the heterologous polypeptide and will include a signal sequence for the DNA-digesting protein and will also include an inducible promoter for the heterologous polypeptide and gene t and an inducible promoter or a non-inducible one with low basal expression for the other lytic enzymes. They also generally include an origin of replication and one or more marker genes.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with bacterial hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species. See, e.g., Bolivar et al., *Gene,* 2: 95 (1977). pBR322 contains genes conferring ampicillin and tetracycline resistance and thus provides an easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, also generally contains, or is modified to contain, promoters that can be used by the bacterial organism for expression of the selectable marker genes.

If the heterologous polypeptide is to be secreted, the DNA encoding the heterologous polypeptide of interest herein contains a signal sequence, such as one at the N-terminus of the mature heterologous polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the heterologous polypeptide DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For bacterial host cells that do not recognize and process the native heterologous polypeptide signal sequence, the signal sequence is substituted by a bacterial signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders.

Expression vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

Expression vectors also generally contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen.

The expression vector for producing a heterologous polypeptide also contains an inducible promoter that is recognized by the host bacterial organism and is operably linked to the nucleic acid encoding the heterologous polypeptide of interest. It also contains a separate inducible or low-basal-expression promoter operably linked to the nucleic acid encoding the lytic enzymes. Inducible promoters suitable for use with bacterial hosts include the β-lactamase and lactose promoter systems (Chang et al., *Nature,* 275: 615 (1978); Goeddel et al., *Nature,* 281: 544 (1979)), the arabinose promoter system, including the ara-BAD promoter (Guzman et al., *J. Bacteriol.,* 174: 7716–7728 (1992); Guzman et al., *J. Bacteriol.,* 177: 4121–4130 (1995); Siegele and Hu, *Proc. Natl. Acad. Sci. USA,* 94: 8168–8172 (1997)), the rhamnose promoter (Haldimann et al., *J. Bacteriol.,* 180: 1277–1286 (1998)), the alkaline phosphatase promoter, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.,* 8: 4057 (1980) and EP 36,776), the $P_{Ltet0-1}$ and $P_{lac/ara-1}$ promoters (Lutz and Bujard, *Nucleic Acids Res.,* 25: 1203–1210 (1997)), and hybrid promoters such as the tac promoter. deBoer et al., *Proc. Natl. Acad. Sci. USA,* 80: 21–25 (1983). However, other known bacterial inducible promoters and low-basal-expression promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled

worker operably to ligate them to DNA encoding the heterologous polypeptide of interest or to the nucleic acids encoding the lytic enzymes (Siebenlist et al., *Cell,* 20: 269 (1980)) using linkers or adaptors to supply any required restriction sites. If a strong and highly leaky promoter, such as the trp promoter, is used, it is generally used only for expression of the nucleic acid encoding the heterologous polypeptide and not for lytic-enzyme-encoding nucleic acid. The tac and P_L promoters could be used for either, but not both, the heterologous polypeptide and the lytic enzymes, but are not preferred. Preferred are the alkaline phosphatase promoter for the product and the arabinose promoter for the lytic enzymes.

Promoters for use in bacterial systems also generally contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the heterologous polypeptide of interest. The promoter can be removed from the bacterial source DNA by restriction enzyme digestion and inserted into the vector containing the desired DNA. The phoA promoter can be removed from the bacterial-source DNA by restriction enzyme digestion and inserted into the vector containing the desired DNA.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) or other strains, and successful transformants are selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Sanger et al., *Proc. Natl. Acad. Sci. USA,* 74: 5463–5467 (1977) or Messing et al., *Nucleic Acids Res.,* 9: 309 (1981), or by the method of Maxam et al., *Methods in Enzymology,* 65: 499 (1980).

Suitable bacteria for this purpose include archaebacteria and eubacteria, especially eubacteria, more preferably Gram-negative bacteria, and most preferably Enterobacteriaceae. Examples of useful bacteria include Escherichia, Enterobacter, Azotobacter, Erwinia, Bacillus, Pseudomonas, Klebsiella, Proteus, Salmonella, Serratia, Shigella, Rhizobia, Vitreoscilla, and Paracoccus. Suitable *E. coli* hosts include *E. coli* W3110 (ATCC 27,325), *E. coli* 294 (ATCC 31,446), *E. coli* B, and *E. coli* X1776 (ATCC 31,537). These examples are illustrative rather than limiting. Mutant cells of any of the above-mentioned bacteria may also be employed. It is, of course, necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli,* Serratia, or Salmonella species can be suitably used as the host when well-known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon.

E. coli strain W3110 is a preferred host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 1A2, which has the complete genotype tonAΔ (also known as ΔfhuA); *E. coli* W3110 strain 9E4, which has the complete genotype tonAΔ ptr3; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype tonAΔ ptr3 phoAΔE15 Δ(argF-lac)169 ompTΔ degP41kan^r; *E. coli* W3110 strain 37D6, which has the complete genotype tonAΔ ptr3 phoAΔE15 Δ(argF-lac)169 ompTΔ degP41kan^r rbs7Δ ilvG; *E. coli* W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant degP deletion mutation; *E. coli* W3110 strain 33D3, which has the complete genotype tonA ptr3 lacIq LacL8 ompT degP kan^r; *E. coli* W3110 strain 36F8, which has the complete genotype tonA phoA Δ(argF-lac) ptr3 degP kan^R ilvG+, and is temperature resistant at 37° C.; *E. coli* W3110 strain 45F8, which has the complete genotype fhuA(tonA) Δ(argF-lac) ptr3 degP41(kanS) Δ omp Δ(nmpc-fepE) ilvG+ phoA+ phoS*(T10Y); *E. coli* W3110 strain 33B8, which has the complete genotype tonA phoA Δ(argF-lac) 189 deoC degP IlvG+(kanS); *E. coli* W3110 strain 43E7, which has the complete genotype fhuA(tonA) Δ(argF-lac) ptr3 degP41 (kanS) ΔopmTΔ(nmpc-fepE) ilvG+ phoA+; and an *E. coli* strain having the mutant periplasmic protease(s) disclosed in U.S. Pat. No. 4,946,783 issued Aug. 7, 1990.

Host cells are transformed with the above-described expression vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing the various promoters if induction is carried out.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or as chromosomal integration. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989), is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO, as described in Chung and Miller, *Nucleic Acids Res.,* 16: 3580 (1988). Yet another method is the use of the technique termed electroporation.

Bacterial cells used to produce the heterologous polypeptide of interest described in this invention are cultured in suitable media in which the promoters can be induced as described generally, e.g., in Sambrook et al., supra.

Any other necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations, introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. The pH of the medium may be any pH from about 5–9, depending mainly on the host organism.

For induction, typically the cells are cultured until a certain optical density is achieved, e.g., a A_{550} of about 80–100, at which point induction is initiated (e.g., by addition of an inducer, by depletion of a repressor, suppressor, or medium component, etc.), to induce expression of the gene encoding the heterologous polypeptide. When about 50% or more of the heterologous polypeptide has accumulated (as determined, e.g., by the optical density reaching a target amount observed in the past to correlate with the desired heterologous polypeptide accumulation, e.g., a A_{550} of about 120–140), induction of the promoter is effected for the lysis enzymes. The induction typically takes place at a point in time post-inoculation about 75–90%, preferably about 80–90%, of the total fermentation process time, as determined from prior experience and assays. For example, induction of the promoter may take place at from about 30 hours, preferably 32 hours, up to about 36 hours post-inoculation of a 40-hour fermentation process.

Gene expression may be measured in a sample directly, for example, by conventional northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA,* 77: 5201–5205 (1980)). Various labels may be employed, most commonly radioisotopes, particularly ^{32}P.

However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like.

For accumulation of an expressed gene product, the host cell is cultured under conditions sufficient for accumulation of the gene product. Such conditions include, e.g., temperature, nutrient, and cell-density conditions that permit protein expression and accumulation by the cell. Moreover, such conditions are those under which the cell can perform basic cellular functions of transcription, translation, and passage of proteins from one cellular compartment to another for the secreted proteins, as are known to those skilled in the art.

After product accumulation, when the cells have been lysed by the lytic enzymes expressed, optionally before product recovery the broth lysate is incubated for a period of time sufficient to release the heterologous polypeptide contained in the cells. This period of time will depend, for example, on the type of heterologous polypeptide being recovered and the temperature involved, but preferably will range from about 1 to 24 hours, more preferably 2 to 24 hours, and most preferably 2 to 3 hours. If there is overdigestion with the enzyme, the improvement in recovery of product will not be as great.

In the second step of this invention, the heterologous polypeptide, as a soluble or insoluble product released from the cellular matrix, is recovered from the lysate, in a manner that minimizes co-recovery of cellular debris with the product. The recovery may be done by any means, but preferably comprises sedimenting refractile particles containing the heterologous polypeptide or collecting supernatant containing soluble product. An example of sedimentation is centrifugation. In this case, the recovery preferably takes place, before EBA or sedimentation, in the presence of an agent that disrupts the outer cell wall to increase permeability and allows more solids to be recovered. Examples of such agents include a chelating agent such as EDTA or a zwitterion such as, for example, a dipolar ionic detergent such as ZWITTERGENT 316™ detergent. See Stabel et al., supra. Most preferably, the recovery takes place in the presence of EDTA. Another technique for the recovery of soluble product is EBA, as described above.

If centrifugation is used for recovery, the relative centrifugal force (RCF) is an important factor. The RCF is adjusted to minimize co-sedimentation of cellular debris with the refractile particles released from the cell wall at lysis. The specific RCF used for this purpose will vary with, for example, the type of product to be recovered, but preferably is at least about 3000×g, more preferably about 3500–6000×g, and most preferably about 4000–6000×g. For the case with t-gene co-expression, about 6000 rpm provides as good a recovery of retractile particles from lysed broth as for intact cells.

The duration of centrifugation will depend on several factors. The sedimentation rate will depend upon, e.g., the size, shape, and density of the refractile particle and the density and viscosity of the fluid. The sedimentation time for solids will depend, e.g., on the sedimentation distance and rate. It is reasonable to expect that the continuous disc-stack centrifuges would work well for the recovery of the released heterologous polypeptide aggregates or for the removal of cellular debris at large scale, since these centrifuges can process at high fluid velocities because of their relatively large centrifugal force and the relatively small sedimentation distance.

The heterologous polypeptide captured in the initial recovery step may then be further purified from the contaminating protein. In a preferred embodiment, the aggregated heterologous polypeptide is isolated, followed by a simultaneous solubilization and refolding of the polypeptide, as disclosed in U.S. Pat. No. 5,288,931. Alternatively, the soluble product is recovered by standard techniques.

The following procedures are exemplary of suitable purification procedures for the soluble heterologous polypeptide released from the periplasm or the cytoplasm: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse-phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, SEPHADEX™ G-75.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all patent and scientific references cited in the specification are expressly incorporated herein by reference.

EXAMPLE I

Co-expression of Lytic Enzymes with Soluble Product

A. Co-expression with T4-lysozyme and endA endonuclease:

Background

rhuMAb CD18 is a recombinant $F(ab')_2$ antibody fragment with two 214-residue light chains and two 241-residue heavy chains. It binds to the MAC-1 (CD11b/CD18) receptor, effectively blocking the binding of neutrophils to the endothelium. In the fermentation process described below, rhuMab CD18 is produced as a $F(ab')_2$-leucine zipper precursor in *E. coli* and secreted into the periplasm. The desired recovery process targets the soluble fraction of the accumulated product and depends on the $F(ab')_2$-leucine zipper being released from the periplasm for initial capture.

T4-lysozyme co-expression was introduced to weaken the peptidoglycan sacculus, and the over-expression of endA protein was introduced to degrade genomic DNA released from the cells under conditions such that the cells are permeabilized or lysed.

In *E. coli,* the gene endA encodes for an endonuclease normally secreted to the periplasm that cleaves DNA into oligodeoxyribonucleotides in an endonucleolytic manner. It has been ;suggested that endA is relatively weakly expressed by *E. coli* (Wackernagel et al., supra). However, one could over-express the endonuclease with the use of a compatible plasmid. By inserting the endA gene with its signal sequence behind the ara-T4-lysozyme cassette in the compatible plasmid pJJ153, now pJJ154, expression of both T4-lysozyme and endonuclease will be induced upon addition of arabinose. While T4-lysozyme is locked inside the cytoplasm, endonuclease is secreted into the periplasmic space and kept away from the genomic DNA located in the cytoplasm during the fermentation process. An effective enzymatic degradation of DNA upon cell lysis is expected to reduce or even eliminate multiple passes through the mechanical disruption device, an operation often needed for both cell disruption and viscosity reduction. Success in doing so would bring significant time and cost reduction to the recovery process.

Materials and Methods

pS1130 Plasmid Construction: Plasmid pS1130 (FIG. **3**) was constructed to direct the production of the rhuMAb CD18 $F(ab')_2$-leucine zipper precursor in *E. coli.* It is based on the well-characterized plasmid pBR322 with a 2138-bp

expression cassette (FIG. **4**; SEQ ID NOS:1 2 and 3) inserted into the EcoRI restriction site. The plasmid encodes for resistance to both tetracycline and beta-lactam antibiotics. The expression cassette contains a single copy of each gene linked in tandem. Transcription of each gene into a single dicistronic mRNA is directed by the *E. coli* phoA promoter (Chang et al., *Gene,* 44: 121–125 (1986)) and ends at the phage lambda t_o terminator (Scholtissek and Grosse, *Nuc. Acids Res.,* 15: 3185 (1987)). Translation initiation signals for each chain are provided by *E. coli* STII (heat-stable enterotoxin) (Picken et al., *Infection and Immunity,* 42: 269–275 (1983)) Shine-Dalgarno sequences.

rhuMAb CD18 was created by humanization of the murine monoclonal antibody muMAb H52 (Hildreth and August, *J. Immunology,* 134: 3272–3280 (1985)) using a process previously described for other antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89: 4285–4289 (1992); Shalaby et al., *J. Exp. Med.,* 175: 217–225 (1992); Presta et al., *J. Immunol.,* 151: 2623–2632 (1993)). Briefly, cDNAs encoding the muMAb H52 variable light (V_L) and variable heavy (V_H) chain domains were isolated using RT-PCR from a hybridoma cell line licensed from Hildreth (Hildreth and August, supra). The complementarity-determining regions (CDRs) of the muMAb H52 were transplanted into the human antibody framework gene encoding huMAb UCHT1-1 (Shalaby et al., supra) by site-directed mutagenesis. Non-CDR, murine framework residues that might influence the affinity of the humanized antibody for its target, human CD18, were identified using molecular modeling. These residues were altered by site-directed mutagenesis and their influence on CD18 binding was tested. Those framework residues that significantly improved affinity were incorporated into the humanized antibody. The expression vector encoding the final humanized version of muMAb H52 in an Fab' format was named pH52-10.0 and it is a derivative of pAK19 (Carter et al., *Bio/Technology,* 10: 163–167 (1992)).

Plasmid pS1130 differs from pH52-10.0 in the heavy-chain coding region. The C-terminus of the heavy chain was extended from CysProPro to the natural hinge sequence CysProProCysProAlaProLeuLeuGlyGly (SEQ ID NO:4) and then fused to the 33-residue leucine zipper domain of the yeast transcription factor GCN4. As described above, the leucine zipper domains dimerize to bring two Fab' molecules together and drive $F(ab')_2$ complex formation. The two cysteine residues in the heavy-chain hinge region then disulfide bond to those from an adjacent Fab' to form a covalently-linked $F(ab')_2$.

Plasmid pS1130 was constructed in a multi-step process outlined below:

First, a filamentous phage (f1) origin of replication was introduced into pH52-10.0 to create plasmid pS0858. Plasmid pS0858 was constructed by ligating the 1977-bp HindIII-HindIII fragment, containing the Fab' expression cassette of pH52-10.0, with the 4870-bp HindIII-HindIII fragment of plasmid pS0191 (referred to as phGHr (1–238) in Fuh et al., *J. Biol. Chem.,* 265: 3111–3115 (1990)).

Second, oligonucleotide-directed mutagenesis was performed on pSO858 to create plasmid zpi1#6. The heavy-chain coding region was extended to include the 2-hinge cysteine residues and pepsin cleavage site: (CysProProCysProAlaProLeuLeuGlyGly; SEQ ID NO:4). A NotI restriction site was also introduced. The sequence of the introduced DNA was confirmed by DNA sequencing.

Third, a DNA fragment encoding the GCN4 leucine zipper domain flanked by NotI and SphI restriction sites was generated (WO98/37200 published Aug. 27, 1998). This 107-bp NotI-SphI DNA fragment was subsequently cloned into similarly-cut zip1#6 to create plasmid ps1111.

Fourth, DNA sequencing of the GCN4 leucine zipper fragment revealed an error in the coding sequence. This error was corrected by oligonucleotide-directed mutagenesis and confirmed by DNA sequencing. The resulting correct plasmid was named pS1117.

The final step in the construction of pS1130 was to restore the tetracycline-resistance gene and remove the f1 origin from pS1117. This was accomplished by ligating the 2884-bp PstI-SphI fragment of pS1117 containing the Fab'-zipper expression cassette with the 3615-bp PstI-SphI fragment of pH52-10.0.

pJJ154 Plasmid Construction: Plasmid pJJ154 (FIG. **5**) is a pACYC177 derivative that is compatible with pBR322 vectors. To construct pJJ154, pJJ153 as described below was digested with MluI and the vector fragment was ligated with PCR-amplified endA gene designed to encode MluI ends. The correct orientation of the plasmid was screened for by restriction digest to produce pJJ154.

The construction of pJJ153 (a pACYC177 derivative that is compatible with pBR322 vectors) is shown in FIG. **5**. The ClaI/AlwNI fragment from pBR322 was inserted into ClaI/AlwNI-digested pBAD18 (Guzman et al., supra) to produce pJJ70. One round of site-directed mutagenesis was then performed, changing HindIII to StuI to obtain pJJ75. A second round of site-directed mutagenesis was done to change MluI to SacII, to produce pJJ76. Then XbaI/HindIII fragments from pJJ76 and from pBKIGF2B were ligated, and XbaI/HindIII fragments from this ligation product and from a T4 lysozyme/tac plasmid were ligated to produce pT4LysAra. Then BamHI (filled in)/ScaI-digested pACYC177 was ligated with ClaI/HindIII (both ends filled in)-digested pT4LysAra to produce pJJ153. The maps for pACYC177, pT4LysAra, and pJJ153 are shown in FIG. **5**.

Bacterial Strains and Growth Conditions: Strain 33B8 (*E. coli* W3110 tonA phoA Δ(argF-lac) 189 deoC degP ilvG+ (kanS)) was used as the production host for the co-expression of T4-lysozyme and DNA-digesting protein from plasmid pJJ154 and the expression of rhuMAb CD18 $F(ab')_2$-leucine zipper from plasmid pS1130. Competent cells of 33B8 were co-transformed with pJJ154 and pS1130 using standard procedures. Transformants were picked from LB plates containing 20 ug/ml tetracycline and 50 ug/ml kanamycin (LB+Tet20+Kan50), streak-purified, and grown in LB broth with 20 ug/ml tetracycline and 50 ug/ml kanamycin in a 37° C. or 30° C. shaker/incubator before being stored in DMSO at −80° C.

For control runs, the host 33B8 was transformed with pS1130 and pJJ96 (analogous to pJJ154 except no nucleic acids encoding the T4-lysozyme and endA product were inserted into the vector) and isolated from similar selective medium.

rhuMAb CD18 $F(ab')_2$-Leucine Zipper Fermentation Process: A shake flask inoculum was prepared by inoculating sterile medium using a freshly thawed stock culture vial. Appropriate antibiotics were included in the medium to provide selective pressure to ensure retention of the plasmid. The shake flask medium composition is given in Table 1. Shake flasks were incubated with shaking at about 30° C. (28° C.–32° C.) for 14–18 hours. This culture was then used to inoculate the production fermentation vessel. The inoculation volume was between 0.1% and 10% of the initial volume of medium.

The production of the $F(ab')_2$-zipper precursor of rhuMAb CD18 was carried out in the production medium given in Table 2. The fermentation process was carried out at about

30° C. (28–32° C.) and about pH 7.0 (6.5–7.9). The aeration rate and the agitation rate were set to provide adequate transfer of oxygen to the culture. Production of the $F(ab')_2$-zipper precursor of rhuMAb CD18 occurred when the phosphate in the medium was depleted, typically 36–60 hours after inoculation.

TABLE 1

Shake Flask Medium Composition

Ingredient	Quantity/Liter
Tetracycline	4–20 mg
Tryptone	8–12 g
Yeast extract	4–6 g
Sodium chloride	8–12 g
Sodium phosphate, added as pH7 solution	4–6 mmol

TABLE 2

Production Medium Composition

Ingredient	Quantity/Liter
Tetracycline	4–20 mg
Glucose[a]	10–250 g
Ammonium sulfate[a]	2–8 g
Sodium phosphate, monobasic, dihydrate[a]	1–5 g
Potassium phosphate, dibasic[a]	1–5 g
Potassium phosphate, monobasic[a]	0.5–5 g
Sodium citrate, dihydrate[a]	0.5–5 g
Magnesium sulfate, heptahydrate[a]	1.0–10 g
FERMAX ™[a] (antifoam)	0–5 ml
Ferric chloride, hexahydrate[a]	20–200 mg
Zinc sulfate, heptahydrate[a]	0.2–20 mg
Cobalt chloride, hexahydrate[a]	0.2–20 mg
Sodium molybdate, dihydrate[a]	0.2–20 mg
Cupric sulfate, pentahydrate[a]	0.2–20 mg
Boric acid[a]	0.2–20 mg
Manganese sulfate, monohydrate[a]	0.2–20 mg
Casein digest	15–25 g
Methionine[a]	0–5 g
Leucine[a]	0–5 g

[a]A portion of these ingredients was added to the fermentor initially and the remainder was fed during the fermentation. Ammonium hydroxide was added as required to control pH.

The timing of arabinose addition ranged from 50 to 65 hours post-inoculation. Bolus additions of 0.1% to 1% (final concentration) arabinose were tested for the induction of co-expression of T4-lysozyme and endA endonuclease.

The fermentation was allowed to proceed for about 65 hours (60 to 72 hours), after which the broth was harvested for subsequent treatment for product recovery.

Assessment of Reduction of Broth Viscosity by endA Endonuclease Over-expression: Aliquots of harvested broth from the rhuMAb CD18 $F(ab')_2$-leucine zipper fermentation with or without the co-expression of endA product in addition to phage lysozyme described above was subjected to one cycle of freeze-thaw. The thawed broth was diluted 1:3 into water or 20 mM $MgCl_2$ before incubation in a 37° C. water bath with agitation. Samples were removed at intervals and the viscosity of the diluted broth was measured by using the Falling Ball viscometer.

Results:

Effect of Over-Expression of EndA in Addition to T4-Lysozyme on Broth Viscosity: As shown in Table 3, diluted freeze-thawed harvest broth from the above fermentation process in the absence of endA over-expression had broth viscosity in excess of 800 cP at the start of the 37° C. incubation. After 60 minutes of incubation, there was little change in the H_2O-diluted freeze-thawed fermentation broth viscosity, while the $MgCl_2$-buffer-diluted broth showed significant reduction in the broth viscosity. Upon extended 37° C. incubation for up to over 2.5 hours, the viscosity of the freeze-thawed diluted harvest broth with no over-expression of endA in addition to T4-lysozyme leveled off at about 40 cP. The viscosity of the diluted freeze-thawed harvest broth with over-expression of endA was less than 20 cP even before any 37° C. incubation.

TABLE 3

Co-expression	Treatment	37° C. Incubation (min)	Broth Viscosity (cP)
T4-lysozyme + endA (pJJ154)	H_2O control +	0	<20
	20 mM $MgCl_2$	0	<20
	H_2O control +	60	<20
	20 mM $MgCl_2$	60	<20
T4-lysozyme only (pJJ153)	H_2O control +	0	>800
	20 mM $MgCl_2$	0	>800
	H_2O control +	60	>800
	20 mM $MgCl_2$	60	36
	H_2O control +	120	41
	20 mM $MgCl_2$	120	36
	H_2O control +	165	40
	20 mM $MgCl_2$	165	42

B. Co-expression of T4-Lysozyme, Gene t, and EndA Endonuclease:

Background

As described in Example IA, over-expression of endA brings significant benefit in lowering the viscosity of permeabilized or lysed broth. It helps in conditioning the fermentation broth for the subsequent product recovery step. By co-expressing T4-lysozyme and t-gene in addition to endA, cells can be biochemically lysed and at the same time yield a well-conditioned broth lysate with fluid viscosity sufficiently low and compatible with product isolation steps such as centrifugation or EBA.

The fermentation process described above was used to produce rhuMAb CD18 as a $F(ab')_2$-leucine zipper precursor directed by plasmid pS1130 in *E. coli,* with the co-expression of lytic enzymes and DNA-digesting protein directed by the plasmid pJJ155. in *E. coli.* The antibody fragment product was secreted and accumulated in the periplasm. The lytic enzymes were compartmentalized away from their substrate until released by the action of the t-gene product. The desired recovery process targets the soluble fraction of the $F(ab')_2$-leucine zipper released from the periplasm for initial capture.

