Extremophile Biotechnology

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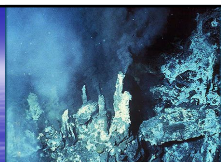
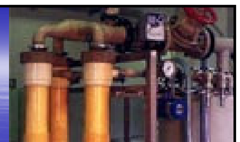




Extremophiles

Microorganisms adapted to harsh environmental conditions including extreme temperatures, high pressure, high salt concentration or extreme pH

Development of new types of production processes based on use of extremophilic organisms is a major challenge in biotechnology.

OUTLINE

Piezophiles = Pressure dependent growth and survival

Psychrophiles = Microorganisms growing at low temperatures

Halophiles = Microorganisms requiring high salt concentrations for growth

Thermo- and hyperthermophiles = Microorganisms growing at high temperatures

Carotenoid filters

Responding to microaerobic conditions:
A Photosynthetic "Purple membrane"

The Halobacterial pigment **bacteriorhodopsin, is used instead of chlorophyll for photosynthesis**.

Halobacteria may have been the starting point for the evolution of photosynthesis.

The **red carotenoid pigments** are similar to that found in **tomatoes**, **flamingos**, and in **autumn leaves**.

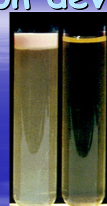
Halophilic microbes are also a source of **Beta-carotene**, an important **antioxidant** and the precursor of **Vitamin A**.

Resisting high levels of solar radiation

- Halobacteria are able to thrive in high levels of solar radiation
- Recent studies have shown that Halobacteria are able to withstand high dosages of ionizing radiation and uv

  (DiRuggiero et al, pers comm).

Gas vesicles: Floatation devices



These are small, **intracellular structures**, made purely of **protein** (unlike standard membranes, which contain lipids as well). They are filled with gas and **enable the cells to float** to the surface of bodies of water.

Some biotechnological applications:

- Production of recombinant gas vesicles for:

  separation

  antigen presentation

  and

  vaccine development

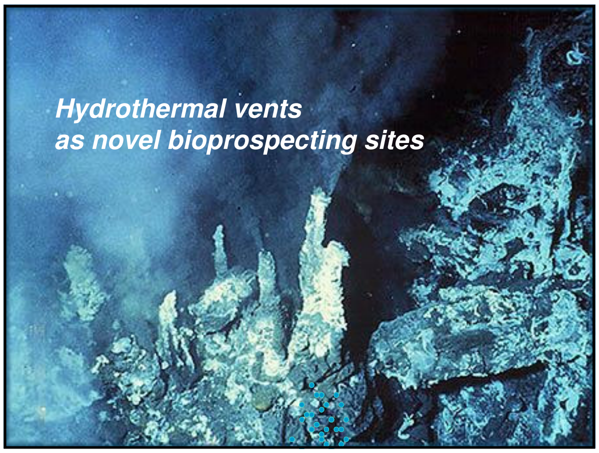
- Large-scale production (in settings such as salterns,) of:

  biopolymers

  novel enzymes and solutes

- Development of stable **enzymes** for catalysis (organic solvents) for:
  sewage treatment
  to increase crude oil recovery from underground wells
- Development of purple membrane films for **holography** -bio-computer chips
- Carotene for use as **food supplements** or **food coloring**

Thanks to Priya and Shil DasSarma for data and information

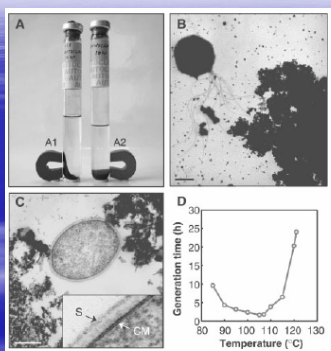


(Hyper)thermophiles

Growth Temperature °C

Species	Optimum	Maximum	Physiology and metabolism
ARCHAEA			
Pyrolobus fumarii	106	113	CO_2, H_2, Nitrate
Pyrococcus furiosus	95	103	Anaerobic, heterotroph
Methanococcus jannaschii	83	86	Anaerobic, methanogen
Sulfolobus solfataricus	75-80	85	Aerobic, acidophile, Sox
BACTERIA			
Aquifex pyrophilus	85	90	Microaerophilic bacterium
Thermotoga maritima	86	90	Anaerobic, heterotrophic