Materials & Methods

PJJ155 Plasmid Construction: Like pJJ154, pJJ155 is a pACYC177 derivative that is compatible with pBR322 vectors. To construct pJJ155, pJJ154 as described above was digested with KpnI and the vector fragment was ligated with PCR-amplified t-gene designed to encode KpnI ends. The correct orientation of the plasmid was screened for by restriction digest to product pJJ155. A map for pJJ155 is shown in FIG. 7.

Bacterial Strains and Growth Conditions: Most experiments were carried out with transformed 33B8 as described above except that pJJ154 was replaced by pJJ155.

Fermentation Process Description: See Example IA.

Results

Cell growth of 33B8 co-transformed with pS1130 and pJJ155 (33B8/pS1130/pJJ155) was not significantly different from that of the control (33B8 transformed with pS1130 only). After addition of 0.5% to 1% arabinose at 50–65 hours to induce the co-expression of the lytic enzymes and DNA-digesting protein, OD550 of the 33B8/pS1130/pJJ155 cul-

ture steadily dropped to 30–40% of peak cell density, suggesting cell lysis.

Table 4 shows the effect of co-expression of T4-lysozyme+endA+t-gene on the release of soluble anti-CD18 antibody fragment into the medium. The supernatant from centrifugation of the harvested broth lysate after incubation in the presence of 25 mM EDTA was assayed by ion-exchange HPLC chromatography for product quantitation. Greater than 80% of the soluble anti-CD18 antibody fragments was found in the supernatant fraction for the experimental condition where co-expression of lytic enzymes and DNA-digesting protein was induced, compared to less than 10% found for the control and the condition with no t-gene (pJJ154) or mechanical disruption.

TABLE 4

Co-Expression	% of Total Product Released
None (control)	<10
T4-Lysozyme + endA (pJJ154)	<10
T4-Lysozyme + endA + t-gene (pJJ155)	>80

Conclusions

Endonuclease degrades DNA and lowers broth viscosity. Over-expression of *E. coli* endogenous endonuclease in addition to T4-lysozyme reduces the need for mechanical cell disruption for the shearing of released DNA. The release of the phage lysozyme from the cytoplasmic compartment mediated by the expressed t-gene protein initiates the biochemical disruption process, resulting in cell lysis and the release of cellular contents including heterologous polypeptide, genomic DNA, and the DNA-digesting protein, which was trapped in either the periplasm or the cytoplasm up to this time. By holding the broth lysate for appropriate digestion of substrates by the lytic enzymes and DNA-digesting protein co-expressed, the broth viscosity and product release from cellular matrix were improved for better product recovery.

EXAMPLE II

Co-expression of Lytic Enzymes with IGF-I

Background

IGF-I was selected as a heterologous polypeptide for evaluation of refractile particle recovery due to large-scale needs. Co-expression of lytic enzymes and DNA-digesting protein was used to improve the release of the IGF-I refractile particles from cell-wall structures in the absence of mechanical disruption.

Upon cell lysis, in addition to releasing T4-lysozyme from the cytoplasmic compartment, genomic DNA released from the cytoplasm would have contributed significant viscosity to the broth lysate fluid. Hence, the co-expression of an *E.coli* endonuclease together with lytic enzymes was useful in reducing fluid viscosity following cell lysis and improving product recovery during centrifugation.

Materials & Methods

pLBIGF57 Plasmid Construction: The plasmid pLBIGF57 for the expression of IGF-I (FIG. **6**) was constructed from a basic backbone of pBR322. The transcriptional and translational sequences required for the expression of nucleic acid encoding IGF-I were provided by the phoA promoter and trp Shine-Dalgarno sequence. Secretion of the protein was directed by a TIR variant of the lamB signal sequence. This TIR variant does not alter the primary amino acid sequence of the lamB signal; however, silent nucleotide sequence changes result, in an increased level of translated protein.

The details of pLBIGF57 construction follow. A codon library of the lamB signal sequence was constructed to screen for translational initiation region (TIR) variants of differing strength. Specifically, the third position of codons 3 to 7 of the lamB signal sequence was varied. This design conserved the wild-type amino acid sequence and yet allowed for divergence within the nucleotide sequence.

As previously described for the screening of the STII signal sequence codon library (S. African Pat. No. ZA 96/1688; Simmons and Yansura, *Nature Biotechnology,* 14: 629–634 (1996)), the phoA gene product served as a reporter for the selection of the lamB TIR variants. The codon library of the lamB signal sequence was inserted downstream of the phoA promoter and trp Shine-Dalgarno and upstream of the phoA gene. Under conditions of low transcriptional activity, the quantity of alkaline phosphatase secreted by each construct was now dependent on the efficiency of translational initiation provided by each TIR variant in the library. Using this method, lamB TIR variants were selected covering an approximate 10-fold activity range. Specifically, lamb TIR variant #57 provides an approximately 1.8-fold stronger TIR than the wild-type lamB codons based on the phoA activity assay.

The vector fragment for the construction of pLBIGF57 was generated by digesting pBK131Ran with XbaI and SphI. This XbaI-SphI vector contains the phoA promoter and trp Shine-Dalgarno sequences. The coding sequences for IGF-I and the lambda t_o transcription terminator were isolated from pBKIGF-2B (U.S. Pat. No. 5,342,763) following digestion with NcoI-SphI. The lamB signal sequence fragment was isolated from pLBPhoTBK#57 (TIR variant #57; generated as described above) following digestion with XbaI-NcoI. These three fragments were then ligated together to construct pLBIGF57.

Bacterial Strains and Growth Conditions: Most experiments were carried out with strain 43E7 (*E. coli* W3110 fhuA(tonA) Δ(argF-lac) ptr3 degP4 (kanS) ΔompTΔ(nmpc-fepE) ilvG+ phoA+). A double-plasmid system involving the product plasmid (pLBIGF57) and pJJ155 for the lytic enzymes was employed. Competent cells of 43E7 were co-transformed with pLBIGF57 and pJJ155 using the standard procedure. Transformants were picked after growth on an LB plate containing 50 μg/mL carbenicillin (LB+ CARB50™) and 50 ug/ml kanamycin, streak-purified and grown in LB broth with 50 μg/mL CARB50™ and 50 ug/ml kanamycin in a 37° C. shaker/incubator before being tested in the fermentor.

For comparison, 43E7 transformed with pLBIGF57 alone was used in the control case conducted under similar conditions. pLBIGF57 confers both carbenicillin and tetracycline resistance to the production host and allows 43E7/pLBIGF57 to grow in the presence of either antibiotic.

Fermentation Process: The fermentation medium composition and experimental protocol used for the co-expression of nucleic acid encoding IGF-I, endA, T4-lysozyme, and t-gene if used were similar to those of the scaled-down high-metabolic rate, high-yield 10-kiloliter IGF-I process. Briefly, a shake flask seed culture of 43E7/pLBIGF57 or 43E7/pLBIGF57/pJJ155 was used to inoculate the rich production medium. The composition of the medium (with the quantities of each component utilized per liter of initial medium) is described below:

Ingredient	Quantity/L
Glucose*	200–500 g
Ammonium Sulfate	2–10 g
Sodium Phosphate, Monobasic Dihydrate	1–5 g
Potassium Phosphate, Dibasic	1–5 g
Sodium Citrate, Dihydrate	0.5–5 g
Potassium Chloride	0.5–5 g
Magnesium Sulfate, Heptahydrate	0.5–5 g
PLURONIC ™ Polyol, L61	0.1–5 mL
Ferric Chloride, Heptahydrate	10–100 mg
Zinc Sulfate, Heptahydrate	0.1–10 mg
Cobalt Chloride, Hexahydrate	0.1–10 mg
Sodium Molybdate, Dihydrate	0.1–10 mg
Cupric Sulfate, Pentahydrate	0.1–10 mg
Boric Acid	0.1–10 mg
Manganese Sulfate, Monohydrate	0.1–10 mg
Hydrochloric Acid	10–100 mg
Tetracycline	4–30 mg
Yeast Extract*	5–25 g
NZ Amine AS*	5–25 g
Methionine*	0–5 g
Ammonium Hydroxide	as required to control pH
Sulfuric Acid	as required to control pH

*A portion of the glucose, yeast extract, NZ Amine AS, and methionine is added to the medium initially, with the remainder being fed throughout the fermentation.

The fermentation was a fed-batch process with fermentation parameters set as follow:

Agitation: Initially at 800 RPM, increased to 1000 RPM at 8 OD

Aeration: 15.0 slpm

pH control: 7.3

Temp.: 37° C.

Back pressure: 0.7 bar

Glucose feed: computer-controlled using an algorithm which regulates the growth rate at approximately 95% of the maximum early in the fermentation and which then controls the dissolved oxygen concentration (DO_2) at 30% of air saturation after the DO_2 drops to 30%.

Complex nitrogen feed: constant feed rate of 0.5 mL/min throughout the run

Run Duration: 40 hours

The timing of arabinose addition ranged from 24 hr to 36 hr. Bolus additions of 0.1% to 1% (final concentration) arabinose were tested to define the induction strength necessary for producing the most preferred amounts of T4-lysozyme for better product recovery at the centrifugation step.

Recovery of Refractile Particles from Harvested Broth: Broth was harvested at the end of fermentation when a target drop in OD_{550} was observed and was either processed soon after or stored briefly at 4° C. prior to use. The test protocol used involved four process steps:

I. Add 1M EDTA to the harvest broth to bring the final concentration of EDTA to 25 mM. EDTA chelates the divalent cations and disrupts the outer cell surface structure. This makes the peptidoglycan layer inside unbroken cells accessible to degradation by T4-lysozyme and weakens the cell wall to promote cell lysis.

II. Hold the lysate at room temperature or incubate at 37° C. for further degradation of cell wall. This step simulates the longer process times associated with the larger-scale process.

III. Recover refractile particles and solids from the lysate by centrifugation. Bench-scale centrifugation in a SORVALL™ GSA rotor at different speeds (3000 rpm to 6000 rpm; equivalent to RCF's of approximately 2500 g to 6000 g at rmax, respectively) was used to collect the solids as pellets.

An additional step to wash the pellet with buffer would remove the lysate entrained by the pellet and minimize the amount of contaminating *E. coli* proteins in the refractile particle preparations.

Samples of the supernatant and pellet from centrifugation of broth, lysate resuspended in buffer were evaluated for product recovery. The amount of product present in the samples was analyzed by a HPLC reverse-phase method. Product recovery efficiency was calculated by expressing the amount of product recovered in the pellet by the process step as a percent of the total product present in the pellet and supernatant combined. To evaluate the quality of the refractile particles recovered, the amount of total protein present in the pellet and the supernatant was measured by the Lowry method (*J. Biol. Chem.,* 193: 265 (1951)).

The contribution of endonuclease activity was assessed by the efficiency of solids recovery during sedimentation from the broth lysate by centrifugation. Also, the amount of nucleic acids present in the pellet and the supernatant was measured by OD_{260} readings.

Results:

IGF-I Fermentation and Product Expression

FIG. **8** shows in general the two-plasmid system employed in this Example for co-expression of lytic enzymes and endA with IGF-I using pJJ155. The initial growth rate of 43E7/pLBIGF57/pJJ155 showed no significant difference from that of the 43E7/pLBIGF57 control. Peak cell densities reached in these broths were similar. However, compared to the control, a significant loss in optical density was observed in cultures after induction for lytic enzyme co-expression, indicating cell lysis. Examination of the harvest broth by phase-contrast microscopy showed that, in comparison to the no-co-expression control, very few intact *E. coli* cells were present and freed refractile particles were evident as a result of the co-expression of lytic enzymes and DNA-digesting protein. See FIGS. **9**A–**9**E.

The respiration rates across this collection of runs looked very similar to the control except for significant continuous loss in oxygen uptake rate (OUR) with a concomitant loss in kla soon after the arabinose addition.

The success of the biochemical cell lysis technique as described in this invention is evident from the differences in the partitioning of nucleic acids and total protein between the solid (pellet) and liquid (supernatant) fractions as a result of the co-expression of the lytic enzymes and DNA-digesting protein versus the control with no co-expression (FIGS. **10**A and **10**B, respectively). The percent of total nucleic acids calculated from A260 readings and the percent of total protein as measured by the Lowry protein assay both increased in the supernatant from the centrifugation of biochemically lysed broth over that from control broth.

The product recovery from the two conditions is summarized in FIG. **11**. With the biochemically-lysed IGF-I broth, IGF-I product was released together with degraded DNA polymer into the broth lysate. The efficiency of recovering the small dense refractile particles increased with the RCF used during centrifugation. As higher g force was used, the percent of the lysed broth recovered as pellet (reported as % pellet) increased (FIG. **12**), and so did the amount of IGF-I product in the pellet. At approximately 6000×g, close to 95% of the product was captured in the pellet.

Conclusion

As disclosed herein, a simple manipulation of gene expression during the fermentation process resulted in a

biochemical cell lysis that could replace the conventional mechanical disruption traditionally used for product recovery at production scale. In vivo co-expression or coordinated expression of T4-lysozyme and t-gene product is a highly effective technique for the disintegration of cells while the over-expressed endA protein degrades the leaked DNA, lowers broth viscosity, and efficiently conditions the broth lysate for product recovery in the initial product capture step. Biochemical cell lysis is applicable to the recovery of soluble as well as insoluble product. The compartmentalization of the co-expressed enzymes away from their substrates until the desired moment for cell lysis is essential and a critical design in the invention. The invention brings significant reduction in process cost, process time, and hence opportunity cost to other products that may be sharing the same production facility.

SEQUENCE LISTING

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<211> LENGTH: 6550
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 1

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tcgcaatatg gcgcaaaatg accaacagcg gttgattgat caggtagagg 200

gggcgctgta cgaggtaaag cccgatgcca gcattcctga cgacgatacg 250

gagctgctgc gcgattacgt aaagaagtta ttgaagcatc ctcgtcagta 300

aaaagttaat cttttcaaca gctgtcataa agttgtcacg gccgagactt 350

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cgatagggtc accatcacct gtcgtgccag tcaggacatc aacaattatc 600

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ggacagcaag gacagcacct acagcctcag cagcaccctg acgctgagca 1050

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What is claimed is:

1. A process for recovering a heterologous polypeptide from bacterial cells comprising:

(a) culturing the bacterial cells which comprise a first nucleic acid encoding phage lysozyme and the gene t product, and a second nucleic acid encoding a protein that displays DNA-digesting activity under control of a signal sequence for secretion of the DNA-digesting protein, wherein the first and second nucleic acids are operatively linked to a first promoter that is the same for both and wherein both are linked on the same nucleic acid construct, and a third nucleic acid encoding the heterologous polypeptide, which third nucleic acid is linked to a second promoter, wherein the second promoter is inducible and the first promoter is different from the second promoter and is either (i) inducible or (ii) a weak constitutive promoter that does not require the addition of an inducer to function as a promoter,

(b) adding an inducer specific for induction of expression of the nucleic acid encoding the heterologous polypeptide from the second inducible promoter,

(c) optionally, when the first promoter driving expression of the nucleic acids encoding the phage lysozyme, the gene t product, and DNA-digesting protein is an inducible promoter, adding an inducer specific for the first promoter after accumulation of about 50% or more of the maximum accumulation of the heterologous polypeptide to be recovered,

(d) lysing the cells, and

(e) recovering accumulated heterologous polypeptide from the broth lysate.

2. The process of claim **1** wherein the heterologous polypeptide is a mammalian polypeptide.

3. The process of claim **2** wherein the mammalian polypeptide is insulin-like growth factor-I (IGF), DNase, vascular endothelial growth factor (VEGF), anti-CD18 antibody or fragment of an anti-CD18 antibody.

4. The process of claim **3** wherein the mammalian polypeptide is IGF-I or an anti-CD18 antibody fragment.

5. The process of claim **1** wherein the culturing is carried out under conditions whereby the heterologous polypeptide is secreted into the periplasm of the bacterial cells.

6. The process of claim **1** wherein the signal sequence is a native sequence of the DNA-digesting protein.

7. The process of claim **1** wherein the DNA-digesting protein is a eukaryotic DNase or bacterial endA.

8. The process of claim **1** wherein the lysozyme is T4-lysozyme.

9. The process of claim **1** wherein the heterologous polypeptide is soluble in the periplasmic space and the recovery step is done using an expanded bed absorption process or sedimentation.

10. The process of claim **9** wherein the heterologous polypeptide is an anti-CD18 antibody or fragment thereof.

11. The process of claim **1** wherein the induction of expression of the nucleic acids encoding the lysozyme, gene t, and DNA-digesting protein is carried out by adding an inducer to the culture medium.

12. The process of claim **1** wherein before recovery the broth lysate is incubated for a time sufficient to release the heterologous polypeptide contained in the cells.

13. The process of claim **1** wherein the recovery comprises sedimenting refractile particles containing the heterologous polypeptide or collecting supernatant containing soluble heterologous polypeptide.

14. The process of claim **1** wherein the bacterial cells are Gram-negative cells.

15. The process of claim **14** wherein the bacterial cells are *E. coli*.

16. The process of claim **1** wherein the recovery step takes place in the presence of an agent that disrupts the outer cell wall of the bacterial cells.

17. The process of claim **16** wherein the agent is a chelating agent or zwitterion.

18. The process of claim **1** wherein one or more of the nucleic acids, including the promoter therefor, is integrated into the genome of the bacterial cells.

19. A process for recovering a heterologous polypeptide from bacterial cells comprising:

(a) culturing the bacterial cells which comprise a first nucleic acid encoding phage lysozyme and a protein that displays DNA-digesting activity under control of a signal sequence for secretion of the DNA-digesting protein, a second nucleic acid comprising gene t, wherein the first nucleic acid encoding the phage lysozyme and DNA-digesting protein is operatively linked to a first promoter that is either (i) inducible or (ii) a weak constitutive promoter that does not require the addition of an inducer to function as a promoter, the second nucleic acid comprising gene t is linked to a second inducible promoter, and the third nucleic acid encoding the heterologous polypeptide is linked to a third inducible promoter and wherein each of the promoters responds to a different inducer,

(b) adding an inducer specific for induction of expression of the nucleic acid encoding the heterologous polypeptide from the third inducible promoter,

(c) optionally when the first promoter driving expression of the nucleic acid encoding the phage lysozyme and the DNA-digesting protein is an inducible promoter, adding inducers specific for the first and second promoters after accumulation of about 50% or more of the maximum accumulation of the heterologous polypeptide to be recovered such that the first promoter driving expression of the nucleic acid encoding phage lysozyme and the DNA-digesting protein is induced before the promoter driving expression of the nucleic acid encoding gene t, or

(c') when the first promoter driving expression of the nucleic acid encoding the phage lysozyme and the DNA-digesting protein is a weak constitutive promoter, adding an inducer specific for the promoter driving expression of gene t after accumulation of about 50% or more of the maximum accumulation of the heterologous polypeptide to be recovered,

(d) lysing the cells, and

(e) recovering the accumulated heterologous polypeptide from the broth lysate.

20. The process of claim **19**, wherein one or more of the nucleic acids, including the promoter therefor, is integrated into the genome of the bacterial cells.

* * * * *

EXHIBIT 11

US005766439A

United States Patent [19]

Eyal et al.

[11] Patent Number: 5,766,439

[45] Date of Patent: Jun. 16, 1998

[54] **PRODUCTION AND RECOVERY OF ORGANIC ACIDS**

[75] Inventors: **Aharon M. Eyal**, Jerusalem, Israel; **William F. Lehnhardt**, Lovington, Ill.

[73] Assignee: **A. E. Staley Manufacturing Co.**, Decatur, Ill.

[21] Appl. No.: **752,803**

[22] Filed: **Nov. 20, 1996**

Related U.S. Application Data

[63] Continuation of Ser. No. 728,836, Oct. 10, 1996, abandoned.

[51] **Int. Cl.**[6] **C12P 7/52**; C25B 3/00; C07C 51/42; C07C 59/08

[52] **U.S. Cl.** **204/524**; 204/519; 204/522; 204/527; 204/530; 204/534; 204/537; 204/637; 210/259; 210/651; 210/654; 435/135; 435/136; 435/139; 435/142; 435/145; 435/146; 435/803; 562/578; 562/580; 562/586; 562/593

[58] **Field of Search** 435/135, 136, 435/139, 142, 145, 146, 803; 562/578, 580, 584, 593; 204/519, 522, 524, 527, 530, 534, 537, 637; 210/259, 651, 654

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Primary Examiner—Herbert J. Lilling
Attorney, Agent, or Firm—Arnold, White & Durkee

[57] **ABSTRACT**

A process is described for producing organic acids such as lactic acid. The process includes the steps of producing lactic acid by fermentation, resulting in an aqueous fermentation broth containing lactic acid, and adding a calcium base, such as calcium carbonate, to the fermentation broth, thereby producing calcium lactate in the broth. Biomass is removed from the broth, thereby leaving an aqueous solution or dispersion of calcium lactate. The calcium lactate is reacted with a source of ammonium ions, such as ammonium carbonate, or a mixture of ammonia and carbon dioxide, thereby producing an ammonium lactate. Contaminating cations can be removed by ion exchange. The free lactic acid or a derivative thereof can be separated from the ammonium ions, preferably by salt-splitting electrodialysis.

58 Claims, 5 Drawing Sheets

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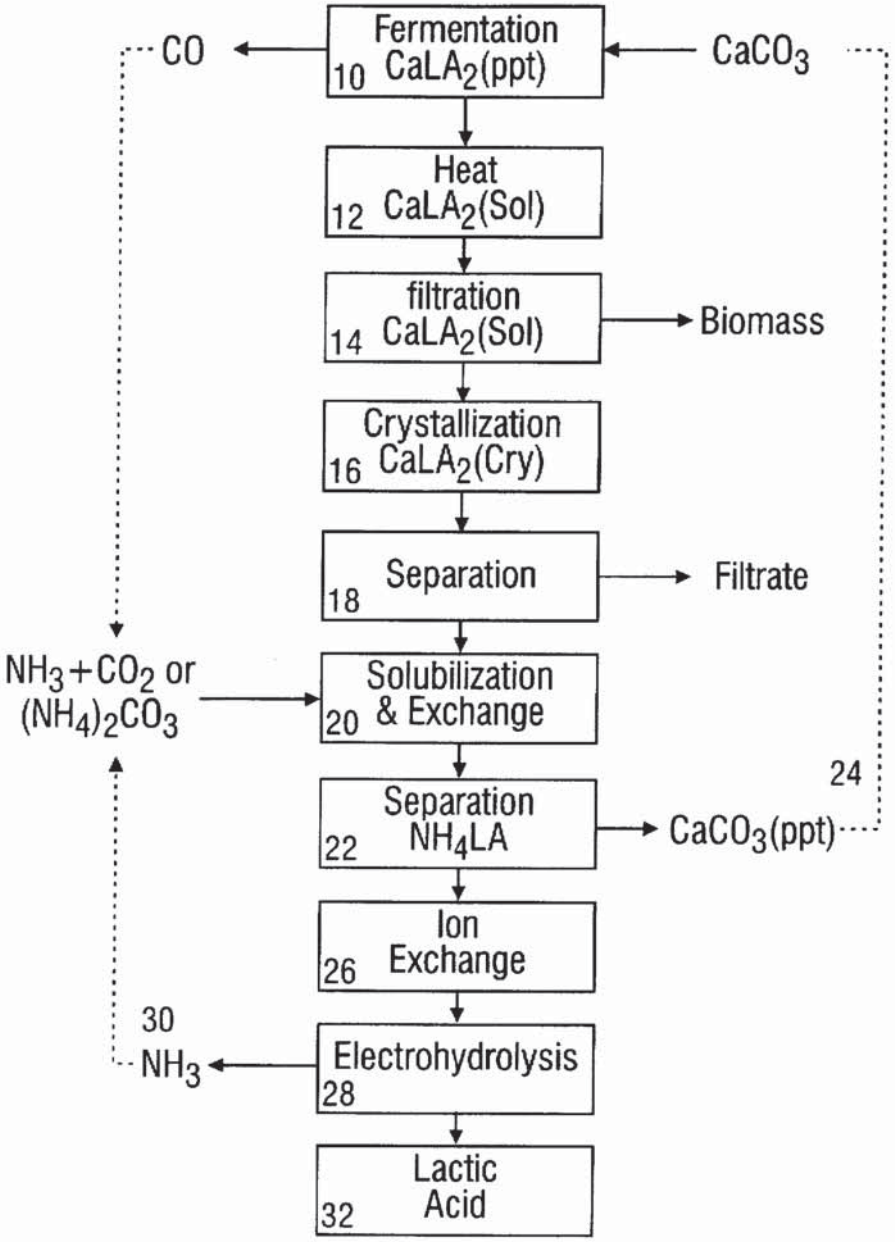
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Heat
12 $CaLA_2$(Sol)
filtration
14 $CaLA_2$(Sol)
Biomass
Crystallization
16 $CaLA_2$(Cry)
Separation
18
Filtrate
NH_3+CO_2 or
$(NH_4)_2CO_3$
Solubilization
20 & Exchange
24
Separation
22 NH_4LA
$CaCO_3$(ppt)
Ion
26 Exchange
30
NH_3
Electrohydrolysis
28
Lactic
32 Acid

FIG. 1

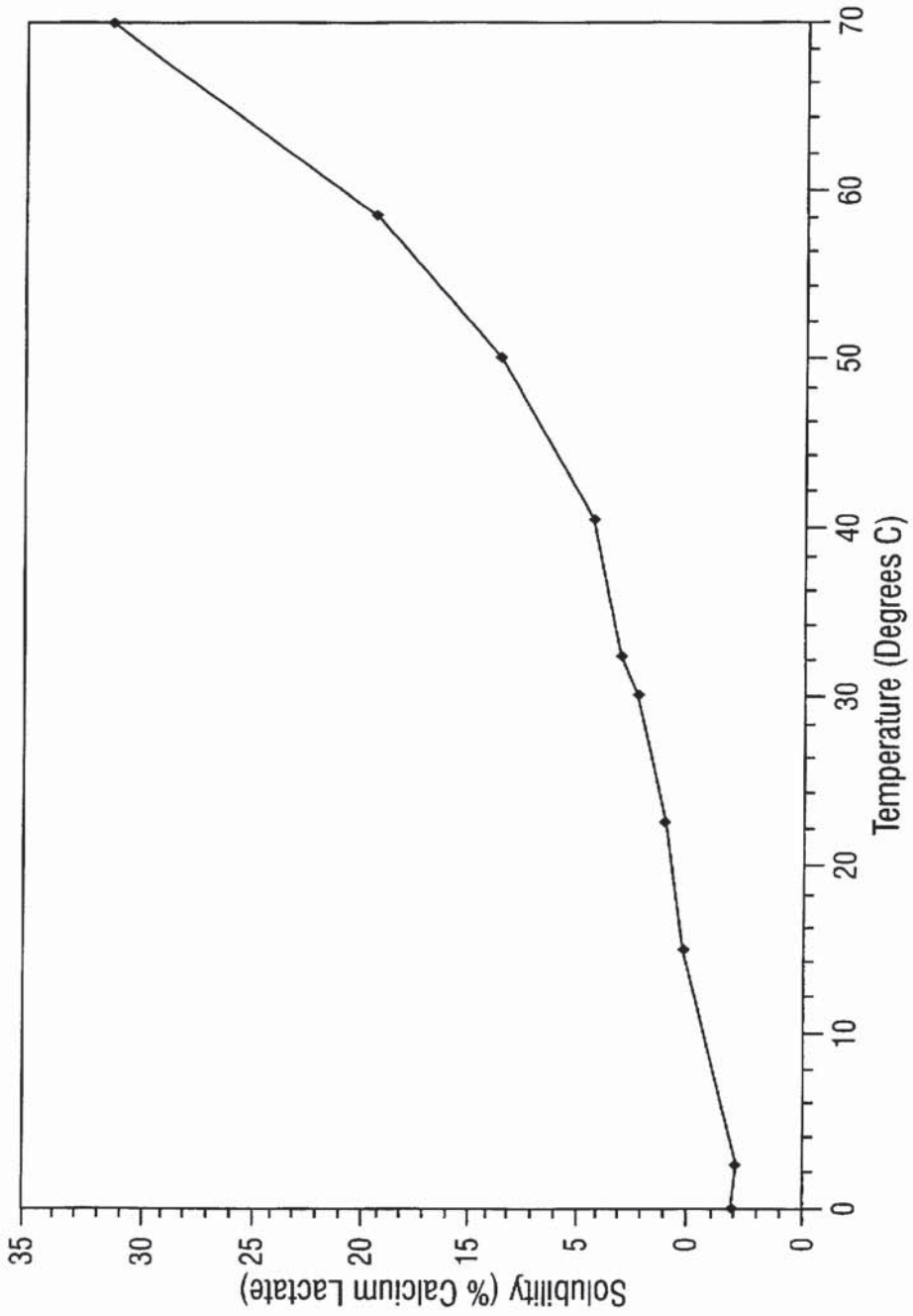
Solubility (% Calcium Lactate)
35
30
25
20
15
5
0
0
10
20
30
40
50
60
70
Temperature (Degrees C)

FIG. 2

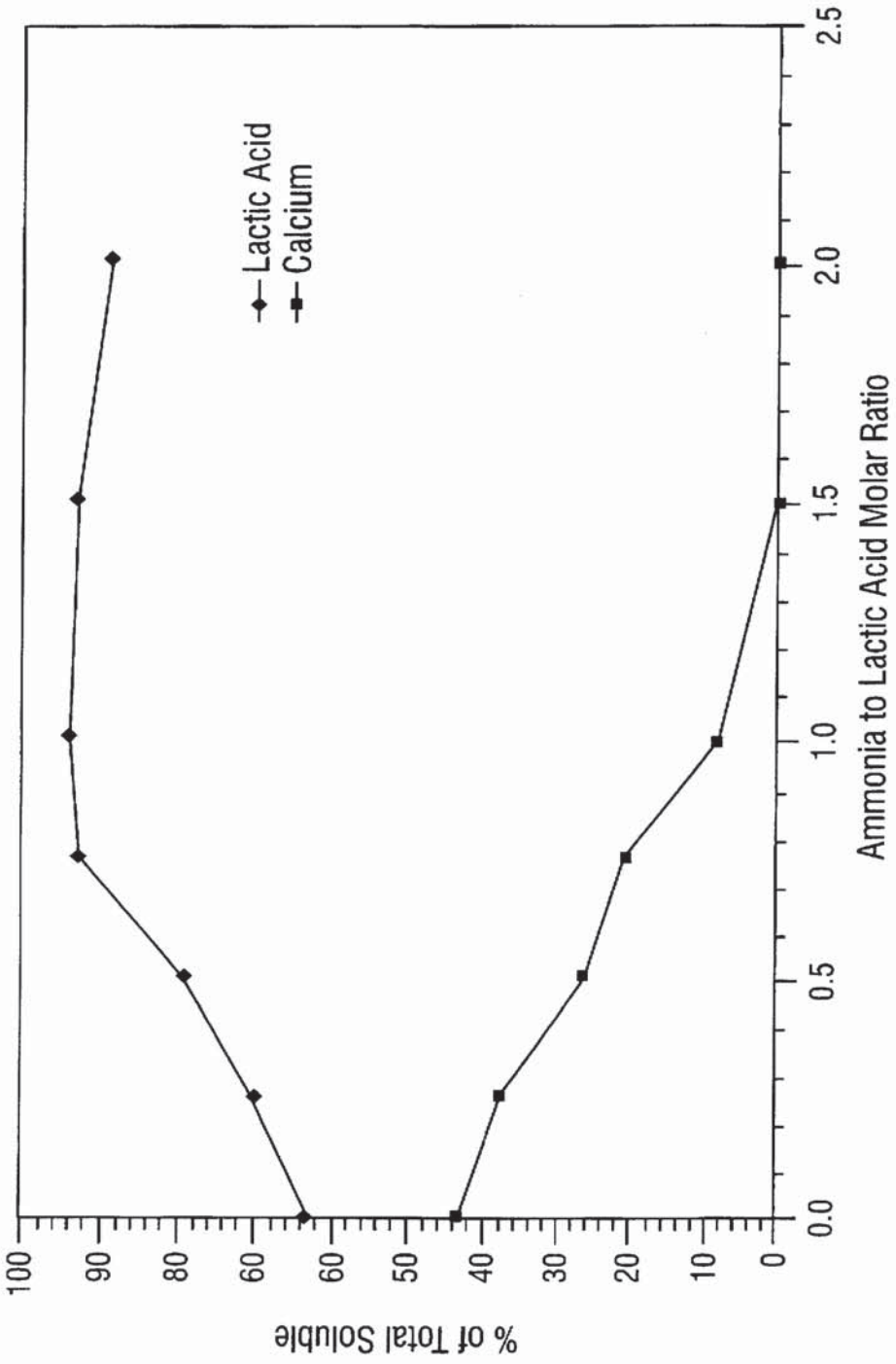
Lactic Acid
Calcium
% of Total Soluble
100
90
80
60
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Ammonia to Lactic Acid Molar Ratio

FIG. 3

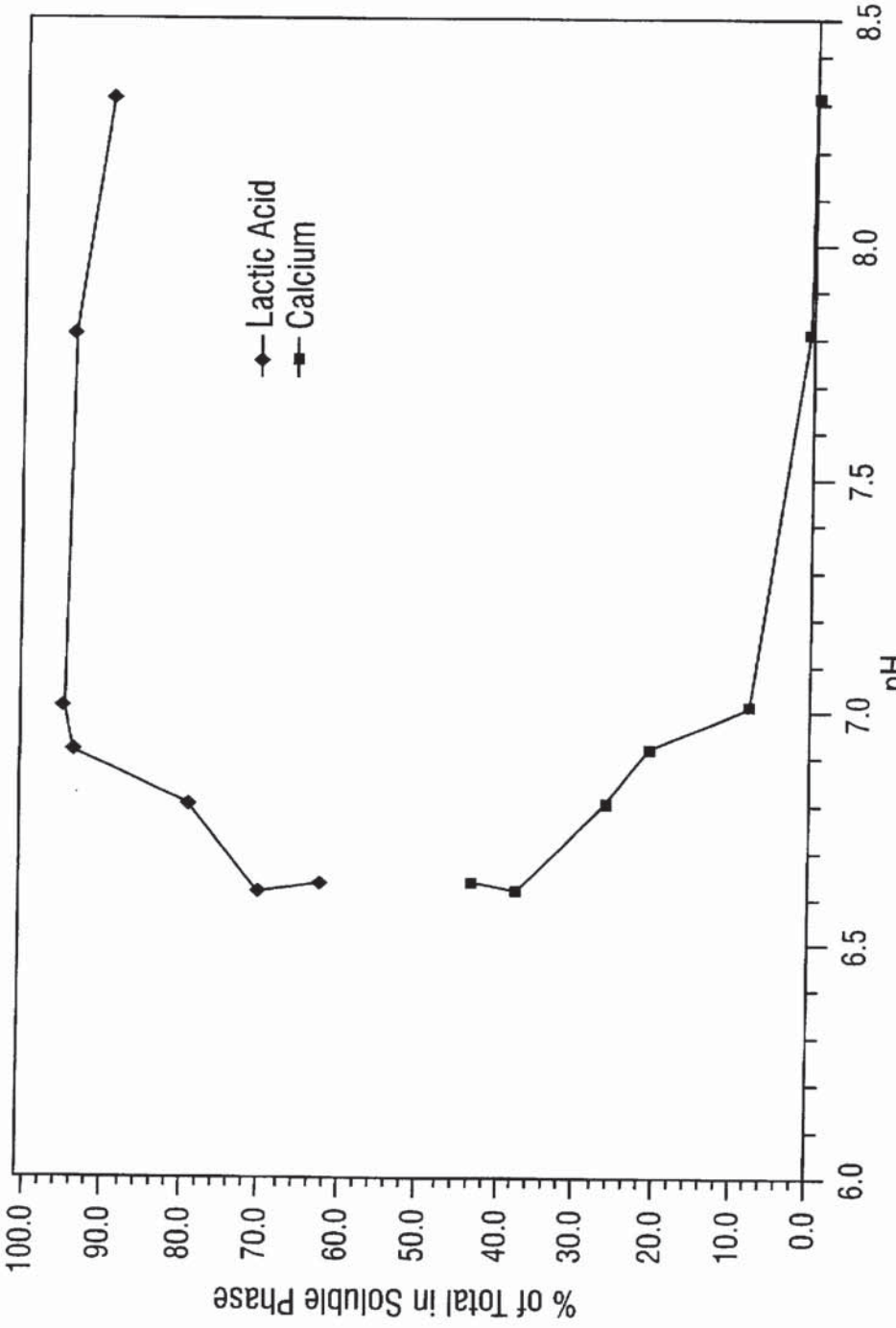
Lactic Acid
Calcium
% of Total in Soluble Phase
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
6.0
6.5
7.0
7.5
8.0
8.5
pH

FIG. 4

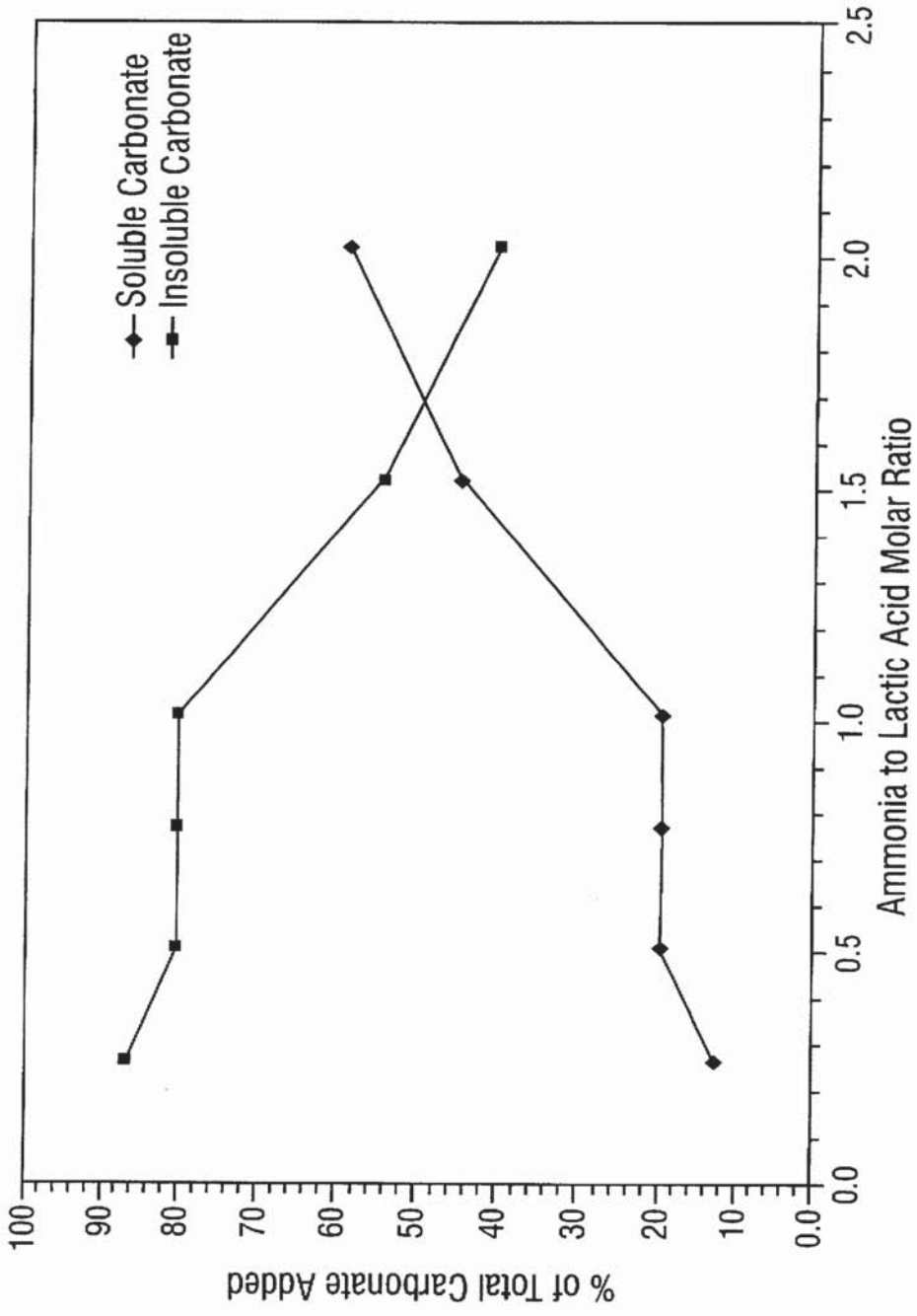
Soluble Carbonate
Insoluble Carbonate
% of Total Carbonate Added
100
90
80
70
60
50
40
30
20
10
0.0
0.0
0.5
1.0
1.5
2.0
2.5
Ammonia to Lactic Acid Molar Ratio

FIG. 5

PRODUCTION AND RECOVERY OF ORGANIC ACIDS

This is a continuation in part of the U.S. application titled "Production and Recovery of Organic Acids," filed on Oct. 10, 1996, application Ser. No. 08/728,836 now abandoned.

BACKGROUND OF THE INVENTION

The present invention relates to carboxylic acids such as lactic acid, and to processes for producing and recovering them.

Lactic acid has a number of commercial uses, for example in food manufacturing, pharmaceuticals, plastics, textiles, and as a starting material in various chemical processes. Lactic acid is commonly produced by fermentation of sugars, starch, or cheese whey, using microorganisms such as *Lactobacillus delbrueckii* to convert monosaccharides such as glucose, fructose, or galactose, or disaccharides such as sucrose or lactose, into lactic acid. The broth that results from fermentation contains unfermented sugars, carbohydrates, amino acids, proteins, and salts, as well as lactic acid. Some of these materials cause an undesirable color. The lactic acid therefore must be recovered from the fermentation broth before it can be put to any substantial use.

During the production of an organic acid such as lactic acid by fermentation, the increasing concentration of the acid in the fermentation broth reduces the pH. As the pH decreases, the growth of the microorganism is inhibited and eventually stops, and therefore acid production stops. To prevent this, the pH of the fermentation broth typically is controlled by adding a base for neutralization. Calcium bases, such as calcium hydroxide, have been preferred for neutralization of lactic acid fermentation broths, but their use results in the production of calcium lactate, which has some undesirable consequences for the ultimate recovery of the lactic acid. In particular, in order to recover free acid from the calcium lactate, a mineral acid such as sulfuric acid is often added. While this does permit recovery of free acid, it also generates gypsum as an unwanted byproduct. Further, the cost of the sulfuric acid is a significant factor in the overall cost of the process.

A need exists for improved processes that will permit production and recovery of organic acids at a desired level of purity and at a reduced cost.

SUMMARY OF THE INVENTION

The present invention relates to a process for producing a carboxylic acid, preferably one selected from the group consisting of mono-, di-, and tricarboxylic acids having 3–8 carbon atoms. Examples include lactic acid, citric acid, malic acid, maleic acid, fumaric acid, adipic acid, succinic acid, tartaric acid, α-ketoglutaric acid, and oxaloacetic acid.

The process includes production of the organic acid by fermentation employing a microorganism, resulting in an aqueous fermentation broth containing the organic acid. An alkaline earth base is added to the fermentation broth during and/or after the fermentation process, in an amount effective to allow growth of the microorganism producing the organic acid, thereby producing an alkaline earth salt of the organic acid in the broth. Biomass can optionally be removed from the broth. Whether the biomass is removed at this point in the process or not, an aqueous solution or dispersion is present that contains the alkaline earth salt of the organic acid. The alkaline earth salt of the organic acid, either with or without biomass, is then reacted with a source of ammonium ions selected from the group consisting of ammonia, ammonium salts, and substituted ammonium salts, thereby producing an ammonium salt or substituted ammonium salt of the organic acid. Free organic acid or a derivative thereof can then be separated and recovered.

The alkaline earth base is preferably a calcium base, for example selected from a group consisting of calcium hydroxide, calcium carbonate, calcium bicarbonate and calcium oxide, with calcium carbonate being especially preferred. The alkaline earth base can be added in an amount effective to control the pH of the broth between about 5.5 and about 7.0.

A preferred method for removal of the biomass from the broth is microfiltration. A preferred method for separating and recovering the free acid is salt-splitting electrodialysis. When salt-splitting electrodialysis is used, it is preferred to first reduce the level of soluble contaminants such as divalent cations in the solution by ion exchange.

When the organic acid produced is lactic acid, the equivalent ratio of ammonia to lactic acid is preferably between about 0.75 and about 2.0, most preferably between about 1.0 and 1.5. The pH after the exchange reaction with a source of ammonium ions is preferably between about 7.0 and about 11.0, most preferably between about 7.5 and 10.0. The optimum pH may vary with the source of the ammonium ions.

In one embodiment of the present invention, a process for producing lactic acid includes the steps of producing lactic acid by fermentation, resulting in an aqueous fermentation broth, adding a calcium base to the fermentation broth during and/or after the fermentation process to maintain the broth pH at a level high enough to allow growth of the microorganism producing the organic acid, thereby producing calcium lactate in the broth, removing biomass from the broth, thereby producing an aqueous solution or dispersion of calcium lactate, reacting the calcium lactate with a source of ammonium ions selected from the group consisting of ammonia, ammonium salts, and substituted ammonium salts, thereby producing ammonium lactate or a substituted ammonium lactate, removing contaminant cations by ion exchange, and separating and recovering free lactic acid by salt-splitting electrodialysis. Optionally the acid may be converted to a derivative, such as an ester, and recovered in that form.

The process can also include the step of heating the fermentation broth prior to removing biomass, to an extent effective to solubilize the majority of the calcium lactate present in the broth. The process can also include the step of concentrating the calcium lactate solution or dispersion prior to reaction with the source of ammonium ions, for example by crystallization of the calcium lactate followed by filtration.

The present invention presents opportunities for recycling streams generated at one point in the process for use at another point in the process. For example, in one embodiment of the present invention, a calcium carbonate precipitate is formed by the reaction of the calcium lactate with the source of ammonium ions, and the precipitate can be recycled to the fermentation broth. As another example, the salt-splitting electrodialysis step can generate ammonia, which can be recycled to the step in which calcium lactate is reacted with the source of ammonium ions.

An especially preferred embodiment of the present invention is a process for producing lactic acid that includes the steps of producing lactic acid by fermentation, resulting in an aqueous fermentation broth containing lactic acid, adding

calcium carbonate to the fermentation broth, thereby producing calcium lactate in the broth, heating the broth to an extent effective to solubilize the majority of the calcium lactate, removing biomass from the broth by filtration, thereby producing an aqueous solution or dispersion of calcium lactate, concentrating calcium lactate by crystallization followed by filtration, adding a source of ammonium ions, selected from the group consisting of ammonium carbonate and a mixture of ammonia and carbon dioxide, to the aqueous solution or dispersion of calcium lactate, thereby producing ammonium lactate and calcium carbonate, separating the calcium carbonate and recycling it to the fermentation broth, removing contaminant cations by ion exchange, and separating and recovering free lactic acid by salt-splitting electrodialysis.

Another embodiment of the present invention concerns an improved process for producing lactic acid or a derivative thereof from a medium comprising calcium lactate salt, which salt is a product of fermentation, where (i) carbohydrates are fermented to lactic acid, (ii) a calcium base is used in the fermentation as neutralizing agent for pH adjustment so that an aqueous solution or dispersion of calcium lactate is formed, and (iii) a product calcium base is formed in the process. The improvement comprises (a) reacting a source of ammonium ions with the aqueous solution or dispersion of calcium lactate to form water soluble ammonium lactate and a product calcium base; (b) converting the water soluble ammonium lactate to lactic acid or a derivative thereof and a by-product that comprises ammonia; (c) separating the by-product that comprises ammonia; (d) using the by-product that comprises ammonia as a source of ammonium ions for step (a); (e) separating the product calcium base formed in step (a); and (f) using the product calcium base from step (e) as a neutralizing agent in the lactic acid fermentation.

In various specific embodiments of this improved process, conversion of the water soluble ammonium lactate to lactic acid or a derivative thereof in step (b) can be done by distillation. The separation of the by-product that comprises ammonia in step (c) can be done by, for example, distillation or crystallization.

The present invention has a number of advantages over prior art processes. It allows the production and recovery of organic acids at reduced cost. Further, it produces solutions of the organic acid having relatively high concentrations, thus reducing or eliminating the need for further concentration steps.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a process flow diagram for an organic acid production and recovery process in accordance with he present invention.

FIG. **2** is a graph of the solubility of calcium lactate in water at various temperatures.

FIGS. **3**, **4**, and **5** are graphs reflecting test results that are described in Example 1.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Referring to FIG. **1**, a process in accordance with the present invention begins with the production of an organic acid by fermentation **10**. Fermentation procedures and starting materials are well known to persons skilled in the art.

If the organic acid to be produced is lactic acid, a suitable fermentation procedure is as follows. The feed to this fermentation can consist of a mix of glucose syrup, light steepwater, corn gluten filtrate, and trace vitamins and minerals. Typical concentrations are about 110 gm. d.s./liter of glucose, 10 gm. d.s./liter of light steepwater, and 7 gm. d.s./liter of corn gluten filtrate. The trace vitamins and minerals can be (on a dry substance basis) 0.5 g/l of diammonium phosphate, 0.04 g/l of manganese sulfate, 68 ppm of choline, 4.4 ppm of riboflavin, and 2.0 ppm of niacin. Residual sulfur dioxide in the light steepwater and corn gluten filtrate can be neutralized with stoichiometric amounts of hydrogen peroxide. The organism can be of the species *L. casei* ssp. *rhamnosus*, for example ATCC 11443. The fermentation can be conducted at 108° F. The fermentation is finished when the glucose is exhausted, typically in about 20 hours. The yield on glucose will typically be about 95%. The fermentation broth will typically contain less than 25% by weight lactic acid. Often at least 80% by weight of the total lactate values in the broth are present as calcium lactate.

In order to maintain the rate of acid production, it is desirable to control the pH of the fermentation broth between about 5.5 and about 7.0. This is preferably done by adding to the broth an alkaline earth carbonate or bicarbonate base, preferably calcium carbonate. The addition of this calcium base is preferably done during the fermentation process. It keeps the pH of the broth from dropping too low, and results in the production of calcium lactate.