Hyperthermophilic Fe III reducer: Strain 121

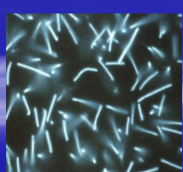


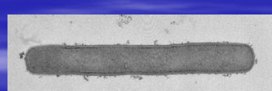
Kashefi and Lovley, 2003

Unofficial record for high temperature growth and survival

Ken Takai, JAMSTEC (Abstract in Thermophiles 2007 conference, Bergen, Norway, September 24-27, 2007)

Methanopyrus kandleri strain 116 was isolated from an in situ colonization device exposed to 365 ˚C black smoker fluid in a vent site of the Kairei field in the Central Indian Ridge. The vent site was located at 2450 m water depth. This is the first *Methanopyrus* strain from the Indian Ocean.



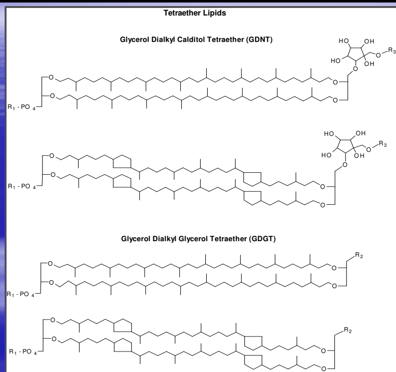


It grows with an apparent Tmax of 116 ˚C in conventional medium at 5 atm. However, under in situ hydrostatic pressure (40 MPa), the growth range is extended to 122 ˚C.

In addition, it was viable after incubation at 130 ˚C for 3 hours under high hydrostatic pressures.

At 122 ˚C and 40 MPa, **isotopically heavy methane** was produced by the methanogen. This revises geochemistry textbook dogma that isotopically light methane is from microoorganisms and geothermal methane is isotopically heavy.

In the hyperthermophilic archaeon *S. acidocaldarius* (85ºC, pH 2-3), a major plasma membrane lipid component is **polar lipid fraction E (PLFE)**. PLFE contains a mixture of bipolar tetraether lipids (~10% GDGT and ~90% GDNT).



R1 = inositol,

R2 = β-D-galactosyl-D-glucose,

R3 = β-glucose

Chains contain up to four cyclopentane rings. The number of rings increases as growth temperature increases.

Data from Parkson Chong Temple University.

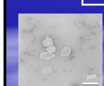
Autoclaving is effective for decontamination of liposome preparations.

- PLFE-based archaeosomes are remarkably stable against multiple autoclaving at pH 4-10 when compared to conventional liposomes.
- Stability was measured in terms of particle size, size distribution and vesicle morphology using dynamic light scattering and transmission electron microscopy

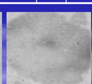
(Z_0 = size before autoclaving, Z_1, Z_2, Z_3 = size after first, second and third autoclaving; all particle sizes in nm)

Conventional lipids used	pH	Z_0	Z_1	Z_2	Z_3
DMPC	4.0	180.0	389.1	307.2	476.2
	7.2	167.3	234.6	322.5	399.7
	10.0	171.7	196.9	258.7	322.5
POPC	4.0	180.5	210.3	241.6	901.0
	7.2	174.5	224.7	196.9	676.3
	10.0	225.7	840.5	>2000	>2000

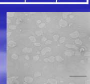
Archael lipids; growth temp	pH	Z_0	Z_1	Z_2	Z_3
PLFE (65 °C)	4.0	156.7	166.3	165.7	163.7
	7.2	159.4	156.4	157.2	159.3
	10.0	152.8	146.4	148.7	158.9
PLFE (78 °C)	4.0	216.8	222.8	207.1	200.7
	7.2	210.2	234.1	202.4	208.3
	10.0	213.4	202.5	230.3	222.6



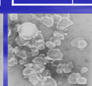
Conventional stealth liposomes before autoclaving average particle size ~200 nm