The broth is heated **12** in order to solubilize the calcium lactate, preferably to a temperature of about 40°–60° C. FIG. **2** shows the solubility of calcium lactate at temperatures ranging from 0° C. to 70° C. Depending on the extent of the heating, some calcium lactate may remain in an undissolved state.

Next, biomass may be (but does not have to be) removed from the broth. If biomass is removed, it is preferably by tight membrane filtration **14**. For example, the broth or liquor can be filtered using ultrafiltration or nanofiltration, for example with ceramic elements having a 0.02 micron pore size. Diafiltration can be used to attain a lactate recovery of at least 95%. The retentate stream, consisting of biomass and other insolubles, can be used as animal feed or in the alternative can be recycled to the fermentation bioreactor.

If the lactate broth is microfiltered, the permeate can then be nanofiltered at 140° F. on a nanofiltration membrane having a molecular weight cut-off of 250 to 1000. Diafiltration can be used to attain a recovery of calcium lactate of at least 95%.

As an alternative to filtration, other procedures such as centrifugation, filter pressing, or rotary vacuum filtration could be used to produce a substantially particulate-free liquor.

If biomass is not removed at this point in the process, then the processing of the broth and its contents can continue as described below with the biomass still present.

It is desirable to concentrate the solution of calcium lactate at this point, and this can be accomplished by crystallizing **16** the calcium lactate and then separating **18** the crystallized material, for example by filtration **18**. Crystallization can be done using standard techniques known to those skilled in the art.

The next step in the process involves an exchange reaction **20**. A source of ammonium ions, and preferably also a source of carbonate ions, are added to the solution. The source of ammonium ions can be in the form of ammonia, an ammonium salt, or a substituted ammonium salt or mixtures thereof. For example, the materials added in this

step can be gaseous ammonia, aqueous ammonium hydroxide, ammonium carbonates or bicarbonates, gaseous mixtures of ammonia and carbon dioxide, or mixtures of one or more of these. If gaseous carbon dioxide is used, it can be taken from the gas stream liberated in the fermentation step **10**.

A preferred process for the formation of the ammonium lactate (or other organic acid salt) is to first react the precipitated calcium lactate with ammonia in an aqueous medium to raise the pH of the reaction media to about 7.0 or higher, and then add ammonium carbonate or other source of ammonium ion to raise the pH to the desired level to form soluble ammonium lactate.

The result of this exchange reaction **20** is the formation of ammonium lactate and calcium carbonate. The calcium carbonate will typically precipitate, so it can be separated **22**, for example by crystallization, and can optionally be recycled **24** to the fermentation step **10**. The remaining solution can then be processed so as to recover the acid as such or as a derivative such as an ester. A preferred way of doing this is by salt-splitting electrodialysis.

Salt-splitting electrodialysis usually requires a relatively high purity solution as its starting material. Therefore, when salt-splitting electrodialysis **28** is used, the ammonium lactate solution is preferably first treated **26** with an ion exchange resin to reduce the residual level of divalent cations such as calcium to less than about 100 ppm, preferably less than about 5 ppm, more preferably less than about 2 ppm, to reduce fouling of the electrodialysis membranes. Exchangers such as Rohm and Haas Duolite C-467, weak acid cation resin, or the like may be used for this purpose. This procedure extends the life of the electrohydrolysis membranes.

The remaining solution can be separated into free lactic acid and ammonia, for example by salt-splitting electrodialysis **28**. Salt splitting electrodialysis (SS-ED) is capable of splitting a salt into its corresponding acid and base. It uses electrical current to drive salt ions through cationic and anionic permeable membranes, and also uses a special bipolar membrane (one side anionic and one side cationic) to split water into H^+ and OH^- to combine with the salt anion and cation respectively. Only charged compounds are transferred to the product streams.

Electrodialysis has the advantage of being able to produce an acid (or base) from a salt solution without forming another salt as a byproduct. Further, salt splitting electrodialysis produces a high quality end product. The major drawbacks of salt splitting electrodialysis are the yield loss, electrical consumption, and the cost and life of the membranes. The yield or lactic acid recovery can be improved at additional electrical and capital costs. Electrodialysis apparatus and conditions are disclosed in U.S. Pat. Nos. 5,198,086, 5,250,159, and 5,268,079, each of which is incorporated here by reference.

The ammonia generated by electrohydrolysis can be recycled **30** to the exchange reaction **20**. This then leaves free lactic acid **32**, which can optionally be further purified, for example by carbon treatment or ion exchange. The free acid optionally may be derivatized, for example to lactic acid esters, or polymerized to form polylactic acid. The product may also be subjected to further purification steps, such as carbon treatment, molecular distillation, or liquid/liquid extraction.

EXAMPLE 1

A series of reactions was performed in 120 ml. screw cap bottles containing the quantities of components listed in Table 1.

TABLE 1

Experimental Design and Data

Bottle Number	Calcium Lactate Grams	Saturated $(NH_4)_2CO_3$ Grams	Water Grams	Total Wt. Grams	Ammonia/Lactic Molar Ratio
1	5.0005	18.05	17.10	40.1505	2.01
2	5.0079	13.51	21.63	40.1479	1.50
3	5.0024	9.03	26.02	40.0524	1.01
4	5.0013	6.79	28.26	40.0513	0.76
5	5.0043	4.50	30.52	40.0243	0.50
6	5.0008	2.28	32.81	40.0908	0.25
7	5.0004	0.00	35.01	40.0104	0.00

Solid calcium lactate (Sigma Chem. Co., Lot 113H2522, 60.23% lactate, 13.92% calcium, 25.85% water) was added to each bottle followed by the appropriate quantity of water and the aqueous, saturated ammonium carbonate solution. The saturated ammonium carbonate solution was prepared by stirring excess ammonium carbonate (Sigma Chem. Co., Lot 26H3613, 32.3% NH_3) with water for 24 hours in a sealed flask at 24° C. and separating the solution by filtration. The pH of this saturated solution was 8.41 and contained 6.46% NH_3. The bottles were sealed and vigorously shaken at 24° C. for 4 hours. The solids were allowed to settle for 20 minutes and the supernatant was clarified through 0.45 micron filters. The pH of the filtered solutions was measured and analyzed for lactic acid, calcium and carbonate. The results of these analyses are shown in Table 2.

TABLE 2

Analytical Data

Bottle Number	Total Water Grams	% of Total Lactic Acid Soluble	% of Total Calcium Soluble	% of Total CO_3 Soluble
1	33.15	89.56	0.21	59.76
2	33.97	93.75	0.30	45.11
3	34.70	94.57	8.26	19.26
4	35.11	93.25	20.45	19.55
5	35.49	79.29	25.89	19.42
6	35.97	70.12	37.46	12.48
7	36.30	62.75	43.15	0.00

Lactic acid and carbonate were determined by High Pressure Liquid Chromatography with a Dionex ion exclusion column (IonPac ICE-AS6) with an eluent flow rate of 1 ml/min with 0.4 mM heptafluorobutyric acid. The detector was a conductivity detector with chemical suppression. The column was regenerated with 0.01M tetrabutylammonium hydroxide at 5 ml/min. Calcium was determined by atomic spectroscopy. The percent of total lactic acid, calcium and CO_3 which is soluble was calculated on the basis of the total grams of water in the system. This is a combination of the water contained in the calcium lactate, saturated ammonium carbonate and added water. The calculated results are shown in Table 3 and are represented in FIGS. **3**, **4**, and **5**.

TABLE 3

	Calculated Data			
Bottle Number	pH Final	% Lactic Acid In Solution	ppm Calcium In Solution	% Carbonate In Solution
1	8.33	7.57	43	3.70
2	7.82	7.76	59	2.04
3	7.01	7.67	1590	0.57
4	6.92	7.48	3893	0.43
5	6.80	6.30	4877	0.28
6	6.62	5.50	6961	0.09
7	6.63	4.88	7945	0.00

At an ammonia to lactic acid mole ratio of one, greater than 94% of the lactic acid was solubilized as the ammonium lactate while greater than 90% of the calcium was insoluble. As this ratio increased toward two, the insoluble calcium increased to greater than 99%.

EXAMPLE 2

Calcium lactate (10.03 g), water (43.12 g) and saturated ammonium carbonate (26.92 g) were placed in a 250 ml screw cap bottle and shaken vigorously for two hours and allowed to stand overnight at 24° C. This is approximately double the quantities of reagents used in Example 1, Bottle 2 where the molar ratio of ammonia to lactic acid is 1.5/1. The mixture was separated using a medium, porous glass filter and the solid fraction was washed three times with about 20 ml of ice cold water. The total weight of the filtrates was 134.38 g and contained 42.5 ppm calcium and 4.60% lactic acid. The recovery of the lactic acid in the soluble phase was 102%. The weight of the precipitate was 4.4140 g and contained 31.0% calcium, a recovery of 98%. A portion of the precipitate (0.5201 g) was extracted with 2.0866 g of hot water and the mixture filtered through a 0.45 micron filter disc. This filtrate contained only 6 ppm of lactic acid. The Infra-red analysis spectrum of the precipitate was consistent with that of carbonate with some slight contaminant of organic matter. This data is consistent with a calcium carbonate precipitate which contains only traces of lactic acid.

The preceding description of specific embodiments of the present invention is not intended to be a complete list of every possible embodiment of the invention. Persons skilled in this field will recognize that modifications can be made to the specific embodiments described here that would be within the scope of the present invention.

We claim:

1. A process for producing an organic acid, comprising the steps of:

producing by fermentation an organic acid selected from the group consisting of mono-, di-, and tricarboxylic acids having 3–8 carbon atoms, resulting in an aqueous fermentation broth;

adding an alkaline earth base to the fermentation broth in amount effective to maintain the broth pH at a level high enough to allow continued production of the organic acid, thereby producing an alkaline earth salt of the organic acid in the broth;

reacting the alkaline earth salt of the organic acid with a source of ammonium ions selected from the group consisting of ammonia, ammonium salts, substituted ammonium salts, and mixtures thereof, thereby producing an aqueous solution or dispersion containing ammonium salt or substituted ammonium salt of the organic acid and divalent cations;

reducing the concentration of divalent cations to a level sufficient to permit salt-splitting electrodialysis;

converting the ammonium or substituted ammonium salt of the organic acid to free organic acid or a derivative thereof; and

recovering the free organic acid or derivative thereof.

2. The process of claim 1, where the organic acid is lactic acid.

3. The process of claim 1, where the alkaline earth base is a calcium base.

4. The process of claim 1, where the alkaline earth base is selected from the group consisting of calcium carbonate, calcium bicarbonate, calcium oxide, and calcium hydroxide.

5. The process of claim 1, where the alkaline earth base is added during the fermentation process.

6. The process of claim 1, where the alkaline earth base is added in an amount effective to control the pH of the broth between about 5.5 and about 7.0.

7. The process of claim 1, where biomass is removed from the broth prior to reacting the alkaline earth salt of the organic acid with the source of ammonium ions.

8. The process of claim 7, where biomass is removed from the broth by microfiltration.

9. The process of claim 1, where the source of ammonium ions comprises ammonia.

10. The process of claim 1, where the source of ammonium ions comprises a mixture of ammonia and at least one ammonium salt or substituted ammonium salt.

11. The process of claim 1, where the source of ammonium ions comprises ammonium carbonate.

12. The process of claim 1, where the alkaline earth salt of the organic acid is reacted with ammonia in an aqueous medium to raise the pH to above about 7, and then ammonium carbonate is added thereto.

13. The process of claim 1, where the derivative of the organic acid is an ester.

14. The process of claim 1, where the ammonium or substituted ammonium salt of the organic acid is converted to the free acid or derivative thereof by salt-splitting electrodialysis.

15. The process of claim 14, where the concentration of divalent cations is reduced prior to salt-splitting electrodialysis by ion exchange.

16. The process of claim 1, where the pH after the reaction with a source of ammonium ions is between about 7.0 and about 11.0.

17. The process of claim 1, where the pH after the reaction with a source of ammonium ions is between about 7.5 and about 10.0.

18. A process for producing lactic acid, comprising the steps of:

producing lactic acid by fermentation, resulting in an aqueous fermentation broth;

adding a calcium base to the fermentation broth in an amount effective to maintain the broth pH at a level high enough to allow continued production of the lactic acid, thereby producing calcium lactate in the broth;

reacting the calcium lactate with a source of ammonium ions selected from the group consisting of ammonia, ammonium salts, substituted ammonium salts, and mixtures thereof, thereby producing aqueous solution or dispersion containing an ammonium lactate or substituted ammonium lactate divalent cations;

reducing the concentration of divalent cations to a level sufficient to permit salt-splitting electrodialysis;

converting the ammonium or substituted ammonium lactate to free lactic acid or a derivative thereof; and

recovering the free lactic acid or derivative thereof.

19. The process of claim **18**, where the calcium base is calcium carbonate.

20. The process of claim **18**, where the calcium base is added in an amount effective to control the pH of the broth between about 5.5 and about 7.0.

21. The process of claim **18**, where biomass is removed from the broth prior to reacting the calcium lactate with the source of ammonium ions.

22. The process of claim **21**, where the biomass is removed from the broth by microfiltration.

23. The process of claim **18**, where the source of ammonium ions comprises ammonia.

24. The process of claim **18**, where the source of ammonium ions comprises a mixture of ammonia and at least one ammonium salt or substituted ammonium salt.

25. The process of claim **18**, where the source of ammonium ions comprises ammonium carbonate.

26. The process of claim **18**, further comprising the step of heating the fermentation broth prior to removing biomass, to an extent effective to solubilize the majority of the calcium lactate present in the broth.

27. The process of claim **18**, where the calcium lactate is reacted with ammonia in an aqueous medium to raise the pH to above about 7, and then ammonium carbonate is added thereto.

28. The process of claim **18**, further comprising the step of concentrating the calcium lactate solution or dispersion prior to reaction with the source of ammonium ions by crystallization of the calcium lactate followed by filtration.

29. The process of claim **18**, where a calcium carbonate precipitate is formed by the reaction of the calcium lactate with the source of ammonium ions, and the precipitate is recycled to the fermentation broth.

30. The process of claim **18**, where the ammonium salt or substituted ammonium salt of lactic acid is converted to free lactic acid or derivative thereof by salt splitting electrodialysis.

31. The process of claim **30**, where the concentration of divalent cations is reduced prior to salt splitting electrodialysis by ion exchange.

32. The process of claim **18**, where the equivalent ratio of ammonia to lactic acid is between about 0.75 and 2.0.

33. The process of claim **18**, where the equivalent ratio of ammonia to lactic acid is between about 1.0 and 1.5.

34. The process of claim **18**, where the pH after the reaction with a source of ammonium ions is between about 7.0 and about 11.0.

35. The process of claim **18**, where the pH after the reaction with a source of ammonium ions is between about 7.5 and about 10.0.

36. The process of claim **18**, where the salt-splitting electrodialysis generates ammonia, which is recycled to the reaction of calcium lactate with the source of ammonium ions.

37. A process for producing lactic acid, comprising the steps of:

producing lactic acid by fermentation, resulting in an aqueous fermentation broth;

adding calcium carbonate to the fermentation broth, thereby producing calcium lactate in the broth;

solubilizing the majority of the calcium lactate;

removing biomass from the broth by filtration, thereby producing an aqueous solution or dispersion of calcium lactate;

adding a source of ammonium ions, selected from the group consisting of ammonium carbonate and a mixture of ammonia and carbon dioxide, to the aqueous solution or dispersion of calcium lactate, thereby producing ammonium lactate and calcium carbonate;

separating the calcium carbonate and recycling it to the fermentation broth;

removing contaminant cations by ion exchange; and

converting the ammonium lactate to free lactic acid by salt-splitting electrodialysis.

38. In a process for producing lactic acid or a derivative thereof from a medium comprising calcium lactate salt, which salt is a product of fermentation, where (i) carbohydrates are fermented to lactic acid, (ii) a calcium base is used in the fermentation as neutralizing agent for pH adjustment so that an aqueous solution or dispersion of calcium lactate is formed, and (iii) a product calcium base is formed in the process, the improvement comprising:

(a) reacting a source of ammonium ions with the aqueous solution or dispersion of calcium lactate to form water soluble ammonium lactate and a product calcium base;

(b) converting the water soluble ammonium lactate to lactic acid or a derivative thereof and a by-product that comprises ammonia;

(c) separating the by-product that comprises ammonia;

(d) using the by-product that comprises ammonia as a source of ammonium ions for step (a);

(e) separating the product calcium base formed in step (a); and

(f) using the product calcium base from step (e) as a neutralizing agent in the lactic acid fermentation.

39. The process of claim **38**, where the conversion of the water soluble ammonium lactate to lactic acid or a derivative thereof in step (b) is done by electrodialysis.

40. The process of claim **39**, where prior to the salt-splitting electrodialysis, contaminant cations are removed by ion exchange.

41. The process of claim **38**, where the conversion of the water soluble ammonium lactate to lactic acid or a derivative thereof in step (b) is done using bipolar membranes.

42. The process of claim **38**, where the conversion of the water soluble ammonium lactate to lactic acid or a derivative thereof in step (b) is done by distillation.

43. The process of claim **38**, where the source of ammonium ions is selected from the group consisting of gaseous ammonia, aqueous ammonium hydroxide, ammonium carbonates, or bicarbonates, mixtures of ammonia with carbon dioxide, and combinations thereof.

44. The process of claim **38**, where the pH in step (a) after the reaction with the source of ammonium ions is between about 7.0 and about 11.0.

45. The process of claim **38**, where the pH in step (a) after the reaction with the source of ammonium ions is between about 7.5 and about 10.0.

46. The process of claim **38**, where the separation of the by-product that comprises ammonia in step (c) is done by distillation.

47. The process of claim **38**, where the separation of the by-product that comprises ammonia in step (c) is done by crystallization.

48. The process of claim 38, where the ammonium lactate is concentrated prior to the conversion in step (b).

49. The process of claim 38, where the product calcium base is selected from a group consisting of calcium hydroxide, calcium carbonate, calcium bicarbonate, and calcium carbonate.

50. The process of claim 38, where prior to the conversion in step (b), an ion exchange is performed to reduce the concentration of divalent cations in solution to less than about 100 ppm, and the conversion of step (b) is then done by salt-splitting electrodialysis.

51. The process of claim 1, where the concentration of divalent cations is reduced prior to the conversion step to less than about 100 ppm.

52. The process of claim 1, where the concentration of divalent cations is reduced prior to the conversion step to less than about 5 ppm.

53. The process of claim 1, where the concentration of divalent cations is reduced prior to the conversion step to less than about 2 ppm.

54. The process of claim 18, where the concentration of divalent cations is reduced prior to the conversion step to less than about 100 ppm.

55. The process of claim 18, where the concentration of divalent cations is reduced prior to the conversion step to less than about 5 ppm.

56. The process of claim 18, where the concentration of divalent cations is reduced prior to the conversion step to less than about 2 ppm.

57. The process of claim 1, where the conversion to the free acid or derivative thereof is done by distillation.

58. The process of claim 18, where the conversion to the free acid or derivative thereof is done by distillation.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO.	:	5,766,439
DATED	:	June 16, 1998
INVENTOR(S)	:	Aharon M. Eyal et al.

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

Claim 1, column 7, line 59, before "amount" insert --an--.

Claim 49, column 11, lines 5-6, change "calcium carbonate, calcium bicarbonate, and calcium carbonate" to --calcium carbonate, and calcium bicarbonate--.

Signed and Sealed this

Twelfth Day of January, 1999

Attest:

Attesting Officer

Acting Commissioner of Patents and Trademarks

EXHIBIT 12


US008722056B2

(12) **United States Patent**
Wang et al.

(10) **Patent No.: US 8,722,056 B2**
(45) **Date of Patent: May 13, 2014**

(54) **METHODS FOR MAKING AND COMPOSITIONS COMPRISING FERMENTATION PRODUCTS OF CORDYCEPS SINENSIS**

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(73) Assignee: **TCM Biotech International Corp.**, Taipei (TW)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 865 days.

(21) Appl. No.: **12/461,304**

(22) Filed: **Aug. 6, 2009**

(65) **Prior Publication Data**

US 2010/0021426 A1 Jan. 28, 2010

Related U.S. Application Data

(60) Continuation-in-part of application No. 12/411,940, filed on Mar. 26, 2009, now abandoned, which is a continuation of application No. 12/003,736, filed on Dec. 31, 2007, now abandoned, which is a division of application No. 10/755,468, filed on Jan. 13, 2004, now abandoned.

(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**
A61K 36/06 (2006.01)

(52) **U.S. Cl.**
USPC **424/195.15**; 424/115

(58) **Field of Classification Search**
None
See application file for complete search history.

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(74) *Attorney, Agent, or Firm* — Finnegan, Henderson, Farabow, Garrett & Dunner LLP.

(57) **ABSTRACT**

The present invention relates to novel and non-obvious methods and media for preparing fermentation products of *Cordyceps sinensis*. The present invention also relates to novel and non-obvious compositions comprising fermentation products of *Cordyceps sinensis* produced by the methods of the invention or obtained from other sources. The present invention also relates to novel and non-obvious methods of treating patients by administering the compositions of the invention. In one embodiment, the *Cordyceps sinensis* mycelia is *Paecilomyces hepiali* mycelia. In another embodiment the compositions of the invention are used to treat patients infected with hepatitis C.

21 Claims, 2 Drawing Sheets

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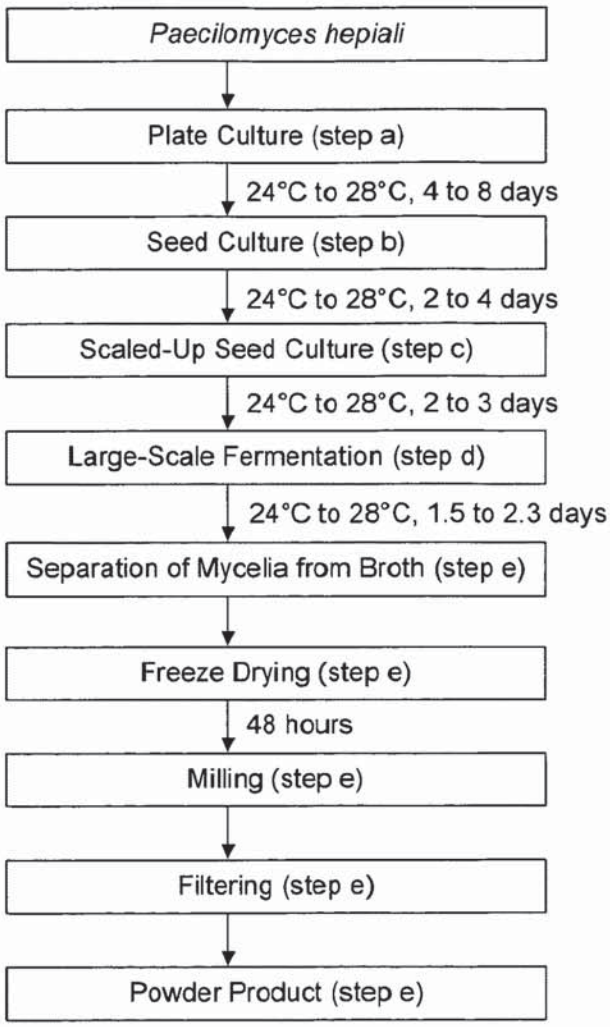
Paecilomyces hepiali
Plate Culture (step a)
24°C to 28°C, 4 to 8 days
Seed Culture (step b)
24°C to 28°C, 2 to 4 days
Scaled-Up Seed Culture (step c)
24°C to 28°C, 2 to 3 days
Large-Scale Fermentation (step d)
24°C to 28°C, 1.5 to 2.3 days
Separation of Mycelia from Broth (step e)
Freeze Drying (step e)
48 hours
Milling (step e)
Filtering (step e)
Powder Product (step e)

FIG. 1

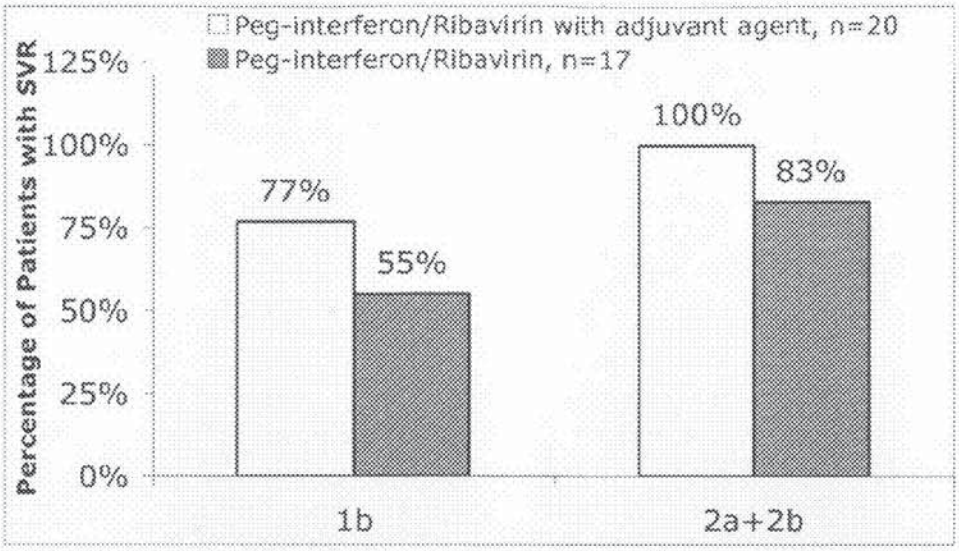
Peg-interferon/Ribavirin with adjuvant agent, n=20
Peg-interferon/Ribavirin, n=17
Percentage of Patients with SVR
125%
100%
75%
50%
25%
0%
77%
55%
100%
83%
1b
2a+2b

FIG. 2

METHODS FOR MAKING AND COMPOSITIONS COMPRISING FERMENTATION PRODUCTS OF CORDYCEPS SINENSIS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. patent application Ser. No. 12/411,940, filed Mar. 26, 2009 now abandoned, which is a continuation of U.S. patent application Ser. No. 12/003,736, filed Dec. 31, 2007 now abandoned, which is a division of U.S. patent application Ser. No. 10/755,468, filed Jan. 13, 2004 now abandoned, which claims priority under 35 U.S.C. §119(a) to Taiwan Patent Application No. 092127864, filed Oct. 3, 2003, all of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to novel and non-obvious methods and media for preparing fermentation products of *Cordyceps sinensis*. The present invention also relates to novel and non-obvious compositions comprising fermentation products of *Cordyceps sinensis* produced by the methods of the invention or obtained from other sources. The present invention also relates to novel and non-obvious methods of treating patients by administering the compositions of the invention. In one embodiment, the *Cordyceps sinensis* mycelia is *Paecilomyces hepiali* mycelia. In another embodiment the compositions of the invention are used to treat patients infected with hepatitis C.

BACKGROUND OF THE INVENTION

A. *Cordyceps sinensis*

Cordyceps sinensis (Berk.) Sacc., also known as Chinese caterpillar fungus and "DongChongXiaCao," is a black, blade-shaped fungus found primarily at high altitudes in the mountains of northwest and southwest China. The fungus is parasitic, growing on and deriving nutrients from the larvae of moths in the genera *Hepialus* and *Thitarodes*. *Cordyceps sinensis* spores infect *Hepialus* and *Thitarodes* caterpillars in late summer or early fall while the caterpillars are hibernating underground. The fungus then multiplies by yeast-like budding and grows in the form of threadlike hyphae, ultimately killing the host. During the following spring, the fruiting body (i.e., the sexual, teleomorphic form) of the fungus grows out of the caterpillar's head and emerges above ground.

Because wild *Cordyceps sinensis* is rare, scientists have attempted to adapt anamorphic (asexual) forms of the fungus for growth under laboratory conditions. However, the isolated fungus is difficult to culture without a host due to its low growth rate and specialized nutritional requirements. In addition, it is difficult to obtain pure isolates from wild *Cordyceps sinensis* and as a result, the various laboratory cultures have not yielded uniform mycelia. Accordingly, the various isolates have been given different anamorph names, including: *Cephalosporium sinensis* (also called *Cephalosporium dongchongxiacao*), *Chrysosporium sinense, Hirsutella hepialid, Hirsutella sinensis, Mortierella hepiali, Paecilomyces hepiali, Paecilomyces sinensis, Scydalilum* sp., *Scytalidium hepiali, Sporothrix insectorum, Stachybotrys* sp., *Tolypocladimn sinensis*, etc. Recent molecular evidence has revealed that *Hirstuella sinensis* is the true anamorph of *Cordyceps sinensis*.

Cordyceps sinensis has a long history of medicinal use in China, and although the various isolates of *Cordyceps sinensis* are morphologically distinct, they share similar chemical compositions and pharmacological properties. A variety of bioactive ingredients have been isolated from *Cordyceps sinensis*, including: proteins, peptides, all essential amino acids, cyclic dipeptides, and polyamines; monosaccharides, polysaccharides, and sugar derivatives; cordycepin (3'deoxyadenosine); cordycepic acid (D-mannitol); sterols, including ergosterol; nucleosides and nucleotides; superoxide dismutase; fatty acids; metal elements; vitamins; and other inorganics.

Cordyceps sinensis has been reported to produce both immuno-stimulating and immunosuppressive effects. Thus, it appears that *Cordyceps sinensis* may be a bi-directional modulator of the immune system. For example, some studies reported that *Cordyceps sinensis* enhances the activities of macrophages and natural killer (NK) cells, while other studies reported that the fungus inhibits these activities under different circumstances. *Cordyceps sinensis* has been shown to suppress or enhance antibody production and the proliferation of T cells, thymocytes, and natural killer cells. *Cordyceps sinensis* has also been shown to suppress or enhance expression of IL1, IL2, IL6, IL10, CD4, CD5, CD8, CD25, tumor necrosis factor, interferons, etc. The combination of *Cordyceps sinensis* with persicae semen (peach seed) has been used to treat severe post-hepatitis cirrhosis by decreasing levels of IgG and IgA, increasing cell-lymphocyte rosette rate, enhancing the efficiency of natural killer cells, and increasing the $CD4^+/CD8^+$ cell ratio. In addition, *Cordyceps sinensis* has been shown to increase $CD4^+/CD8^+$ ratios and reduce the amount of viral proteins expressed in patients chronically infected with the hepadnavirus, hepatitis B.

In view of these potential therapeutic uses for *Cordyceps sinensis*, a need exists for a consistent and plentiful supply of highly bioactive material. Conventional cultivation methods for *Cordyceps sinensis* include solid media stationary incubation, liquid media rotating shaking incubation, liquid-state fermentation, and submerged liquid-state fermentation. However, current methods for producing *Cordyceps sinensis* fermentation products require substantial time and labor, and generally produce low yields of product with poor bioactivity.

For example, U.S. application Ser. No. 11/450,747 and International Application No. PCT/CN98/00258, which are incorporated herein by reference, disclose liquid culture methods for producing *Cordyceps sinensis* fermentation products. The method disclosed in the '747 application requires the following separate steps:

1. Isolating new fungal strains from nature;
2. Selecting fungal strains that are capable of developing stromata;
3. Plate-culturing on solid media for more than 10 generations, at least 5 generations of which are grown under low temperatures of 0-10° C.;
4. Performing a second cultivation in liquid culture media at a temperature of 12° C. on a rocking device for 12 days; and
5. Fermenting at a temperature of 12° C. in a starter vat for 10 days and then expanding the volume of culture media 10-fold until the needed quantity is reached.

The media used in the third step of the method disclosed in the '747 application contains beef tea, lactalbumin hydrolysate, yeast powder, glucose, milk, nucleic acid, magnesium sulfate, sodium dihydrogen phosphate, and vitamins. The media used in the fourth and fifth steps of the method disclosed in the '747 application contains silk worm chrysalis powder, protein pep-

tone, corn flour, wheat gluten, glucose, magnesium sulfate, and dipotassium hydrogen phosphate.

The method disclosed in PCT/CN98/00258 also requires five separate culture steps involving:

1. Culturing a slant strain for 5-6 days;
2. Culturing a primary seed culture for 2-4 days;
3. Culturing a secondary seed culture for 2-4 days;
4. Culturing a scaled up seed culture for 3 days; and
5. Culturing a large scale fermentation culture for 5-6 days.

The media used in PCT/CN98/00258 contains glucose, sucrose, peptone, bran, KH_2PO_4, and $MgSO_4$.

From start to finish, large-scale fermentations according to these methods require long periods of time and generally do not yield highly bioactive product. The present invention improves upon these current large scale fermentation methods for manufacturing *Cordyceps sinensis* fungal mycelia, thereby satisfying longstanding needs in the art.

B. Hepatitis C Virus

Hepatitis C virus (HCV), also known as "non-A non-B hepatitis," is a contagious blood-borne virus. HCV is a member of the Flaviviridae family of viruses and comprises a single-stranded, positive-sense RNA genome that encodes a long polyprotein precursor of about 3,000 amino acids, which is processed by both cellular and viral proteases to yield at least 10 individual proteins. An estimated 150 to 200 million people worldwide are infected with hepatitis C, including nearly 4 million in the United States. The virus is cleared spontaneously in less than 20% of HCV-infected individuals, and in the majority of patients, the virus persists and causes chronic hepatitis that may lead to end-stage liver diseases, cancer, or even death.

Hepatitis C viruses have a high level of genetic heterogeneity and thus have been grouped by their degree of sequence identity into six separate genotypes (types 1 through 6), and further divided into numerous subtypes. Geographic distribution and responses to therapeutic treatments differ between genotypes. Genotypes 1a and 1b are the most prevalent in the United States and Western Europe, followed by genotypes 2 and 3. Among hepatitis C patients in northern Taiwan, approximately 58% to 73% are infected with genotype 1b and approximately 7.4% to 16.5% are infected with genotype 2a, while in southern Taiwan, around 48% to 64.3% are infected with genotype 1b and around 35% to 41% are infected with genotype 2a.

Hepatitis C therapy traditionally includes treatment with interferons, such as interferon-α 2A (ROFERON-A, Roche). Current hepatitis C therapy uses a combination of pegylated interferon-α, for example, PEGINTRON (Schering-Plough) or PEGASYS (Roche), and the guanosine analog, ribavirin, for example, REBETOL (Schering-Plough) or COPEGUS (Roche). The current standard therapy for treating chronic hepatitis C is a combination of weekly injections of pegylated interferon (IFN)-α 2A and daily oral doses of ribavirin for a period of 24 or 48 weeks. Both drugs are indirect antivirals because they do not target a specific HCV protein or genome element. A sustained viral response (SVR), which is defined as patients remaining HCV-free (undetectable levels of virus) for 6 months after the termination of therapy, is achieved in only half of the treated patients and in less than half of patients with high levels of virus. The SVR is higher in patients infected with genotypes 2 and 3, but much lower in patients infected with genotypes 1 and 4 through 6. In addition, the standard therapy is associated with considerable adverse effects, including: depression, fatigue, and "flu-like" symptoms such as fever, headache, muscle soreness, and nausea, caused by IFN-α; and hemolytic anemia, cough, rash, and insomnia, caused by ribavirin. These symptoms tend to disappear after two to three weeks, but then reappear, often with new side effects, at the end stage of therapy. Such late-stage adverse effects Include: severe fatigue and muscle soreness, leucopenia, anemia, dysphoria, weight loss, and hair loss.

Due to the side effects, the cost, and the inefficiency of the current standard therapy, there is a large unmet need for safe, effective HCV therapies. The instant invention solves some of the problems associated with current HCV therapy, thereby satisfying longstanding needs in the art.

SUMMARY OF THE INVENTION

The present invention relates to novel and non-obvious methods and media for preparing fermentation products of *Cordyceps sinensis*. The invention also relates to novel and non-obvious compositions comprising *Cordyceps sinensis* fermentation products and methods for treating various diseases or disorders by administering an effective amount of such compositions.

In one embodiment, the invention relates to a novel and non-obvious nutrient medium comprising:

- about 1% to about 5% (w/w) of at least one carbon source;
- about 0.1% to about 3% (w/w) of at least one nitrogen source;
- about 0.05% to about 1% of at least one inorganic salt; and
- about 0.01% to about 0.2% (w/w) manganese, and/or about 0.001% to about 0.01% (w/w) copper, and/or about 0.01% to about 0.2% (w/w) iron, and/or about 0.02% to about 0.2% cobalt, and/or about 0.05% to about 0.5% (w/w) calcium, and/or about 0.0003% to about 0.003% (w/w) selenium;
- wherein the pH of the medium is about 5 to about 7.

In some embodiments the carbon source is about 1% to about 5% (w/w) sucrose and/or about 1% to about 5% (w/w) glucose. In other embodiments, the nitrogen source is about 0.2% to about 1.2% (w/w) yeast extract, and/or about 1.5% to about 3% (w/w) soy bean powder, and/or about 0.1% to about 0.35% (w/w) $(NH_4)_2HPO_4$. In other embodiments, the inorganic salt is about 0.1% to about 0.15% (w/w) KH_2PO_4, and/or about 0.5% to about 1% (w/w) $MgSO_4.7H_2O$, and/or about 0.1% to about 0.25% (w/w) K_2HPO_4, and/or about 0.05% to about 0.06% (w/w) KCl.

In another embodiment, the invention relates to a novel and non-obvious method for producing a *Cordyceps sinensis* fermentation product comprising:

a. inoculating a solid nutrient medium (i.e., a plate culture) with at least one strain of *Cordyceps sinensis* and incubating at about 18° C. to about 28° C. for about 4 to 8 days;
b. inoculating a flask comprising a first liquid nutrient medium (i.e., a seed culture) with at least a portion of the culture from (a) and incubating at about 18° C. to about 28° C. for about 2 to 4 days;
c. inoculating a second flask comprising liquid nutrient medium (i.e., a second, or scaled up seed culture) with at least a portion of the culture from (b) and incubating at about 18° C. to about 28° C. for about 2 to 3 days;
d. inoculating a vat chamber comprising liquid nutrient medium (i.e., a fermentation culture) with at least a portion of the culture from (c) and incubating at about 18° C. to about 28° C. for about 1 to 3 days; and
e. recovering the *Cordyceps sinensis* fermentation product.

In one embodiment, the *Cordyceps sinensis* is *Paecillomyces hepiali.*

In another embodiment, the invention relates to a novel and non-obvious method for producing a *Cordyceps sinensis* fermentation product comprising:

a. inoculating a solid nutrient medium with at least one strain of *Cordyceps sinensis* and incubating at about 18° C. to about 28° C. for about 4 to 8 days;
b. inoculating a flask comprising a first liquid nutrient medium (i.e., a seed culture) with at least a portion of the culture from (a) and incubating at about 18° C. to about 28° C. for about 2 to 4 days;
c. inoculating a second flask comprising liquid nutrient medium (i.e., a second, or scaled up seed culture) with at least a portion of the culture from (b) and incubating at about 18° C. to about 28° C. for about 2 to 3 days;
d. inoculating a vat chamber comprising liquid nutrient medium (i.e., a fermentation culture) with at least a portion of the culture from (c) and incubating at about 18° C. to about 28° C. for about 3 to 7 days; and
e. recovering the *Cordyceps sinensis* fermentation product;

wherein the method does not comprise additional culture steps between steps (a) and (b), (b) and (c), and/or (c) and (d).

In one embodiment, the *Cordyceps sinensis* is *Hirsutella sinensis*.

The invention also relates to novel and non-obvious adjuvant compositions comprising a *Cordyceps sinensis* fermentation product. In some embodiments, the *Cordyceps sinensis* fermentation products used in the compositions of the invention are produced by the methods of the invention. In other embodiments, the *Cordyceps sinensis* fermentation products are obtained from other sources.

In some embodiments, the adjuvant compositions of the invention further comprise extracts from *Astragalus membranaceus*. In some embodiments, the adjuvant compositions of the invention further comprise zinc. In some embodiments, the adjuvant compositions comprise about 50% to about 90% (w/w) of a *Cordyceps sinensis* fermentation product, about 10% to about 50% (w/w) of *Astragalus membranaceus* extract, and/or about 5% to about 10% (w/w) zinc.

In some embodiments, the adjuvant compositions of the invention further comprise a conventional hepatitis C therapy. In some embodiments the conventional hepatitis C therapy comprises an interferon and a guanosine analog. In other embodiments, the interferon is interferon-α and the guanosine analog is ribavirin. In other embodiments, the interferon-α is pegylated.

The invention also relates to methods for treating patients infected with or who may have been exposed to hepatitis C comprising administering an effective amount of a composition comprising a conventional hepatitis C therapy and an adjuvant composition of the invention. In other embodiments, the invention relates to methods for treating patients infected with or who may have been exposed to hepatitis C comprising administering an effective amount of an adjuvant composition of the invention, and separately administering an effective amount of at least one conventional hepatitis C therapy. In some embodiments, the adjuvant compositions of the invention and conventional hepatitis C therapies are administered simultaneously. In other embodiments, the adjuvant compositions of the invention and conventional hepatitis C therapies are administered sequentially. In other embodiments, the adjuvant compositions of the invention and conventional hepatitis C therapies are administered spaced out over a period of time.

In some embodiments, the adjuvant compositions of the invention increase the effect and reduce the negative side effects of conventional hepatitis C therapies. In other embodiments, the adjuvant compositions of the invention increase the effect of conventional hepatitis C therapies by at least two-fold.

The invention also relates to methods for treating patients infected with or who may have been exposed to hepatitis B, comprising administering a therapeutically effective amount of an adjuvant composition of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Schematic representation of one embodiment of the method of the invention for *Paecilomyces hepiali* fermentation.

FIG. **2**. Results from treating patients infected with HCV genotype 1b or 2a and 2b with pegylated-interferon-α at 180 mcg/vial weekly for 24 weeks in combination with ribavirin at 1000 (if body weight was <75 kg) or 1200 mg (if body weight was ≥75 kg) daily for 24 weeks, with or without adjuvant agent comprising *Paecilomyces hepiali* mycelia and *Astragalus membranaceus* extract. The data are presented as the percentage of patients showing a sustained virus response (SVR), defined as undetectable HCV RNA virus at 6 months after the end of conventional treatment with interferon and ribavirin.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

In the context of the present invention the following terms are to be understood as follows:

The term "*Cordyceps sinensis* fermentation product" as used herein refers to the product of a process of mycelial fermentation of one or more laboratory isolates of *Cordyceps sinensis*, including but not limited to: *Cephalosporium maydis, Cephalosporium sinensis* (also called *Cephalosporium dongchongxiacao*), *Chrysosporium sinense, Hirstuella hepialid, Hirsutella sinensis, Mortierella hepiali, Paecilomyces hepiali, Paecilomyces sinensis, Paecilomyces variotii, Scytalidium hepiali, Sporothrix insectorum, Stachybotrys chartarum, Stachybotrys clindrospora, Stachybotrys dichroa, Tolypocladium calcdonica, Tolypocladimn sinensis*, and *Verticillium bulbillosum*. The fermentation products of the invention are generally a light yellow to dark brown powder with a slightly bitter taste and aromatic odor.

The term "*Paecilomyces hepiali*" as used herein refers to the Chen et Dai strain of the *Cordyceps sinensis* fungus. Morphologically, when cultivated on potato dextrose agar (PDA) at 26° C. for about one month, the strain produces synnemata either singly or in clusters from the loose cottony aerial mycelia. The synnemata is white in color and turns to yellow upon aging. The aerial mycelia are yellow to orange in color. Under the microscope, the branching mycelia is transparent, septated, and about 2.5 to 4 μm in diameter. The conidiospores are arranged singly, alternately, or oppositely. Some flask-shaped conidiospores are arranged as a simple brush-like structure. The conidia on the conidiospores are near spherical in shape, smooth, and arranged in a single chain. In liquid culture grown under continuous agitation, the conidia may have a spherical or elliptical shape.

The term "*Astragalus membranaceus*" as used herein refers to the perennial leguminosae herbaceous plant also known as milk-vetch root. The plant is mainly cultivated in southern China, and the root has been used to prepare extracts for several therapeutic purposes. Numerous bioactive constituents of *Astragalus membranaceus* have been discovered, including polysaccharides, monosaccharides, flavonoids,

alkaloids (such as choline and betaine), amino acids, glucuronic acid, folic acid, metallic elements (such as selenium), and diatomite materials.

The phrases "therapeutically effective amount" and "effective amount" as used herein mean an amount sufficient to provide a benefit in the treatment of a disease, or in the improvement of one's general health and sense of well-being. Therapeutically effective amounts will vary depending on the patient and symptoms being treated. In general, a therapeutically effective amount of *Cordyceps sinensis* fermentation product is about 0.1 g to about 30 g administered in single or divided daily doses. In some embodiments, the dose is about 1 g to about 10 g. In other embodiments, the dose is about 2 g to about 5 g. In other embodiments, the dose is 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, or 4.5 g. In general, a therapeutically effective amount of *Astragalus membranaceus* extract is about 0.1 g to about 30 g administered in single or divided daily doses. In some embodiments, the dose is about 0.1 g to about 5 g. In other embodiments, the dose is about 3 g to about 7 g. In other embodiments, the dose is 1.0, 1.1, 1.2, 1.3, 1.4, or 1.5 g.

As used herein, the terms "mammal" and "patient" include, but are not limited to, domestic pets, farm animals and livestock, and humans.

As used herein, the terms "sustained viral response" and "SVR" are defined as patients remaining HCV-free (undetectable levels of virus) for 6 months after the termination of therapy.

As used herein, the term "interferon" includes recombinant and non-recombinant forms of interferon-α, as well as pegylated and non-pegylated forms of interferon-α.

As used herein, the term "conventional therapy" with respect to Hepatitis C refers to therapies comprising an interferon and a guanosine analog.

As used herein, the term "slant culture" refers to any culture made on the slanting surface of solid medium that has been solidified in a tube that has been inclined from the perpendicular in order to provide a greater surface area for growth.

As used herein, the term "seed culture" refers to any liquid culture incubated in a flask using a rocking device (shaker) before being transferred into a fermentor (vat) chamber.

As used herein, the term "fermentor culture" refers to any liquid culture incubated in a vat chamber used for growing large amounts of mycelia.

As used herein, the term "flask" refers to any container for holding a liquid of a volume at or below 2000 ml.

As used herein, the term "vat chamber" or "fementor chamber" refers to any container for holding a liquid of a volume at or greater than 10 L. Vat/fementor chambers often contain a temperature control system (such as a heater and/or cooler system) to provide a constant temperature, and an agitator for aeration.

As used herein, the term "about" with respect to any numerical value or range of values means±10%. All numerical values and ranges recited herein are to be understood as being modified by the term "about" even where not expressly stated.

B. Methods and Media for Producing *Cordyceps sinensis* Fermentation Products

The invention provides novel and non-obvious methods and media for making *Cordyceps sinensis* fermentation products. The methods and media of the invention produce greater yields of *Cordyceps sinensis* fermentation products having improved potency in a time span much shorter than prior art methods. For example, the methods of the invention reduce the time required for fermentation by about 50% to about 90% compared to some prior art methods, and the resulting mycelia contain about 30% to about 80% more active ingredients than mycelia produced by some prior art methods. In addition, the methods and media of the invention eliminate the need for multiple seed cultures, substantially reducing the time and labor involved in producing *Cordyceps sinensis* mycelia compared to some prior art methods.

1. Nutrient Media

The novel and non-obvious nutrient media used in the methods of the invention may be solid and/or liquid. The media may comprise sources of carbon, nitrogen, inorganic salts, and trace elements. The media may further comprise any additional nutritional material that supports the growth, reproduction, fermentation catabolism, and anabolism of *Cordyceps sinensis* mycelia. The composition of the fermentation media of the invention may be varied depending on the strain of *Cordyceps sinensis* used.

Sources of carbon for the nutrient media of the invention may include carbohydrates such as sugars, for example, glucose, fructose, dextrose, maltose, mannitol, sucrose, xylose, and the like, and/or starches such as grains, for example, cornstarch, corn meal, oats, ryes, wheat germ, and the like. In general, the amount of carbohydrate usually varies from about 0.5% to about 10% by weight and often from about 1% to about 5%. The carbon sources may be used individually, or several carbon sources may be combined in the media. For example, in some embodiments, the media may contain about 1% to about 5% of sucrose and/or about 1% to about 5% of glucose. In other embodiments, the media may contain about 1.5% to about 2.5% of sucrose and/or about 1.5% to about 2.5% of glucose. In some embodiments, the media may contain 1.5%, 2%, 2.5%, 3%, 3.5%, or 4.5% of sucrose and/or 1.5%, 2%, 2.5%, 3%, 3.5%, or 4.5% of glucose.

Nitrogen sources for the nutrient media of the invention may include, for example, ammonium nitrate, ammonium sulfate, corn steep liquor, cottonseed flour, diammonium hydrogen phosphate, distiller's solubles, fish meal, hydrolysates of casein, meat extract, peanut meal, peptone, primary yeast, rice bran, sodium nitrate, soybean cake, soybean meal, soybean powder, tomato paste, yeast hydrolysates, and the like. In general, the amount of nitrogen source usually varies from about 0.05% to about 10% by weight and often from about 0.1% to about 5%. The nitrogen sources may be used individually, or several nitrogen sources may be combined in the media. For example, in some embodiments, the media contains about 0.1% to about 5% yeast powder and/or about 1% to about 5% soy bean powder and/or about 0.01% to about 0.5% diammonium hydrogen phosphate ($(NH_4)_2HPO_4$). In other embodiments, the media contains about 0.2% to about 1.2% yeast powder and/or about 1.5% to about 3% soy bean powder and/or about 0.01% to about 0.035% $(NH_4)_2HPO_4$. In some embodiments, the media contains 0.35% yeast powder and/or other nitrogen sources. In other embodiments, the media contains 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, or 1.2% yeast powder and/or 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, or 3% soy bean powder and/or 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, or 0.035% $(NH_4)_2HPO_4$, and/or other nitrogen sources.