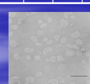
Conventional stealth liposomes after 1 autoclaving average particle size ~1700 nm



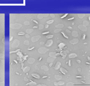
PLFE archaeosomes before autoclaving



PLFE archaeosomes after 6 cycles of autoclaving



PLFE-based stealth archaeosomes before autoclaving



PLFE-based stealth archaeosomes after 6 cycles of autoclaving

Compatible solutes:

Stabilizers of biological structures

Provide protection from extreme environmental conditions

High temperature, osmotic stress, radiation *bitop - top in biotech*

Cosmaceuticals

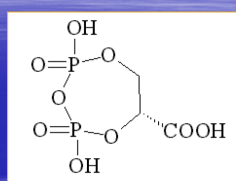
bitop was founded in 1993 by researchers from the Witten/Herdecke University.

"Stress protection through compatible solutes"

bitop focuses on development and production of new compatible solutes and stress proteins from Extremophiles with applications in research, cosmetics, and, potentially, pharmaceuticals.

Cyclic DPG

DGP



From the hyperthermophile *Archeoglobus fulgidus* (Topt 87^{0}C)

DGP (diglycerol phosphate) affords protection against temperature and salt stress.

Approach

bitop´s strategy for development is based on the principle that “hypersolutes” are effective in stabilizing proteins and membranes.

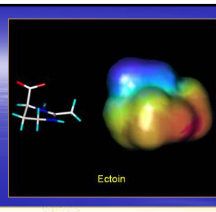
Current technology mainly in the areas of skin protection, dermatology, oncology and diagnostics.

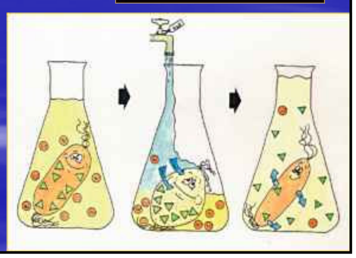
Compatible solutes in cosmetics – “Cosmaceuticals”

Since novel compatible solutes protect microorganisms from extreme environmental conditions, application potential exists in the area of skin protection.

- Antiageing
- Moisture regulation
- Protection from environmental poisons
- Cell protection
- Protection of skin structure
- Microencapsulation of cosmaceuticals and additives
- Stabilization of liposomes
- Protection against radicals and UV radiation
- Protection against osmotic stress

In 2000 bitop AG received the Ruhrgebiet Innovation Prize for the development of the solute Ectoine on the basis of a new biotechnological procedure: “bacterial milking“.





“Bacterial milking”:
A quick decrease in extracellular salt concentration many microorganisms triggers release of compatible solutes in the medium through so called **mechanosensitive channels (MSC).**

Upon achievement of maximal cell density - and also maximal solute content - the biomass is concentrated by tangential-flow filtration. Then diluted rapidly with water to the original volume.

This hypoosmotic shock causes the cells to release the product through MSCs.
The cells are resuspended in high salt growth medium and accumulate solutes.

3-4 fermentation cycles per week.

Hyperthermophiles are “secret” halophiles.

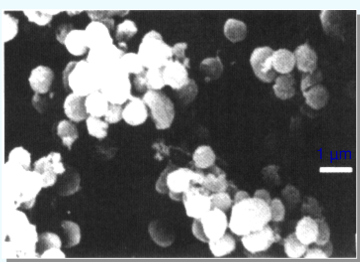
Their cytoplasms contain molar concentrations of salt

Examples:

Pyrococcus furiosus 0.7 M KCl

Methanopyrus furiosus 3.3 M K, 1 M Phosphate

Thermococcus aggregans - anaerobic thermophilic Archaeon



- *Guaymas basin, Gulf of California*
- *Hydrothermal vent*
- *88° C, pH 6.9*

Grows optimally on starch and secretes thermoactive amylases and pullulanases

Starch – Process using extremozymes; one step process

(all enzymes active at same pH and temperature, savings in time and energy)

Steeping – pH 4.0-4.5, 50° C, 10 h

Liquefaction – pH 4.5, 105° C 0.1 h, 95° C 1-2 h; Ca-independent, low pH α-amylase from Archaea (active at 100 C)