Among the inorganic salts which may be used in the nutrient media of the invention are the customary salts capable of yielding ammonium, calcium, carbonate, chloride, magnesium, potassium, phosphate, sodium, sulfate, and like ions. Exemplary inorganic salts include KH_2PO_4, $MgSO_4.7H_2O$, K_2HPO_4, and KCl. In general, the amount of inorganic salt usually varies from about 0.01% to about 1% % by weight and

often from about 0.1% to about 0.5%. The inorganic salts may be used individually, or several inorganic salts may be combined in the media. For example, in some embodiments, the media contains about 0.01% to about 0.5% KH_2PO_4 and/or about 0.01% to about 1% $MgSO_4.7H_2O$ and/or about 0.02% to about 0.5% K_2HPO_4 and/or about 0.01% to about 0.5% KCl. In other embodiments, the media contains about 0.1% to about 0.15% KH_2PO_4 and/or about 0.05% to about 0.6% $MgSO_4.7H_2O$ and/or about 0.1% to about 0.25% K_2HPO_4 and/or about 0.05% to about 0.06% KCl. In other embodiments, the media contains 0.1%, 0.11%, 0.12%, 0.13%, 0.14%, or 0.15% KH_2PO_4 and/or 0.05%, 0.15%, 0.25%, 0.35%, 0.5%, 0.75%, or 1%, $MgSO_4.7H_2O$ and/or 0.1%, 0.13%, 0.2%, 0.23%, or 0.25% K_2HPO_4 and/or 0.05%, to 0.052%, 0.054%, 0.056%, 0.058%, or 0.060% KCl.

Among the trace elements which may be used in the nutrient media of the invention are barium, cadmium, calcium, chromium, cobalt, copper, iron, lead, manganese, molybdenum, nickel, selenium, strontium, zinc, etc. Sources of such trace elements may include, for example, $MnSO_4$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CoSO_4.7H_2O$, $CaCl_2$, KH_2PO_4, $MgSO_4$, Na_2SeO_3, etc. In general, the amount of trace element usually varies from about 0.0001% to about 5% of the media and often from about 0.001% to about 0.5%. The trace elements may be used individually, or several trace elements may be combined in the media. For example, in some embodiments, the media contains about 0.01% to about 0.2% manganese and/or about 0.001% to about 0.01% copper and/or about 0.01% to about 0.2% iron and/or about 0.02% to about 0.2% cobalt and/or about 0.05% to about 0.5% calcium and/or about 0.0003% to about 0.003% selenium. In other embodiments, the media contains 0.02%, 0.03%, 0.04%, or 0.05% manganese (e.g., $MnSO_4$) and/or 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, or 0.006% copper (e.g., $CuSO_4.5H_2O$) and/or 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, or 0.1% iron (e.g., $FeSO_4.7H_2O$) and/or 0.02%, 0.05%, 0.1%, or 0.2% cobalt (e.g., $CoSO_4.7H_2O$) and/or 0.07%, 0.1%, 0.12%, or 0.15% calcium (e.g., $CaCl_2$) and/or 0.0003%, 0.0006%, 0.001%, or 0.0026% selenium (e.g., Na_2SeO_3).

The pH of the nutrient media of the invention may vary from about 5.0 to about 7.0. In some embodiments, the pH is within the range of about 6.5 to about 6.7. In some embodiments, the pH is 6.5, 6.6, or 6.7.

2. Fermentation Methods

The media compositions described above may be used in the novel and non-obvious fermentation methods of the invention. The methods of the invention may be carried out using either a solid media or a liquid media, or both. Various modifications of the nutrient media and methods disclosed herein may be made by those skilled in the art, in view of practical and economic considerations, such as the scale of fermentation and local supply of components.

The fermentation method of the invention may be carried out at temperatures ranging from about 18° C. to about 37° C. In some embodiments, the temperatures range from about 18° C. to about 22° C. or from about 24° C. to about 28° C. In some embodiments, the temperatures are 18° C., 20° C., 22° C., 24° C., 26° C., or 28° C.

The fermentation method of the invention may be performed by surface or submerged culture, or a combination of both. In some embodiments, the fermentation is carried out in liquid culture with aeration and agitation. In some embodiments, an anti-foaming agent, such as silicon oil, vegetable oil (e.g., 0.01% soybean oil), or other surfactants, may be added to the liquid cultures of the invention. In some embodiments, the liquid cultures of the invention may be agitated at speeds of about 30 rpm to 130 rpm, and ventilation rates of about 200 to 800 m^3/h. In some embodiments, the liquid cultures of the invention may be agitated at speeds of 50, 75, 85, or 100 rpm, and ventilation rates of 300, 500, 650, or 750 m^3/h.

The fermentation method of the invention may be initiated in a sterilized flask of media from a slant culture, plate culture, or other source. If a plate culture is used, the culture may be initiated by streaking, spreading, or otherwise plating fungus from a slant culture or other source. The plate culture may be incubated at about 18° C. to about 28° C. for about 4 to 8 days, or until the growth of the fungus is satisfactory, before initiating a liquid seed culture. In some embodiments, the plate culture may be incubated at 18° C. for 4, 5, 6, 7, or 8 days, at 22° C. for 4, 5, 6, 7, or 8 days, at 24° C. for 4, 5, 6, 7, or 8 days, at 26° C. for 4, 5, 6, 7, or 8 days, or at 28° C. for 4, 5, 6, 7, or 8 days. An isolated fungal strain may be used to inoculate the first seed culture, or a mixture of fungal strains may be used.

The method of the invention may involve one or more seed cultures before initiating a large-scale fermentation culture. In some embodiments, the method of the invention involves a single seed culture and a scaled up seed culture. In some embodiments, the seed culture is grown at a temperature of about 18° C. to about 28° C. for about 2 to 10 days, depending on the strain of *Cordyceps sinensis* used, or until growth of the fungus is satisfactory. In some embodiments, the seed culture is incubated at about 18° C. to about 28° C. for about 2 to 4 days. In some embodiments, the seed culture is grown at 18° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, at 22° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, at 24° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, at 24° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, or at 28° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days.

The seed culture may then be scaled up, and the scaled up seed culture may be incubated at about 18° C. to about 28° C. for about 2 to 10 days, depending on the strain of *Cordyceps sinensis* used, or until the growth of fungus is satisfactory. In some embodiments, the scaled up seed culture is incubated at about 18° C. to about 28° C. for about 2 to 3 days. In some embodiments, the scaled up seed culture is grown at 18° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, at 22° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, at 24° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days, or at 28° C. for 2, 3, 4, 5, 6, 7, 8, 9, or 10 days. The scaled up seed culture may then be used to initiate the large-scale fermentation step.

In some embodiments, the seed culture steps may be repeated until there is a sufficient amount of fungus to start the large scale fermentation. Such intermediate stage seed cultures, when used, are developed in essentially the same manner as described above. That is, part of the contents of the flask from the previous seed culture is used to inoculate the medium of the next seed culture and the seed cultures are incubated at about 18° C. to about 28° C. for about 2 to 10 days. However, an advantage of the method of the invention is that it produces large quantities of highly active fungal mycelia using only a single seed culture and scaled up seed culture, as opposed to some prior art methods, which require at least a primary, secondary, and scaled up seed culture before inoculating the large-scale fermentation.

The conditions of the large-scale fermentation step will depend on the particular *Cordyceps sinensis* strain used in the fermentation. For example, if *Paecilomyces hepiali* is used, the large-scale fermentation step may involve incubating the fungus at about 18° C. to about 28° C., 20° C. to 26° C., or 22° C. to 24° C. for any of about 0.5 to 10 days, 1 to 5 days, or 1.5 to 2.5 days. In some embodiments, the large-scale fermentation of *Paecilomyces hepiali* is incubated at 22° C. for 1.8 days, at 24° C. for 1.8 days, at 26° C. for 1.8 days, at 22° C. for

2 days, at 24° C. for 2 days, at 26° C. for 2 days, at 22° C. for 2.3 days, at 24° C. for 2.3 days, or at 26° C. for 2.3 days. Alternatively, if *Hirsutella sinensis* is used, the large-scale fermentation step may involve incubating the fungus at about 18° C. to about 28° C., 20° C. to 26° C., or 22° C. to 24° C. for any of about 1 to 10 days, 3 to 8 days, or 3.5 to 7.5 days. In some embodiments, the large-scale fermentation of *Hirsutella sinensis* is incubated at 18° C. for 3.5 days, at 18° C. for 4.5 days, at 18° C. for 5.5 days, at 18° C. for 6.5 days, at 18° C. for 7.5 days, at 20° C. for 3.5 days, at 20° C. for 4.5 days, at 20° C. for 5.5 days, at 20° C. for 6.5 days, at 20° C. for 7.5 days, at 22° C. for 3.5 days, at 22° C. for 4.5 days, at 22° C. for 5.5 days, at 22° C. for 6.5 days, or at 22° C. for 7.5 days. Alternatively, if *Paecilomyces sinensis* is used, the large scale fermentation step may involve incubating the fungus at about 18° C. to about 28° C., 20° C. to 26° C., or 22° C. to 24° C. for any of about 0.5 to 10 days, 1 to 5 days, or 1.5 to 2.5 days. In some embodiments, the large-scale fermentation of *Paecilomyces sinensis* is incubated at 22° C. for 1.7 days, at 24° C. for 1.8 days, at 26° C. for 1.9 days, at 22° C. for 1.9 days, at 24° C. for 2 days, at 26° C. for 2.1 days, at 22° C. for 2.3 days, at 24° C. for 2.4 days, or at 26° C. for 2.5 days. The resulting mycelia may then be harvested, freeze dried, milled, and/or filtered for use in the formulations of the invention.

In some embodiments, the large scale fermentation may be allowed to continue until a sufficient amount of mycelia is produced. However, an advantage of the method of the invention is that it produces large quantities of highly active fungal mycelia in substantially shorter fermentation times than prior art methods. For example, if *Paecilomyces hepiali* is used, the large scale fermentation may take only 1.5 to 2.3 days, as opposed to prior art methods, which typically require at least 5 to 7 days of large scale fermentation. Similarly, if *Hirsutella sinensis* is used, the large scale fermentation may take only 3 to 8 days, as opposed to prior art methods, which typically require at least 35 to 60 days of large scale fermentation.

The large scale fermentation may be conducted in a suitable container, such as, for example, a stirred tank reactor with means for maintaining a constant temperature, sterilizing the fermentation media and the container, and aerating the fermentation media. In some embodiments, the culture media of the invention is made up in the reactor container and sterilized by heating at temperatures above about 120° C. Upon cooling the container and media to about 18° C. to about 28° C., the sterilized media is inoculated with all or part of the scaled up seed culture and the fermentation is permitted to proceed for a period of time until a sufficient amount of mycelia is produced, while aerating the media.

At the end of the fermentation process, the *Cordyceps sinensis* fermentation products may be isolated and recovered from the culture by any suitable methods known in the art. For example, in one embodiment, the fermentation broth may be drained and discarded, and the solid residue comprising the fungal mycelia may be sterilized by heat, for example, by high pressure steam, and then dried and crushed into a powder. In another embodiment, the mycelia may be freeze dried for about 48 hours and then milled. The resulting powder may be used directly in the various compositions and formulations provided by the present invention. Alternatively, the powder may be further processed, for example, by extraction with organic solvents, such as, 75% ethanol. After evaporation to dryness, the extract may then be used in the various compositions and formulations of the present invention. Alternatively, the organic solvent-extracted fermentation product may be further extracted with water, hot water, organic solvents such as, for example, ether or ethyl acetate, or polar solvents such as, for example, acetone or alcohol. Residual impurities may be removed with solvents such as, for example, petroleum ether or hexane, by adsorptive chromatography with active carbon or silica gel, or by gel filtration through, for example, a SEPHADEX column (Pharmacia). Other methods for removing impurities are known in the art.

Accordingly, in one embodiment, the fermentation method of the invention comprises:

a. inoculating a solid nutrient medium (i.e. a plate culture) with at least one strain of *Cordyceps sinensis* and incubating at about 18° C. to about 28° C. for about 4 to 8 days;

b. inoculating a first liquid nutrient medium (i.e. a seed culture) with at least a portion of the culture from (a) and incubating at about 18° C. to about 28° C. for about 2 to 4 days;

c. inoculating a second liquid nutrient medium (i.e. a scaled up seed culture) with at least a portion of the culture from (b) and incubating at about 18° C. to about 28° C. for about 2 to 3 days;

d. inoculating a third liquid nutrient medium (i.e. a large-scale fermentation culture) with at least a portion of the culture from (c) and incubating at about 18° C. to about 28° C. for about 1 to 3 days or for about 3 to 7 days; and

e. recovering the *Cordyceps sinensis* fermentation product.

An exemplary process of the fermentation method of the invention using *Paecilomyces hepiali* is depicted in FIG. **1**.

C. Uses for *Cordyceps sinensis* Fermentation Products

The *Cordyceps sinensis* fermentation products produced by the methods and media of the invention, or obtained by other sources, may be used for treating a mammal afflicted with a variety of diseases, disorders, ailments, and/or symptoms. The fermentation products of the invention may also be used for treating a mammal that may have been exposed to a variety of diseases, disorders, ailments, and/or symptoms.

1. Uses and Formulations for *Cordyceps sinensis* Fermentation Products

The inventors have discovered that *Cordyceps sinensis* fermentation products produced using the methods and media of the invention have increased bioactivities compared to *Cordyceps sinensis* fermentation products produced by other methods. Thus, the *Cordyceps sinensis* fermentation products produced using the method and media of the invention may be used in any therapeutic method known in the art involving *Cordyceps sinensis* fermentation products. The fermentation products, or extracts thereof, may be used in pharmaceutical and dietary compositions containing one or more other components or biologically active agents, such as adjuvants, pharmaceutically acceptable surfactants, excipients, carriers, diluents, vehicles, binding and filling agents, lubricants, disintegrants, wetting agents, suspending agents, emulsifying agents, preservatives, and/or other suitable ingredients. If desired, sweetening, flavoring and/or coloring agents may also be added.

Examples of adjuvants include, for example, alum, MPL, and QS21. Examples of surfactants include, for example, glycerides. Examples of suitable excipients include, but are not limited to, lactose. Examples of binding and filling agents may include, for example, pregelatinized maize starch, polyvinylpyrrolidone, hydroxypropyl methylcellulose, lactose, pentosan, and microcrystalline cellulose or calcium hydrogen phosphate. Examples of lubricants may include, for example, magnesium stearate, talc, or silica. Examples of disintegrants may include, for example, potato starch or sodium starch glycolate. Examples of wetting agents may include, for

example, sodium lauryl sulfate. Examples of suspending agents may include, for example, sorbitol syrup, cellulose derivatives, or hydrogenated edible fats. Examples of emulsifying agents may include, for example, lecithin, acacia, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils. Examples of preservatives may include, for example, methyl or propyl-p-hydroxybenzoates or sorbic acid. Examples of other suitable ingredients may include, but are not limited to, vitamins, antioxidants, amino acids, metal salts, minerals, meat extracts, vegetable extracts, and flavor enhancers.

Pharmaceutical compositions comprising the *Cordyceps sinensis* fermentation products of the invention may be formulated in any dosage form including, for example, powders, granules, capsules, cachets, tablets, dispersions, aqueous or non-aqueous solutions or suspensions, and oil-in water or water-in-oil emulsions. The compositions may be formulated as discrete unit dosage forms with each dose containing a predetermined amount of *Cordyceps sinensis* fermentation product. In some embodiments, the compositions are prepared by uniformly and intimately admixing the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation. In some embodiments, the formulations are present as a dry product for constitution with water or other suitable vehicle before use. Any methods known to those skilled in the art may be used to formulate and administer the compositions of the invention.

Suitable routes of administration for the pharmaceutical compositions of the invention may include intradermal, transdermal (e.g., slow release polymers), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural, and intranasal routes. Any other convenient route of administration can be used including, for example, infusion or bolus injection, or absorption through epithelial or mucocutaneous linings. In some embodiments, the compositions may be added directly to foods and ingested during normal meals.

2. Formulations and Uses of *Cordyceps sinensis* Fermentation Products for Treating Hepatitis C Infection or Exposure

Hepatitis C therapy traditionally includes treatment with interferons, such as interferon-α2A (ROFERON-A, Roche). Current hepatitis C therapy uses a combination of pegylated interferon-α, for example, PEGINTRON (Schering-Plough) or PEGASYS (Roche), and the guanosine analog, ribavirin, for example, REBETOL (Schering-Plough) or COPEGUS (Roche). However, this conventional combination therapy is not fully effective (with only approximately 55% of patients showing a sustained virological response) and its frequent side-effects reduce health-related quality of life in many patients.

The inventors have discovered that *Cordyceps sinensis* fermentation products have a potent adjuvant effect for enhancing the effectiveness of conventional hepatitis C therapies. In one embodiment, the invention relates to adjuvant compositions comprising an effective amount of a *Cordyceps sinensis* fermentation product. The *Cordyceps sinensis* fermentation product used in the adjuvant compositions of the invention may be obtained from any suitable source, or may be produced using the fermentation methods and media described above.

In some embodiments, the *Cordyceps sinensis* fermentation products used in the adjuvant compositions of the invention are from *Paecilomyces hepiali*. In other embodiments, the *Cordyceps sinensis* fermentation products are from *Hirsutella sinensis*. In some embodiments, the adjuvant compositions of the invention further comprise an effective amount of *Astragalus membranaceus* root extract. In some embodiments, the adjuvant compositions of the invention further comprise an effective amount of zinc.

In some embodiments, the adjuvant agent comprises about 50% to about 90% (w/w) of *Cordyceps sinensis* fermentation product, about 10% to about 50% (w/w) of *Astragalus membranaceus* root extract, and/or about 5 to about 10% (w/w) zinc. In some embodiments, the adjuvant agent comprises about 70% to about 80% (w/w) of *Cordyceps sinensis* fermentation product, about 10% to about 30% (w/w) of *Astragalus membranaceus* root extract, and/or about 5% to about 10% (w/w) zinc. In some embodiments, the adjuvant agent comprises about 70% to about 80% (w/w) of *Cordyceps sinensis* fermentation product, about 10% to about 20% (w/w) of *Astragalus membranaceus* root extract, and/or about 5% to about 10% (w/w) zinc. In some embodiments, the adjuvant agent comprises 80% (w/w) of *Cordyceps sinensis* fermentation product, 18% (w/w) of *Astragalus membranaceus* root extract, and 2% (w/w) zinc. In some embodiments, the adjuvant agent comprises 70% (w/w) of *Cordyceps sinensis* fermentation product, 28% (w/w) of *Astragalus membranaceus* root extract, and 2% (w/w) zinc. The adjuvant compositions of the invention may be formulated and administered by any of the methods discussed above.

In other embodiments, the invention relates to methods of treating patients who are infected with hepatitis C and/or patients who may have been exposed to hepatitis C comprising administering to the patient a conventional hepatitis C therapy and an effective amount of an adjuvant composition of the invention. In some embodiments, the conventional hepatitis C therapy comprises an interferon or a pegylated interferon. In some embodiments, the conventional hepatitis C therapy further comprises a guanosine analog such as ribavirin. In some embodiments, the dosage of interferon may be about 50 to about 150 mcg per week and the dosage of ribavirin may be about 800 to about 1200 mg per day.

In some embodiments, the adjuvant compositions of the invention may be formulated in a separate dosage form from the conventional hepatitis C therapeutic agents. In some embodiments, the adjuvant compositions of the invention may be combined with the conventional hepatitis C therapeutic agents into a single dosage form. If the adjuvant agents of the invention and the conventional hepatitis C therapeutic agents are formulated in separate dosage forms, the separate dosage forms may be administered simultaneously, sequentially, or spaced out over a period of time.

The inventors have found that the adjuvant compositions of the invention increase the sustained virologic response attained with conventional hepatitis C therapy and reduce the number and type of adverse effects. This enhancing effect of the adjuvant agents of the invention was particularly pronounced in patients with cirrhosis. Without being bound by theory, it is believed that the interferon component of conventional therapy kills hepatitis C virus and the adjuvant agent serves to boost the immune system by increasing levels of IgG and IgA, and by increasing the $CD4^+/CD8^+$ cell ratio.

EXAMPLES

Reference will now be made in detail to specific embodiments of the invention. While the invention will be described in conjunction with these embodiments, it will be understood that the invention is not limited to those embodiments. On the contrary, the invention is intended to cover alternatives, modifications, and equivalents, which may be included within the invention as defined by the appended claims.

A. Adjuvant Preparation

1. *Hirsutella sinensis* Fermentation

The *Hirsutella sinensis* mycelia used in the experiments discussed below were produced by the following method.

The *Hirsutella sinensis* strain was purified from natural fresh *Cordyceps sinensis* obtained from Mainland China. The strain was plate-cultured for 30 days at 18° C. on solid medium containing the components discussed below for the seed culture media plus 2.0% agar.

A series of sequential liquid cultures and fermentations were then performed over a period of about 27 days.

1. The seed culture was prepared as follows:

Culture media (a total of 0.6 L in water):

sucrose	24 g
$(NH_4)_2HPO_4$	0.15 g
KH_2PO_4	0.6 g
K_2HPO_4	1.38 g
$MgSO_4 \cdot 7H_2O$	0.3 g
KCl	0.3 g
$MnSO_4$	0.6 g
$CuSO_4 5 \cdot H_2O$	0.0096 g
$FeSO_4 \cdot 7H_2O$	0.6 g
$CaCl_2$	0.9 g
$NaSeO_3$	0.0072 g

6 flasks (size 250 mL) were each filled with 100 mL culture media. The flasks containing the media were sterilized, cooled to 18° C., and adjusted to pH 6.5. Each flask was inoculated with *Hirsutella sinensis* strain from a plate culture. The inoculated cultures were incubated for 10 days at 18° C., in a shaking incubator at a speed of 175 rpm.

2. The scaled up seed culture was prepared as follows:

Culture media (a total of 6 L in water):

sucrose	240 g
$(NH_4)_2HPO_4$	1.5 g
KH_2PO_4	6 g
K_2HPO_4	13.8 g
$MgSO_4 \cdot 7H_2O$	3 g
KCl	3 g
$MnSO_4$	6 g
$CuSO_4 \cdot 5H_2O$	0.096 g
$FeSO_4 \cdot 7H_2O$	6 g
$CaCl_2$	9 g
$NaSeO_3$	0.072 g

6 flasks (size 3 L) were each filled with 1000 mL culture media. The flasks containing the media were sterilized, cooled to 18° C., and adjusted to pH 6.5. Each flask was inoculated with the *Hirsutella sinensis* strain from the seed culture. The inoculated cultures were incubated for 10 days at 18° C., in a shaking incubator at a speed of 130 rpm.

3. The fermentation steps were carried out as follows (note, however, that the mycelium product produced in any of the following fermentation steps may be used as raw material for the adjuvant agents of the invention, since the main difference among the three fermentation steps is the amount of raw material produced):

Small Scale Fermentation Culture: Fermentation media (a total of 40 L in water in a 100 L fermentor chamber):

sucrose	1600 g
$(NH_4)_2HPO_4$	10 g
KH_2PO_4	40 g
K_2HPO_4	92 g
$MgSO_4 \cdot 7H_2O$	20 g
KCl	20 g
MnSO4	40 g
$CuSO_4 \cdot 5H_2O$	0.64 g
$FeSO_4 \cdot 7H_2O$	40 g
$CaCl_2$	60 g
$NaSeO_3$	0.48 g

The fermentation chamber containing the culture media was sterilized, cooled to 18° C., and adjusted to pH 6.5. The media was inoculated with the *Hirsutella sinensis* strain from the scaled up seed culture by adding the 6 L scale-up seed culture to the fermentor chamber. The inoculated cultures were incubated for 7 days at 18° C. with a stir-speed of 80-200 rpm.

Middle Scale Fermentation Culture: Fermentation media (a total of 500 L in water in a 1000 L fermentor chamber):

sucrose	20 kg
$(NH_4)_2HPO_4$	125 g
KH_2PO_4	500 g
K_2HPO_4	1150 g
$MgSO_4 \cdot 7H_2O$	250 g
KCl	250 g
MnSO4	500 g
$CuSO_4 \cdot 5H_2O$	8 g
$FeSO_4 \cdot 7H_2O$	500 g
$CaCl_2$	750 g
$NaSeO_3$	6 g

The fermentation chamber containing the culture media was sterilized, cooled to 18° C., and adjusted to pH 6.5. The media was inoculated with the *Hirsutella sinensis* strain from the 100 L fermentor chamber. The inoculated cultures were incubated for 5 days at 18° C. with a stir-speed of 50-80 rpm.

Large-Scale Fermentation Culture: Fermentation media (a total of 8000 L in water in a 10000 L fermentor chamber):

sucrose	320 kg
$(NH_4)_2HPO_4$	2000 g
KH_2PO_4	8000 g
K_2HPO_4	18.4 kg
$MgSO_4 \cdot 7H_2O$	4000 g
KCl	4000 g
MnSO4	8000 g
$CuSO_4 \cdot 5H_2O$	128 g
$FeSO_4 \cdot 7H_2O$	8000 g
$CaCl_2$	12 kg
$NaSeO_3$	96 g

The fermentation chamber containing the culture media was sterilized, cooled to 18° C., and adjusted to pH 6.5. The media was inoculated with the *Hirsutella sinensis* strain from the 1000 L fermentor chamber. The inoculated cultures were incubated for 4 days at 18° C. with a stir-speed of 40-70 rpm. The fermented culture medium (broth) was separated from the solid product, and a mycelia cake was produced using a plate and frame filter press. The water was then removed from the mycelia cake by freeze-drying.

A comparison between the method of the invention for producing *Hirsutella sinensis* mycelia and the method disclosed in U.S. application Ser. No. 11/450,747 is shown as Table 1.

TABLE 1

Comparison between the method of the invention for producing *Hirsutella sinensis* mycelia and the method disclosed in U.S. Application No. 11/450,747

	Method of the Invention	U.S. Application No. 11/450,747
Plate culture	3 generations (10 days/generation) 30 days 18° C. pH 6.5 Solid medium as shown above	>10 generations (10 days/generation) >100 days >5 generations at 0-10° C. pH 8 Solid medium containing beef tea, lactalbumin hydrolysate, yeast powder, glucose, milk, nucleic acid, magnesium sulfate, sodium dihydrogen phosphate, and vitamins
Seed cultures in rocking device (shaker) (liquid medium)	Culturing a seed culture for 10 days; Culturing a scaled up seed culture for 10 days; 18° C. pH 6.5 Liquid medium as shown above	Culturing a seed culture for 12 days The culture volume is then expanded 8-fold and fermented stepwise before transfer into a vat fermentor 18° C. pH 7.5 Liquid medium containing silk worm chrysalis powder, protein peptone, corn flour, wheat gluten, glucose, magnesium sulfate, and dipotassium hydrogen phosphate
Fermentation culture in vat fermentor (liquid medium)	3-7 days for each fermentation culture expansion step Small Scale Fermentation Culture: 7 days Middle Scale Fermentation Culture: 5 days Large Scale Fermentation Culture: 4 days (Total: 16 days) 18° C. pH 6.5 Liquid medium as shown above Expansion: 15-20 times the seed culture media volume	12 days for each fermentation culture step Small Scale Fermentation Culture: 12 days Middle Scale Fermentation Culture: 12 days Large Scale Fermentation Culture: 12 days (Total: 36 days) 18° C. pH 7.5 Liquid medium containing silk worm chrysalis powder, protein peptone, corn flour, wheat gluten, glucose, magnesium sulfate, and dipotassium hydrogen phosphate Expansion: 8 times the seed culture media volume

The advantages of the method of the invention over prior art methods are as follows:

a) the method of the invention involves growing the fungus for only 3 generations in the plate culture stage, whereas the method of the '747 application requires more than 10 generations; and

b) the method of the invention involves fermenting the fungus for only 3-7 days in media that is 15 to 20 times the volume of the serial fermenting cultures, whereas the fermentation step of the '747 application requires 12 days in media that is only 8 to 10 times the volume of the serial fermenting cultures.

Without being bound by theory, it is believed that the advantages of the method of the invention are due to the operating pH and the novel formulation of the media used, including the use of specialized trace elements. The advantages of the method of the invention over prior art methods reduce the cost and time of producing *Cordyceps sinensis* mycelia and also increase the bioactivity of the mycelia, as discussed in greater detail below.

2. *Paecilomyces hepiali* Fermentation

The *Paecilomyces hepiali* mycelia used in the clinical trials discussed below were produced by the following method.

The *Paecilomyces hepiali* strain was purified from natural fresh *Cordyceps sinensis* obtained from Mainland China. The strain was plate-cultured for 7 days at 24° C. on solid medium containing the components discussed below for the seed culture media plus 2.0% agar.

A series of sequential liquid cultures and fermentations were then performed over a period of about 9 days.

1. The seed culture was prepared as follows:

Culture media (a total of 0.8 L in water):

sucrose	16 g
glucose	16 g
yeast powder	2.4 g
KH_2PO_4	0.8 g
$MgSO_4 \cdot 7H_2O$	4 g
KCl	0.4 g
$MnSO_4$	0.6 g
$CuSO_4 \cdot 5H_2O$	0.0256 g
$FeSO_4 \cdot 7H2O$	0.6 g
$CaCl_2$	0.24 g
$NaSeO_3$	0.0096 g

8 flasks (size 250 mL) were each filled with 100 mL culture media. The flasks containing the media were sterilized, cooled to 24° C., and adjusted to pH 6.5. Each flask was inoculated with a *Paecilomyces hepiali* strain from a plate culture. The inoculated cultures were incubated for 4 days at 24° C., in a shaking incubator at a speed of 175 rpm.

2. The scaled up seed culture was prepared as follows:

Culture media (a total of 8 L in water):

sucrose	160 g
glucose	160 g
yeast powder	24 g
KH_2PO_4	8 g
$MgSO_4 \cdot 7H_2O$	40 g
KCl	4 g
$MnSO_4$	6 g
$CuSO_4 \cdot 5H_2O$	0.256 g
$FeSO_4 \cdot 7H_2O$	6 g
$CaCl_2$	24 g
$NaSeO_3$	0.096 g

8 flasks (size 3 L) were each filled with 1000 mL culture media. The flasks containing the media were sterilized, cooled to 24° C., and adjusted to pH 6.5. Each flask was inoculated with the *Paecilomyces hepialistrain* from the seed culture. The inoculated cultures were incubated for 3 days at 24° C., in a shaking incubator at a speed of 145 rpm.

3. The large scale fermentation was carried out as follows:

Fermentation media (a total of 8000 L in water in a 10000 L fermentor chamber):

sucrose	160000 g
glucose	160000 g
yeast powder	24000 g
KH_2PO_4	8000 g
$MgSO_4 \cdot 7H_2O$	40000 g
KCl	4000 g
$MnSO_4$	6000 g
$CuSO_4 \cdot 5H_2O$	256 g
$FeSO_4 \cdot 7H_2O$	6000 g
$CaCl_2$	24000 g
$NaSeO_3$	96 g

The fermentor chamber containing the culture media was sterilized, cooled to 24° C., and adjusted to pH 6.5. The media

was inoculated with the *Paecilomyces hepiali* strain from the scaled up seed culture by adding the 6 L scaled up seed culture to the fermentor chamber. The inoculated cultures were incubated for 2 days at 24° C. with a stir-speed of 50-100 rpm. The fermented culture medium (broth) was separated from the solid product, and a mycelia cake was produced using a plate and frame filter press. The water was then removed from the mycelia cake by freeze-drying.

A comparison between the method of the invention for producing the *Paecilomyces hepiali* mycelia and the method disclosed in PCT/CN98/00258 is shown as Table 2.

TABLE 2

Comparison between the method of the invention for producing the *Paecilomyces hepiali* mycelia and the method disclosed in PCT/CN98/00258

	Method of the Invention	PCT/CN98/00258
plate culture	7 days 24° C. pH 6.5 Solid medium as shown above	5-6 days 25° C. pH 6.4 Compostion of culture media not avaliable
Seed cultures in rocking device (shaker) (liquid medium)	Culturing a seed culture for 4 days; Culturing a scaled up seed culture for 3 days; 24° C. pH 6.5 Liquid medium as shown above	Culturing a primary seed culture for 5-6 days; Culturing a secondary seed culture for 4 days; Culturing a scaled up seed culture for 4 days; and 25° C. pH 6.4 Liquid medium containing glucose, sucrose, peptone, bran, potassium dihydrogen phosphate, magnesium sulfate
Fermentation culture in vat fermentor (liquid medium)	2 days in a 10000 L vat fermentor directly from seed culture 24° C. pH 6.5 Liquid medium as shown above Expansion: 1300~1400 times the seed culture media volume	5-6 days for each fermentation culture expansion step The culture volume is expanded and fermented step-wise until a 2000 L vat fermentor volume is reached 24-25° C. pH 6.4 Liquid medium containing glucose, sucrose, powder of soya-bean cake, bran, potassium dihydrogen phosphate, magnesium sulfate, soya-bean oil Expansion: 10 times the seed culture media volume

The major advantages of the method of the invention over prior art methods are as follows:

a) the method of the invention involves fermenting the fungus in a vat for only 2 days, whereas the method of the PCT/CN98/00258 requires 5-6 days; and

b) the method of the invention allows the total volume to be expanded about 1300-1400 times, whereas the method of the PCT/CN98/00258 involves only a 10-fold expansion of culture volume.

The *Paecilomyces hepiali* mycelia produced by the foregoing method ("Mycelia") were compared to a commercially available *Paecilomyces hepiali* mycelia preparation, CordyMax Cs-4, NUSKIN ("CordyMax"). The total nucleosides, polysaccharide, superoxide dismutases, and total free amino acids were analyzed by HPLC, ethanol participation, spectrophotometer, and amino acid analyzer, respectively. Table 3 presents the results of this analysis.

TABLE 3

Comparison between the compositions of mycelium produced by the method of the invention and CordyMax Cs-4 mycelium

Component	Mycelia	CordyMax
Total nucleoside (Adenosine)	1.8% (0.4%)	0.7% (0.1%)
Total Polysaccharide	9%	6%
Superoxide dismutases (SOD)	4×10^3 U/g	2×10^3 U/g
Total Free Amino Acid (mg/100 g)	20300	3800
L-Threonine*	620	75
L-Valine*	720	330
L-Methionine*	350	35
L-Isoleucine*	670	110
L-Leucine*	1400	110
L-Phenylalanine*	860	30
Tryptophan*	260	28
L-Lysine*	1400	250
L-Arginine#	1000	120
L-Histidine#	N.D.	100
γ-Aminobutyric Acid	1830	250
o-Phosphorserine	80	N.D.
Taurine	18	70
o-Phosphoethanolamine	62	70
Urea	N.D.	N.D.
L-Aspartic Acid	740	130
L-Serine	720	64
Asparagine	650	N.D.
L-Glutamic acid	670	800
Sarcosine	N.D.	N.D.
L-2-Aminoadipic Acid	160	26
Glycine	530	97
L-Alanine	1500	400
L-Citrulline	1100	N.D.
DL-2-Aminobutyric Acid	40	25
L(−)-Proline	1600	250
L(−)-Cystine	140	N.D.
L-Cystathionine	95	57
L-Tyrosine	680	140
β-Alanine	N.D.	30
DL-3-Aminoisobutyric Acid	74	19
Ethanolamine	100	18
DL-plus allo-δ-Hydroxylysine	56	15
L-Ornithine	530	69
L-1-Methylhistidine	400	N.D.
L-3-Methylhistidine	N.D.	N.D.
L-Anserine	1300	100
L-Carnosine	170	N.D.
L-Hydroxyproline	N.D.	N.D.

*Essential amino acid
#Semi-Essential amino acid
N.D. = not determined

These data demonstrate that the *Paecilomyces hepiali* mycelia produced by the method of the invention contain greater quantities of functional ingredients than *Paecilomyces hepiali* mycelia produced by prior art methods.

3. *Astragalus membranaceus* Extraction

Dried root of *Astraglus membranaceus* (Fisch.) Bge. (Shaanxi, China) was extracted with hot water twice at a 1:10 (v/v) ratio and centrifuged to remove the particulate material. The extraction fluids were combined, concentrated, and spray-dried. The extraction ratio, i.e., the weight ratio of raw material (dried of *Astraglus membranaceus*) to the extracted powder, was about 2 to 5.

4. Adjuvant Formulation

The adjuvant compositions used in the examples discussed below were prepared as follows.

50-90% (w/w) of fermented *Cordyceps sinensis* powder, 10-50% of extracted *Astraglus membranaceus* powder, and/or 5-10% of zinc were placed into a U-type granulator and mixed for 3 minutes. Water was added and the combination was kneaded to produce a wet granule. After drying the wet granule in an oven at 50° C. for 8 hrs, the granule was sealed in a drum and cooled to room temperature. The dry granule was sieved through an 0.8 mm mesh and mixed in a tumbler for 6 minutes with the following excipients: Povidone K-90 (Polyvinylpyrrolidone K-90; Poly[1-(2-oxo-1-pyrrolidinyl) ethylene], CORUM), calcium phosphate dibasic (Sigma), Crospovidone XL (CORUM), magnesium stearate (Merck), Opadry II (Colorcon), and ariable yellow (BASF). The mixed powder was tableted and packaged with press through packaging (PTP, U.M. GRAVURE).

B. Animal Safety Studies

1. 14 Day Subacute Toxicity Study

Rats were administered water extracts of the *Hirsutella sinensis* mycelia described above ("Mycelia") or vehicle control ("Control") once daily by oral gavage for 14 days. The amount of extract used in this study (5 g/kg) was equivalent to 10-times the recommended dose for humans. The dependent variables examined in this study were: (1) behavior (active contraction); (2) body weight and intake of food and water; (3) hematology parameters, such as hemochrome, thrombocyte, red blood cell, hemoleukocyte, and clotting time; (4) clinical chemistry, such as bilirubin, AST, ALT, BUN, creatinine, glucose, total protein, albumin, K, Na, Ca; (5) urinalysis, such as settling rate, protein, electrolytic containing pH value; and (6) histopathology, such as heat, liver, lung, kidney, stomach, and cholecyst. The results are shown in Table 4 (data are presented as the average of each test group±the standard deviation).

TABLE 4

Results of 14 day subacute toxicity study examining the effects of *Hirsutella sinensis* mycelia on rats

Variable	Control	Mycelia
Body weight change	36.9 g ± 3.3 g	41.8 g ± 9.0 g
Food consumption	26.7 g ± 0.7 g	26.6 g ± 2.0 g
Water intake	46.2 g ± 1.1 g	42.7 g ± 5.0 g
Hb	12.8 g/dL ± 0.2 g/dL	12.9 g/dL ± 0.3 g/dL
WBC	8.9×10^3/μL ± 0.5×10^3/μL	9.8×10^3/μL ± 0.8×10^3/μL
ALB	3.2 mg/dL ± 0.2 mg/dL	3.2 mg/dL ± 0.1 mg/dL
BUN	18 mg/dL ± 0.05 mg/dL	18.9 mg/dL ± 1.5 mg/dL

Among the dependent variables, including the body weight and intake of food and water, there was no statistically significant difference between the treatment and the control groups. These results indicate that under the conditions used in this experiment, the water extracts of *Hirsutella sinensis* mycelia were not toxic to rats.

2. 90 Day Subacute Toxicity Study

Rats were administrated water extracts of the *Paecilomyces hepiali* mycelia described above or vehicle control once daily by oral gavage for 90 days. Three experimental groups were examined: (1) control ("Control"); (2) 2500 mg/kg dosing ("Mycelia 2500"); and (3) 7500 mg/kg dosing ("Mycelia 7500"). The amount of extract used in experimental groups 2 and 3 was equivalent to 100 times and 300 times the recommended dose for humans, respectively. The dependent variables examined in this study were: (1) mortality; (2) clinical observations; (3) body weight; (4) food consumption; (5) hematology parameters, such as Hb, HCT, PLT, RBC, and WBC; (6) clinical chemistry, such as ALT, TP, BUN, Cre, and Glu; and (7) histopathology, such as heart, liver, lung, kidney, and stomach. The results are shown in Table 5 (data are presented as the average of each test group±the standard deviation).

TABLE 5

Results of 90 day subacute toxicity study examining the effects of *Paecilomyces hepiali* mycelia on rats

Variable	Control	Mycelia 2500	Mycelia 7500
Body weight change	414 g ± 11.5 g	416 g ± 21.5 g	412.5 g ± 25 g
Hb	13.6 g/dL ± 0.25 g/dL	14.35 g/dL ± 0.2 g/dL	13.85 g/dL ± 0.3 g/dL
HCT	38.58% ± 0.83%	40.5% ± 0.5%	40.25% ± 0.7%
PLT	843.5 sec ± 37.5 sec	921 sec ± 44 sec	781 sec ± 19 sec
RBC	7.45×10^6/μL ± 0.06×10^6/μL	7.8×10^6/μL ± 0.04×10^6/μL	7.65×10^6/μL ± 0.06×10^6/μL
WBC	4.6×10^3/μL ± 0.6×10^3/μL	4×10^3/μL ± 0.5×10^3/μL	$5.15\ 10^3$/μL ± $0.45\ 10^3$/μL
ALT	54.5 U/L ± 4.25 U/L	52.5 U/L ± 4.45 U/L	60 U/L ± 4.5 U/L
TP	6.46 g/dL ± 0.35 g/dL	6.5 g/dL ± 0.3 g/dL	6.52 g/dL ± 0.35 g/dL
BUN	22 mg/dL ± 3.05 mg/dL	18.55 mg/dL ± 2.95 mg/dL	18 mg/dL ± 0.315 mg/dL
Cre	0.43 mg/dL ± 0.02 mg/dL	0.4 mg/dL ± 0.02 mg/dL	0.44 mg/dL ± 0.03 mg/dL
Glu	155.5 mg/dL ± 6.45 mg/dL	144 mg/dL ± 5.05 mg/dL	152.5 mg/dL ± 4.4 mg/dL

Among the dependent variables, there was no significant difference between the treatment and the control groups. These results indicate that under the conditions used in this experiment, the water extracts of *Paecilomyces hepiali* mycelia were not toxic to rats.

C. Bioassay Experiments

Human peripheral blood mononuclear cells (PBMCs) were suspended in RPMI (6×10^6 cells/mL; Sigma) supplemented with 5% fetal calf serum (FCS) and penicillin/streptomycin/glutamine (PSG) and were incubated at 37° C. and 5% CO_2 for 16 hours with 5 μg/mL Brefeldin A and 50 μg/mL of *Paecilomyces hepiali* mycelia obtained from five different sources: (1) Pharmanex; (2) Formosa Kingstone Bioproducts

Int'l Corp.; (3) GeneFerm Biotechnology Co. Ltd.; (4) Green Strong Corp.; and (5) the method of the invention. Negative control cells were incubated with PBS, and positive control cells were incubated with phorbol myristate acetate (PMA) plus ionomycine. After incubation, the PBMCs were harvested, suspended in two 1.5 mL tubes, and incubated with anti-CD4 antibodies (anti-CD4 clone RPA-T4, BD Pharmingen) or anti-CD8 antibodies (anti-CD8 clone HIT8a, 8D Pharmingen) at 4° C. for 30 min in the dark. The cells were washed with 5% FCS and then incubated with Cytofix/Cytoperm™ (BD Pharmingen) for 20 minutes at 4° C. The cells were then washed once with 250 μL of 1×Perm/Wash (BD Pharmingen). Intracellular cytokines were stained with anti-human IL-2 antibody (clone MQ1-17H12, BD Pharmingen) or anti-IFN-γ antibody (clone 4S.B3, BD Pharmingen) at 4° C. for 30 minutes in the dark. After washing with 5% FCS, the number of IL-2 or IFN-γ positive cells (as a % of $CD4^+$ or $CD8^+$ cells, respectively) was detected by flow cytometry. Table 6 presents the results of this experiment (control data was set to 1 and all other data is shown relative to the control).

TABLE 6

Relative number of IL-2 and IFN-γ positive cells after treatment with *Paecilomyces hepiali* mycelia from different sources

	Negative Control	Positive Control	Pharmanex	Formosa	GeneFerm	Green Strong	Method of Invention
$CD4^+/IL\text{-}2^+$	1	15	5	7	10	6	19
$CD8^+/IFN\text{-}\gamma^+$	1	20	3	4	6	6	10

The $CD4^+$/IL-2 data demonstrate that *Paecilomyces hepiali* mycelia produced by the method of the invention show the highest potency for immune-stimulation and are around 2 to 4 times more potent than *Paecilomyces hepiali* mycelia from other sources. The CD8+/IFNγ data confirm that the mycelia of the invention have the highest potency for immune-stimulation, and are around 1.5 to 3 times more potent than mycelia from other sources. Since $CD4^+$/IL-2 and CD8+/IFNγ data are good indicators of the ability to kill hepatitis C virus in the liver, these results suggest that the mycelia of the invention have greater antiviral activity than mycelia from other sources.

The experimental protocol described above was used to compare the bioactivities of *Paecilomyces hepiali* mycelia produced by the method of the invention with *Hirsutella sinensis* mycelia produced by the method of the invention. The results are shown in Table 7 (control data was set to 1 and all other data is shown relative to the control).

TABLE 7

Relative number of IL-2 and IFN-γ positive cells after treatment with *Hirsutella sinensis* or *Paecilomyces hepiali* mycelia

	PBS Control	Positive Control	P.h.	H.s.
$CD4^+/IL\text{-}2^+$	1.00	34.6	9.4	2.9
$CD8^+/IFN\text{-}\gamma^+$	1.00	42.6	10.4	3.8

The results demonstrate that *Paecilomyces hepiali* mycelia produced by the method of the invention have a higher potency for immune-stimulation than *Hirsutella sinensis* mycelia produced by the method of the invention.

In order to investigate the therapeutic effects of *Paecilomyces hepiali* mycelia combined with *Astragalus membranaceus* extract, two experiments were performed. In the first study, the experimental protocol described above was used to examine the immune-stimulation of *Paecilomyces hepiali* mycelia produced by the method of the invention compared to the immune-stimulation of *Paecilomyces hepiali* mycelia produced by the method of the invention combined with *Astragalus membranaceus* extract. The results of this experiment are presented in Table 8 (control data was set to 1 and all other data is shown relative to the control).

TABLE 8

Relative number of IL-2 and IFN-γ positive cells after treatment with *Paecilomyces hepiali* mycelia with or without *Astragalus membranaceus* extract

	PBS Control	Positive Control	Mycelia Alone	Mycelia + Extract
$CD4^+/IL\text{-}2^+$	1	21	15	17
$CD8^+/IFN\text{-}\gamma^+$	1	19	10	11

The $CD4^+$/IL-2 data demonstrate that the combination of *Paecilomyces hepiali* mycelia produced by the method of the invention and *Astragalus membranaceus* extract has a higher potency for immune-stimulation than *Paecilomyces hepiali* mycelia alone.

The second experiment examined the superoxide scavenging activity of *Paecilomyces hepiali* mycelia produced by the method of the invention compared to the superoxide scavenging activity of *Paecilomyces hepiali* mycelia produced by the method of the invention combined with *Astragalus membranaceus* extract. Superoxide scavenging activity is indicative of an ability to prevent liver damage caused by free radicals. The superoxide scavenging activity was analyzed by nitroblue tetrazolium chloride (NBT) assay. NBT absorbs superoxide and changes the color of the reaction solution to purple, which is absorbed at 560 nm. 25 μl of test sample or superoxide dismutase (SOD) standard ("positive control") was added to each well in a 96-well plate and 63 μM NBT, 300 μM xanthine, and 0.03 U/ml xanthine oxidase (each obtained from Sigma) were added. The absorbance at 560 nm was measured at 1 minute intervals for 5 minutes total. Table 9 presents the results of this study (control data was set to 1 and all other data is shown relative to the control).

TABLE 9

Relative levels of superoxide dismutase activity after treatment with *Paecilomyces hepiali* mycelia with or without *Astragalus membranaceus* extract

	Positive Control	Mycelia Alone	Mycelia + Extract
SOD (U/mg)	1.00	0.468	2.384

These results show that the combination of *Paecilomyces hepialimycelia* produced by the method of the invention and

Astragalus membranaceus extract has a 5-fold higher superoxide scavenging activity (antioxidant activity) than *Paecilomyces hepiali* mycelia alone. Thus, adjuvant agent of the invention comprising *Astragalus membranaceus* and *Paecilomyces hepiali* mycelia has higher potency, and may exhibit therapeutic synergy compared to adjuvant agents comprising *Paecilomyces hepiali* mycelia without *Astragalus membranaceus*.

D. Hepatitis C Clinical Trials 1-3

Three double-blinded clinical trials were conducted to examine the effects of the adjuvant agent of the invention comprising *Hirsutella sinensis* mycelia and *Astragalus membranaceus* extract for treating hepatitis C patients. Patients were treated with α-interferon, with or without ribavirin, for one week and on the second week, the patients were randomly assigned into two groups. The two groups were treated with the adjuvant agent of the invention or placebo concurrently with α-interferon, with or without ribavirin, for 24 weeks, and then with the adjuvant agent of the invention without α-interferon and ribavirin for another 24 weeks. Blood samples were collected and any side effects were documented throughout the experiment period. The dependent variables examined were: (1) blood chemistry: WBC, hemoglobin, platelet, BUN, creatinine, SGOT/SGPT, and HCV-RNA; and (2) occurrence of side effects.

Experimental conditions and results of the individual clinical trials are described below.

1. Clinical Trial #1

A total 28 patients infected with hepatitis C were treated with interferon-α 2A (ROFERON-A, Roche, "IFN") at 3 MU subcutaneously 3 times per week for 6 months. All patients recruited in this study had not been previously treated with ROFERON-A. On the second week, the patients were randomly assigned into two groups and either treated with the adjuvant agent of the invention (N=10) at 3180 mg (about 70% to 80% (w/w) of *Hirsutella sinensis* mycelia, about 10% to 20% (w/w) of *Astragalus membranaceus* extract, and about 5% to 10% of zinc) per day or placebo (N=18, adjuvant agent in which the active raw materials were replaced with microcrystalline cellulose) concurrently with ROFERON-A until the treatment was completed. After treatment was finished, the patients were examined again in follow-up visits at 6 and 12 months.