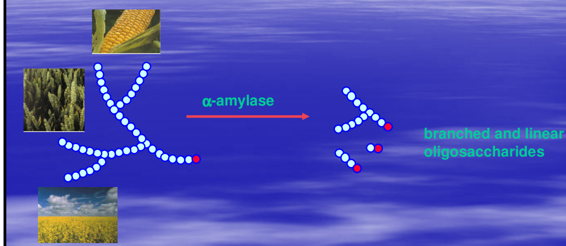
Saccharification – pH 4.0-4.5, 90-100° C, 30-60 h; Pullulanase and GA from extremophiles

Na_2CO_3

Isomerization – Isomerase

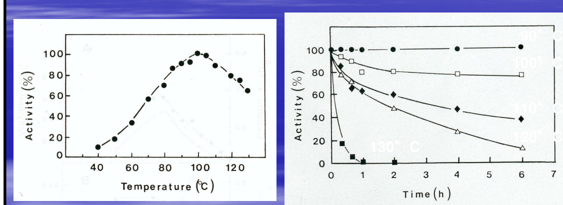
Thanks to
Garo Antranikian
President, International Society
of Extremophiles

Mode of action of α-amylase
(thermoactive enzymes for the starch industry)



(EC 3.2.1.1) α-amylase, glucanotransferase, α-1,4-glucan-4-glucanohydrolase; family 13 of glycosyl hydrolases

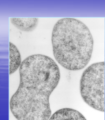
Temperature optimum and stability of the α-amylase from marine Archaea e.g. Thermococcus aggregans



- *the addition of Ca^{2+} did not influence enzymatic activity*
- *active with 10 mM EDTA*
- *Zn^{2+} (2 mM) caused complete inhibition*
- *Size 50 kDa*

Pyrococcus furiosus

Vulcano Island, Italy



Growth temperature: 95-103⁰C. Optimum 100⁰C
Motile cocci with about 50 flagella
Requires 3.5% NaCl (range 0.5-5% w/v)
Utilize peptide, proteins and several carbohydrates
Grows with or without elemental sulfur
Genome size: 2.1 Mb (38.5 %GC)

Heat Shock Proteins

- Occur in almost all organisms, in all three domains
- Expression increased under stress (heat, desiccation).
- Required for acquired thermotolerance.
- Many heat shock proteins are molecular chaperones.
  - Promote folding or unfolding of other proteins.
  - Promote proteolysis of denatured proteins.
  - Prevent denatured proteins from aggregating.

Comparison of *P. furiosus* and *M.jannaschii* heat shock regulons

	M. jannaschii	*P. furiosus*
Thermosome (Hsp60)	Yes	Yes
HspX/HtpX	No	Yes
sHSP (Hsp20)	Yes	Yes
Prefoldin	Yes	No
Proteasome & Lon protease	Only protease regulatory subunit	Yes
CRISPR-associated genes	Yes	Yes
DNA repair protein, RadA	Yes	Yes
Other DNA repair proteins (helicase, ligase, endonuclease)	No	Yes

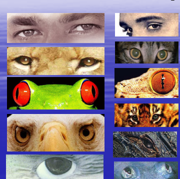
Comparison of heat shock regulons

	M. jannaschii	*P. furiosus*
Reverse gyrase	No	Yes
RNA polymerase, subunit D	Yes	Yes
ABC transporter	Yes	Yes
Ribosomal subunits	Yes (some induced/repressed)	Yes (some early-induced)
AAA+ ATPase	Yes	Yes
Cobalamin biosynthesis	Yes	Yes

Small Heat Shock Proteins

- Modular amino acid sequence similarity with α-crystallins
- Found in ALL Archaea
- Form large complexes: 200 kDa – 1 MDa
- Functions of α-crystallins and sHSPs are similar: holdase function

Eye lens proteins must achieve a high refractive index

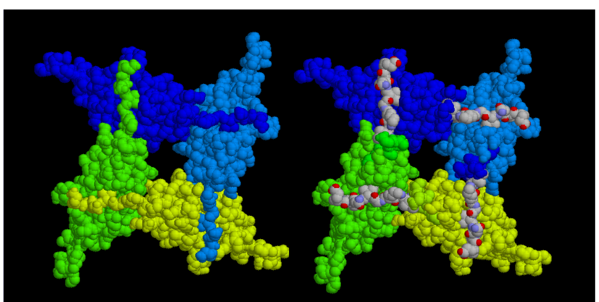


Total protein concentration
Human: 450 mg/ml
Fish: 490 mg/ml

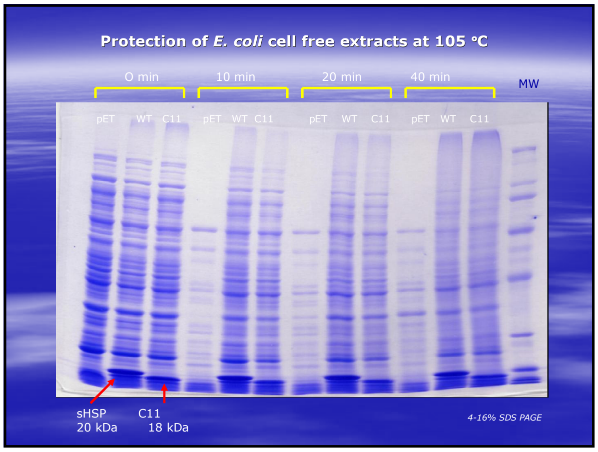
Crystallins contribute up to 90% of total protein content in eye lenses (Horwitz, 2000)

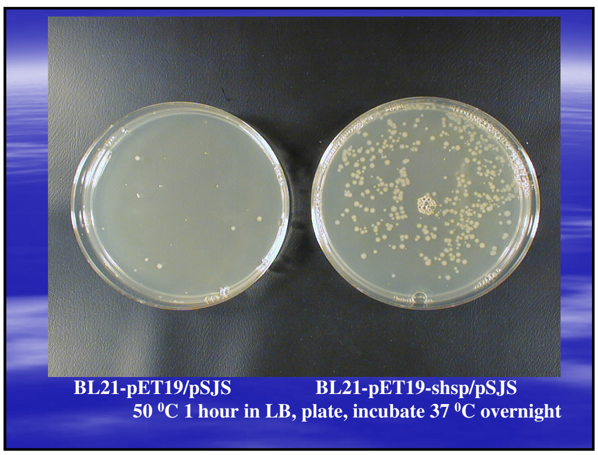
Proteins in eye lenses function throughout life

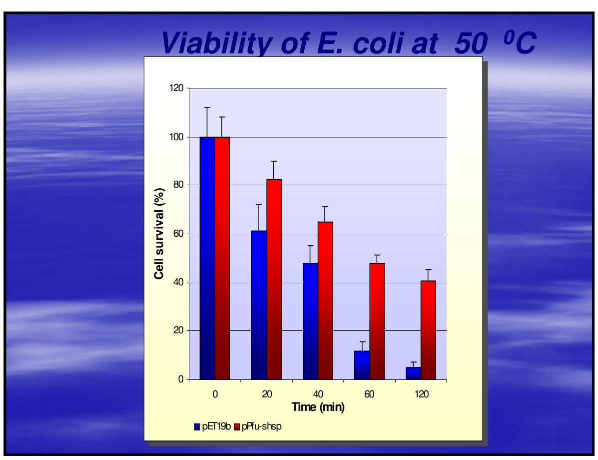
Aggregation results in cataract formation



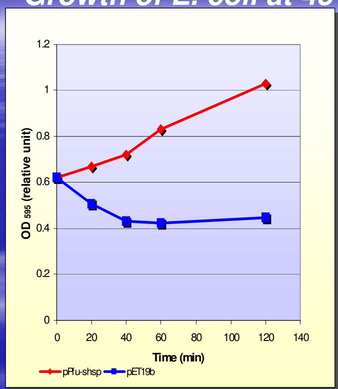
The Archaeal small Heat Shock Protein:
An alpha-crystallin homolog with C-terminal
Extension (C-terminus to body contact 140-147)