Serum samples from each patient were analyzed for the presence of HCV RNA using a LightCycler-RNA Amplification Kit SYBR Green I (Roche Diagnostics GmbH). Each 20 μL of the PCR mixture contained LightCycler-RT-PCR Buffer, SYBR Green (Roche), 5 mM $MgCl_2$, 0.25 μM forward primer (5'-GAGGAACTACTGTCTTCACGCAGAA-3'), 0.5 μM reverse primer (5'-CTTTCGCGACCCAACAC-TACTC-3'), LightCycler-RT-PCR Enzyme Mix, and template RNA. RT-PCR amplification was performed using a 10 min cycle at 55° C. to allow for reverse transcription, followed by a 30 sec cycle at 95° C. to allow for denaturation, followed by 45 cycles at 95° C. for 5 sec, 55° C. for 10 sec, 72° C. for 13 sec, and 85° C. for 5 sec to allow for amplification. All reactions were performed in a LightCycler (Roche Diagnostics GmbH).

Table 10 summarizes the results for these patients. "Response" is defined as the percentage of patients who showed no detectable levels of HCV RNA.

TABLE 10

Patients showing no detectable levels of HCV RNA after treatment with IFN with adjuvant agent of the invention or placebo

	Response			
Group	3 Months	6 Months	6 Months Post Treatment	12 Months Post Treatment
IFN + adjuvant	4/10 (40%)	7/10 (70%)	7/10 (70%)*	7/10 (70%)
IFN + placebo	10/18 (55.5%)	6/18 (33.3%)	2/18 (11.1%)*	2/18 (11.1%)

*SVR

These results show that: (1) the adjuvant agent enhanced the therapeutic effect of IFN; and (2) the recurrence of hepatitis C was lower in the group treated with the adjuvant agent than the placebo group.

2. Clinical Trial #2

A total 20 patients infected with hepatitis C were treated with interferon-α 2A (ROFERON-A, Roche, "IFN") at 3 MU, three times a week subcutaneously and ribavirin (COPEGUS, Roche, "ribavirin") at 1000 mg, twice a week for 6 months. All patients recruited in this study had not been previously treated with ROFERON-A or COPEGUS. On the second week, the patients were randomly assigned into two groups treated with the adjuvant agent of the invention at 3180 mg (about 70% to 80% (w/w) of *Hirsutella sinensis* mycelia, about 10% to 20% (w/w) of *Astragalus membranaceus* extract, and about 5% to 10% of zinc) per day (N=8) or placebo (N=12, adjuvant agent in which the active raw materials were replaced with microcrystalline cellulose) concurrently with ROFERON-A and COPEGUS until the treatment was completed. The patients were examined again in follow-up visits at 6 and 12 months.

Serum samples from each patient were analyzed for the presence of HCV RNA as discussed above. Table 11 summarizes the results for these patients ("response" is defined as the percentage of patients who showed no detectable levels of HCV RNA).

TABLE 11

Patients showing no detectable levels of HCV RNA after treatment with IFN and ribavirin with adjuvant agent of the invention or placebo

	Response			
Group	3 Months	6 Months	6 Months Post Treatment	12 Months Post Treatment
IFN + ribavirin + adjuvant	3/8 (7.5%)	7/8 (87.5%)	7/8 (87.5%)*	7/8 (87.5%)
IFN + ribavirin + placebo	0/12 (0%)	2/12 (16.7%)	2/12 (16.7%)*	0/12 (0%)

*SVR

These results show that: (1) the adjuvant agent enhanced the therapeutic effect of the combination therapy of IFN and ribavirin; (2) the recurrence of hepatitis C was lower in the group treated with the adjuvant agent than the placebo group; (3) in the group treated with adjuvant agent, the patients who exhibited an SVR remained HCV-free for one year after the treatment was completed; and (4) ribavirin did not appear to improve the therapeutic effect for hepatitis C in this study.

3. Clinical Trial #3

A total 32 patients infected with hepatitis C were treated with interferon-α 2A (ROFERON-A, Roche, "IFN") at 3

MU, three times a week subcutaneously and ribavirin (COPEGUS, Roche, "ribavirin") at 1000 mg, twice a week for 6 months. All patients recruited in this study had not been previously treated with ROFERON-A or COPEGUS. On the second week, the patients were randomly assigned into two groups and either treated with the adjuvant agent of the invention at 3180 mg (about 70% to 80% (w/w) of *Hirsutella sinensis* mycelia, about 10% to 20% (w/w) of *Astragalus membranaceus* extract, and about 5% to 10% of zinc) per day (N=16) or placebo (N=16, adjuvant agent in which the active raw materials were replaced with microcrystalline cellulose) concurrently with ROFERON-A and COPEGUS until the treatment was completed. The patients were examined again in follow-up visits at 6 and 12 months.

Serum samples from each patient were analyzed for the presence of HCV RNA as discussed above. Table 12 summarizes the results for these patients. "Recurrence" is defined as patients who exhibited undetectable levels of HCV RNA at the end of conventional treatment but exhibited detectable levels of HCV RNA at 6 months post conventional treatment ("response" is defined as the percentage of patients who showed no detectable levels of HCV RNA).

TABLE 12

Patients showing no detectable levels of HCV RNA after treatment with IFN and ribavirin with adjuvant agent of the invention or placebo

	Response			
Group	3 Months	6 Months	6 Months Post Treatment	Recurrence
IFN + ribavirin + adjuvant	16/16 (100%)	16/16 (100%)	15/16 (64%)*	1/16 (6%)
IFN + ribavirin + placebo	10/16 (63%)	10/16 (63%)	3/16 (19%)*	7/16 (44%)

*SVR

Table 13 summarizes the adverse events observed for these patients. The percentages indicate the number of patients who experienced a particular adverse event during the treatment period.

TABLE 13

Patients experiencing an adverse event during treatment with INF and ribavirin with adjuvant agent of the invention or placebo

Adverse Event	INF + Ribavirin + Adjuvant	INF + Ribavirin + Placebo
Fever, headache, myalgia	83%	90%
Fatigue	75%	75%
Arthralgia	83%	75%
Irregular bowel movement	63%	83%
Mild hair loss	56%	63%
Rash	69%	69%
Depression	63%	69%
Irritability	13%	19%
Insomnia	75%	83%
Weight loss	56%	90%
Anemia		
10 g/dl < hemoglobin < 11 g/dl	31%	56%
9 g/dl < hemoglobin < 10 g/dl	6%	13%
Hemoglobin < 9 g/dl	6%	0%
Leukopenia		
3,000/UL < leukocytes < 3,500/UL	19%	31%
2,500/UL < leukocytes, 3,000/UL	0%	0%
Leukocytes < 2,500/UL	19%	6%

Taken together, these results show that: (1) the combination therapy of interferon, ribavirin, and the adjuvant agent increased the sustained virological response rate and decreased the rate of relapse of hepatitis C; and (2) the adjuvant agent decreased several of the adverse events caused by conventional treatment with interferon and ribavirin, such as irregular bowel movement, weight loss, anemia and leukopenia.

E. Hepatitis C Clinical Trials 4 and 5

Two open label clinical trials were conducted to examine the effects of the adjuvant agent of the invention comprising *Paecilomyces hepiali* mycelia and *Astragalus membranaceus* extract for treating hepatitis C patients. For trial 4, patients were treated with interferon α-2a with ribavirin and the adjuvant agent described above for 24 weeks, and then with the adjuvant agent without α-interferon and ribavirin for another 24 weeks. For trial 5, the patients were assigned into two groups. The two groups were treated with or without (control group) the adjuvant agent of the invention concurrently with peginterferon α-2a and ribavirin for 24 weeks, and then with or without (control group) the adjuvant agent without α-interferon and ribavirin for another 24 weeks. Blood samples were collected and any side effects were documented throughout the experiment period. The dependent variables examined were: (1) blood chemistry: WBC, hemoglobin, platelet, BUN, creatinine, SGOT/SGPT, and HCV-RNA; and (2) occurrence of side effects.

1. Clinical Trial #4

A total 32 patients infected with hepatitis C were treated with interferon-α2A (ROFERON-A, Roche, "IFN") at 3 MU, three times a week subcutaneously and ribavirin (COPEGUS, Roche, "ribavirin") at 1000 (if body weight was <75 kg) or 1200 mg (if body weight was ≥75 kg) daily. All patients recruited in this study had not been previously treated with ROFERON-A or COPEGUS. The patients were infected with HCV genotype 1b (N=24) or HCV genotype 2a+2b (N=8). The patients were treated with the adjuvant agent of the invention at 3180 mg (about 70% to 80% (w/w) of *Paecilomyces hepiali* mycelia, about 10% to 20% (w/w) of *Astragalus membranaceus* extract, and about 5% to 10% of zinc) per day concurrently with ROFE RON-A and COPEGUS for 24 weeks (3 months), and then with the adjuvant agent without ROFERON-A and COPEGUS for another 24 weeks (6 months) until the treatment was completed. The patients were examined again in a follow-up visit at 6 months ("6 months post INF treatment").

Serum samples from each patient were analyzed for the presence of HCV RNA as discussed above. Table 14 summarizes the results for these patients ("response" is defined as the percentage of patients who showed no detectable levels of HCV RNA).

TABLE 14

Patients showing no detectable levels of HCV RNA after treatment with IFN and ribavirin and adjuvant agent of the invention

	Response		
HCV Genotype	3 Months	6 Months	6 Months Post INF Treatment
1b	21/24 (87.5%)	19/24 (79.2%)	12/24 (50%)*
2a + 2b	8/8 (100%)	8/8 (100%)	7/8 (87.5%)*

*SVR

Table 15 summarizes the undesirable effects for these patients. The percentages indicate the number of patients that experienced a particular adverse event during the first and sixth months of treatment.

TABLE 15

Patients experiencing an adverse event during treatment with INF and ribavirin and adjuvant agent of the invention or placebo

Effect	1st Month	6th Month
Fever	97%	0%
Fatigue	94%	72%
Rigors	60%	0%
Nausea, Vomiting	9%	3%
Myalgia	81%	34%
Asthenia	59%	0%

Table 16 compares the anemia and leucopenia side effects for these patients to those listed in the REBETOL package insert for treatment with REBETOL and interferon α without the adjuvant composition of the invention. The percentages indicate the number of patients that experienced a particular adverse event during the treatment period.

TABLE 16

Patients experiencing anemia or leucopenia adverse events after treatment with IFN and ribavirin and the adjuvant agent of the invention

Effect	REBETOL Package Insert	Clinical Trial Patients
Hemoglobin concentration <10 mg/dl	14%	9.4%
	WBC	
WHO grade 3 (1000-1999 cells/μl)	21%	0%
WHO grade 4 (<1000 cells/μl)	7%	0%

These results show: (1) the combination therapy of interferon, ribavirin, and the adjuvant agent increased the sustained virological response rate for hepatitis C genotypes 1b and 2a+2b compared to published trial results of 36% for genotype 1b and 59% for genotype 2a+2b over a treatment period of 48 weeks, from Roche's package insert of ROFERON-A; and (2) the adjuvant agent exhibits a trend towards reducing some of the side effects caused by conventional treatment comprising interferon and ribavirin.

2. Clinical Trial #5

A total 37 patients infected with hepatitis C were treated with pegylated interferon-α 2A (PEGASUS, Roche, "Peg-IFN") at 180 mcg, once per week subcutaneously and ribavirin (COPEGUS, Roche, "ribavirin") at 1000 (if body weight was <75 kg) or 1200 mg (if body weight was ≥75 kg) daily. All patients recruited in this study had not been previously treated with PEGASUS or COPEGUS. The patients were assigned into two groups. The two groups were treated with or without the adjuvant agent of the invention at 3180 mg (about 70% to 80% (w/w) of *Paecilomyces hepiali* mycelia, about 10% to 20% (w/w) of *Astragalus membranaceus* extract, and about 5% to 10% of zinc) per day concurrently with PEGASUS and COPEGUS for 24 weeks, and then with (N=20) or without (N=17) the adjuvant agent without PEGASUS or COPEGUS for another 24 weeks.

FIG. **2** summarizes the response rates for these patients. The figure shows the SVR for in patients infected with HCV genotypes 1b and 2a+2b with and without adjuvant.

These results show that the combination therapy of peg-interferon α-2a, ribavirin, and the adjuvant agent of the invention increased the sustained virological response rate for hepatitis C genotype 1b by 22% (from 55% to 77%) and by 17% (from 83% to 100%) for genotypes 2a+2b compared to peg-interferon and ribavirin alone.

F. Hepatitis B Experiment

The hepatitis B virus producing cell lines, 1.3ES8 and HepG2.2.15, which express the adw and ayw genotypes of Hepatitis B, respectively, were used to evaluate the effect of the mycelia of the invention on the levels of HBs and HBe surface antigens. The 1.3ES8 and HepG2.2.15 cell lines are human hepatoma cells that have been stably transfected with the HBV genome (see Journal of Virology, 2005 February; 79(3): 1813-1823, and Journal of Virology, 2003 May; 77(9): 5503-5506). These cells produce infectious HBV viral particles and secrete the HBV HBs and HBe antigens into the culture medium.

The 1.3ES8 and HepG2.2.15 cells were cultured for two and four days (day 2 and day 4), respectively, with or without the adjuvant agent of the invention (200 μg/ml in DMSO) (about 70% to 80% (w/w) of *Paecilomyces hepiali* mycelia, about 10% to 20% (w/w) of *Astragalus membranaceus* extract, and about 5% to 10% of zinc). The culture medium was collected and stored at −20° C. for testing. 50 μl of the cell culture media was added to ELISA plates coated with anti-HBs or anti-HBe and analyzed according to manufacturer's instructions (SURASE B-96 (TMB) (GENERAL BIOLOGICALS, 4SGE3) was used for HBs measurement, and EASE BN-96 (TMB) (GENERAL BIOLOGICALS, 4BNE3) was for HBe measurement). Briefly, the plates were incubated at 40° C. for 2 hours for the HBs test, or at 40° C. overnight for HBe test. The wells were washed six times with washing buffer and peroxidase conjugated antibody was added. 50 μl of anti-HBs-peroxidase and 100 μl of anti-HBe-peroxidase was added, respectively. After a 1 hour incubation at 40° C., the wells were washed six times with washing buffer. 100 μl of tetramethylbenzidine (TMB) substrate was added and the plates were incubated in the dark for 30 minutes. Finally, 100 μl of 2M H_2SO_4 was added to stop the reaction. The absorbance was read at a wavelength of 450 nm in an ELISA plate reader.

Table 17 summarizes the results of these experiments. "Inhibition rate" refers to the ability of the adjuvant agent of the invention to inhibit the secretion of HBs and HBe antigen by the 1.3ES8 and HepG2.2.15 cells (percentages indicate the amount of inhibition relative to control cells that were not treated with the adjuvant agent of the invention).

TABLE 17

Relative level of inhibition of HBV surface antigen expression in cells treated with adjuvant agent of the invention

	Inhibition Rate			
	Day 2		Day 4	
	1.3ES8 Cells	HepG2.2.15 Cells	1.3ES8 Cells	HepG2.2.15 Cells
HBs antigen	22%	7%	22%	20%
HBe antigen	62%	0%	62%	26%

These results show that the adjuvant agent of the invention inhibits secretion of hepatitis B HBs and HBe antigens. The results also show that the adjuvant agent has greater inhibitory effects in 1.3ES8 cells than in HepG2.2.15 cells. Thus, the data demonstrate that the adjuvant agent of the invention has a greater anti-viral effect on the adw genotype of hepatitis B than the ayw genotype.

Based on the in vitro, in vivo, and human studies described above, it is clear that *Cordyceps sinensis* fermentation prod-

ucts have a number of beneficial therapeutic uses. The bioassay studies revealed that *Cordyceps sinensis* fermentation products produced by the method of the invention show much higher potency for immune-stimulation than mycelia from other sources. In addition, potency can be enhanced by adding *Astragalus membranaceus* extracts to the *Cordyceps sinensis* fermentation products. The bioassay studies also indicate that the adjuvant agents of the invention comprising *Cordyceps sinensis* fermentation products have a strong ability to kill hepatitis C virus in human liver cells in vitro. The human studies confirm these in vitro and in vivo results by demonstrating that the adjuvant agent of the invention not only increases the cure rate of HCV patients treated with conventional treatment, but also decreases the adverse effects caused by conventional treatments. Thus, the adjuvant agents of the invention have novel and non-obvious therapeutic utility.

The invention claimed is:

1. A method for producing *Cordyceps sinensis* fermentation product comprising:

(a) inoculating a plate culture comprising solid nutrient medium with at least one strain of *Cordyceps sinensis* and incubating the inoculated plate culture at about 18° C. to about 28° C. for about 4 to 8 days;

(b) inoculating a first seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (a) and incubating the first seed culture at about 18° C. to about 28° C. for about 2 to 4 days;

(c) inoculating a second seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (b) and incubating the second seed culture at about 18° C. to about 28° C. for about 2 to 3 days;

(d) inoculating a fermentation culture comprising liquid nutrient medium with a least a portion of the incubated seed culture from step (c) and incubating the fermentation culture at about 18° C. to about 28° C. for about 1 to 3 days; and

(e) recovering the *Cordyceps sinensis* fermentation product from the incubated fermentation culture of step (d), wherein the nutrient media in steps (a) through (d) each comprise: about 0.001% to about 0.01% (w/w) copper and about 0.0003% to about 0.003% (w/w) selenium.

2. A pharmaceutical composition comprising a *Cordyceps sinensis* fermentation product produced by a method comprising:

(a) inoculating a plate culture comprising solid nutrient medium with at least one strain of *Cordyceps sinensis* and incubating the inoculated plate culture at about 18° C. to about 28° C. for about 4 to 8 days;

(b) inoculating a first seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (a) and incubating the first seed culture at about 18° C. to about 28° C. for about 2 to 4 days;

(c) inoculating a second seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (b) and incubating the second seed culture at about 18° C. to about 28° C. for about 2 to 3 days;

(d) inoculating a fermentation culture comprising liquid nutrient medium with a least a portion of the incubated seed culture from step (c) and incubating the fermentation culture at about 18° C. to about 28° C. for about 1 to 3 days; and

(e) recovering the *Cordyceps sinensis* fermentation product from the incubated fermentation culture of step (d), wherein the nutrient media in steps (a) through (d) each comprise: about 0.001% to about 0.01% (w/w) copper and about 0.0003% to about 0.003% (w/w) selenium;

and a pharmaceutically acceptable carrier.

3. A method for treating a patient infected with or exposed to hepatitis C comprising administering effective amounts of:

the composition of claim **2**;

an interferon; and

a guanosine analog;

to a patient in need thereof.

4. The method of claim **3**, further comprising administering an effective amount of an extract from *Astragalus membranaceus.*

5. A method for treating a patient infected with or exposed to hepatitis B, comprising administering a therapeutically effective amount of the composition of claim **2** to a patient in need thereof.

6. A method for producing *Cordyceps sinensis* fermentation product comprising:

(a) inoculating a plate culture comprising solid nutrient medium with at least one strain of *Cordyceps sinensis* and incubating the inoculated plate culture at about 18° C. to about 28° C. for about 4 to 8 days;

(b) inoculating a first seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (a) and incubating the first seed culture at about 18° C. to about 28° C. for about 2 to 4 days;

(c) inoculating a second seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (b) and incubating the second seed culture at about 18° C. to about 28° C. for about 2 to 3 days;

(d) inoculating a fermentation culture comprising liquid nutrient medium with a least a portion of the incubated seed culture from step (c) and incubating the fermentation culture at about 18° C. to about 28° C. for about 3 to 7 days; and

(e) recovering the *Cordyceps sinensis* fermentation product from the incubated fermentation culture of step (d), wherein the method does not comprise additional culture steps between steps (a) and (b), (b) and (c), and/or (c) and (d), and wherein the nutrient media in steps (a) through (d) each comprise: about 0.001% to about 0.01% (w/w) copper and about 0.0003% to about 0.003% (w/w) selenium.

7. A pharmaceutical composition comprising a *Cordyceps sinensis* fermentation product produced by a method comprising:

(a) inoculating a plate culture comprising solid nutrient medium with at least one strain of *Cordyceps sinensis* and incubating the inoculated plate culture at about 18° C. to about 28° C. for about 4 to 8 days;

(b) inoculating a first seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (a) and incubating the first seed culture at about 18° C. to about 28° C. for about 2 to 4 days;

(c) inoculating a second seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (b) and incubating the second seed culture at about 18° C. to about 28° C. for about 2 to 3 days;

(d) inoculating a fermentation culture comprising liquid nutrient medium with a least a portion of the incubated seed culture from step (c) and incubating the fermentation culture at about 18° C. to about 28° C. for about 3 to 7 days; and

(e) recovering the *Cordyceps sinensis* fermentation product from the incubated fermentation culture of step (d),

wherein the method does not comprise additional culture steps between steps (a) and (b), (b) and (c), and/or (c) and (d), and wherein the nutrient media in steps (a) through (d) each comprise: about 0.001% to about 0.01% (w/w) copper and about 0.0003% to about 0.003% (w/w) selenium; and a pharmaceutically acceptable carrier.

8. A method for treating a patient infected with or exposed to hepatitis C comprising administering effective amounts of:

the composition of claim **7**;

an interferon; and

a guanosine analog;

to a patient in need thereof.

9. The method of claim **8**, further comprising administering an effective amount of an extract from *Astragalus membranaceus*.

10. A method for treating a patient infected with or exposed to hepatitis B, comprising administering a therapeutically effective amount of the composition of claim **7** to a patient in need thereof.

11. An adjuvant composition for use with a conventional therapy for treating a patient infected with or exposed to hepatitis C comprising:

about 50% to about 90% (w/w) of a *Cordyceps sinensis* fermentation product; and about 10% to about 50% (w/w) of an *Astragalus membranaceus* extract; and

a pharmaceutically acceptable carrier,

wherein the adjuvant composition enhances the sustained virological response of the convention therapy, and wherein the *Cordyceps sinensis* fermentation product is produced by a method comprising:

(a) inoculating a plate culture comprising solid nutrient medium with at least one strain of *Cordyceps sinensis* and incubating the inoculated plate culture at about 18° C. to about 28° C. for about 4 to 8 days;

(b) inoculating a first seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (a) and incubating the first seed culture at about 18° C. to about 28° C. for about 2 to 4 days;

(c) inoculating a second seed culture comprising liquid nutrient medium with at least a portion of the inoculated plate culture from step (b) and incubating the second seed culture at about 18° C. to about 28° C. for about 2 to 3 days;

(d) inoculating a fermentation culture comprising liquid nutrient medium with a least a portion of the incubated seed culture from step (c) and incubating the fermentation culture at about 18° C. to about 28° C. for about 1 to 3 days; and

(e) recovering the *Cordyceps sinensis* fermentation product from the incubated fermentation culture of step (d), wherein the nutrient media in steps (a) through (d) each comprise: about 0.001% to about 0.01% (w/w) copper and about 0.0003% to about 0.003% (w/w) selenium.

12. The composition of claim **11**, wherein the *Cordyceps sinensis* is *Paecilomyces hepiali*.

13. The composition of claim **11**, further comprising zinc.

14. The composition of claim **13**, wherein the concentration of *Cordyceps sinensis* fermentation product is about 70% to about 80% (w/w), the concentration of *Astragalus membranaceus* extract is about 10% to about 20% (w/w), and the concentration of zinc is about 5% to about 10%.

15. A method for treating a patient infected with or exposed to hepatitis C comprising administering effective amounts of:

the composition of claim **11**;

an interferon; and

a guanosine analog;

to a patient in need thereof.

16. The method of claim **15**, wherein the interferon is a pegylated interferon-α 2A and the guanosine is ribavirin.

17. The method of claim **15**, wherein the composition of claim **11**, the interferon, and the ribavirin are administered simultaneously.

18. The method of claim **15**, wherein the composition of claim **11**, the interferon, and the ribavirin are administered sequentially.

19. The pharmaceutical composition of claim **2**, wherein the nutrient media in steps (a) through (d) each further comprise: about 0.01% to about 0.2% (w/w) manganese and/or about 0.01% to about 0.2% (w/w) iron and/or about 0.02% to about 0.2% (w/w) cobalt and/or about 0.05% to about 0.5% (w/w) calcium and/or zinc.

20. The pharmaceutical composition of claim **7**, wherein the nutrient media in steps (a) through (d) each further comprise: about 0.01% to about 0.2% (w/w) manganese and/or about 0.01% to about 0.2% (w/w) iron and/or about 0.02% to about 0.2% (w/w) cobalt and/or about 0.05% to about 0.5% (w/w) calcium and/or zinc.

21. The adjuvant composition of claim **11**, wherein the nutrient media in steps (a) through (d) each further comprise: about 0.01% to about 0.2% (w/w) manganese and/or about 0.01% to about 0.2% (w/w) iron about 0.02% to about 0.2% (w/w) cobalt and/or about 0.05% to about 0.5% (w/w) calcium and/or zinc.

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EXHIBIT 13

REDACTED IN ITS ENTIRETY

EXHIBIT 14

REDACTED IN ITS ENTIRETY

EXHIBIT 15

REDACTED IN ITS ENTIRETY

EXHIBIT 16

REDACTED IN ITS ENTIRETY

EXHIBIT 17

REDACTED IN ITS ENTIRETY

EXHIBIT 18

REDACTED IN ITS ENTIRETY

EXHIBIT 19

REDACTED IN ITS ENTIRETY

EXHIBIT 20

REDACTED IN ITS ENTIRETY

EXHIBIT 21

REDACTED IN ITS ENTIRETY