Protection of E. coli cell free extracts at 105 °C
0 min
10 min
20 min
40 min
MW
pET WT C11 pET WT C11 pET WT C11 pET WT C11
sHSP
20 kDa
C11
18 kDa
4-16% SDS PAGE


BL21-pET19/pSJS
BL21-pET19-shsp/pSJS
50 ⁰C 1 hour in LB, plate, incubate 37 ⁰C overnight


Viability of E. coli at 50 ⁰C
Cell survival (%)
120
100
80
60
40
20
0
0
20
40
60
120
Time (min)
pET19b
pPfu-shsp

Growth of E. coli at 45 ^{0}C



Conclusion:

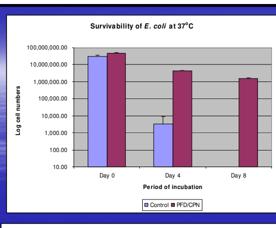
Hyperthermophile chaperones can improve bacterial performance during stress and long term storage

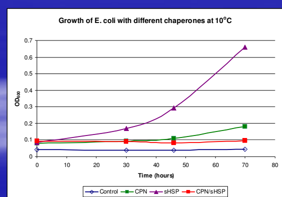
Potential technology: Express hyperchaperones to improve the storage of live vaccines, avoiding the need for the "cold chain" in developing countries.

They call it the Cold Chain.

Attenuated, live bacterial strains are ideal oral vaccines in developing countries, BUT they have storage issues.

"For aid agencies who have made a mission of distributing vaccines, the world has become a tangle of electrical cords connecting refrigerators to remote villages around the globe."





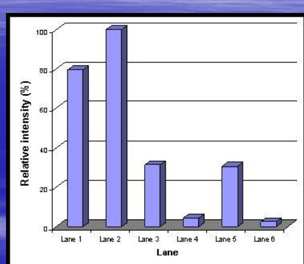
Hyperthermophile chaperones improve growth and survival at during long term storage

Potential application:
To improve the storage of live, attenuated bacterial vaccines, avoiding the need for the "cold chain" in developing countries

Stabilization of taq DNA polymerase by Pfu-sHSP

Temperature cycling takes taq polymerase close to its Tm during denaturation phase

control 5-fold 10-fold
Polymerase Dilutions



control 5-fold 10-fold
Dilutions

Robb and Laksanalamai, US Patent 20020077459

Hyperthermophilic Archaea:

Minimal protein folding systems?

- Small genome size: 1.5 – 2.9 Mb

Exception: M. acetivorans (5.75 Mb) and N. equitans (0.5 Mb)

- Hyperthermophiles have elevated genome copy number (7-13 chromosomes per cell)

Laksanalamai et al, Nature Rev Micro (2004) 2(4): 315 – 324.

Robb and Laksanalamai (2003)
"Enhanced protein thermostability and temperature resistance"
(US #6,579,703).

Summary

sHSPs are induced by heat stress in *P. furiosus* and *M. jannaschii*

Pfu-sHSP functions primarily as a passive chaperone, and complements *E. coli* to confer increased durability

Subunit assembly is critical for chaperone functions of Pfu-sHSP in vivo

Bioprospecting

DNA extraction and molecular techniques:
access genetic material from uncultured organisms that account for over 99% of the Earth's untapped biodiversity

Technology: DNA extraction
cloning of large inserts of "environmental DNA",
state-of-the-art screening
gene evolution, shuffling

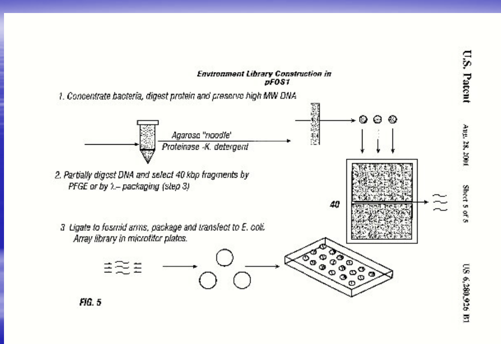


FIG. 5

Diversa Corp. Products

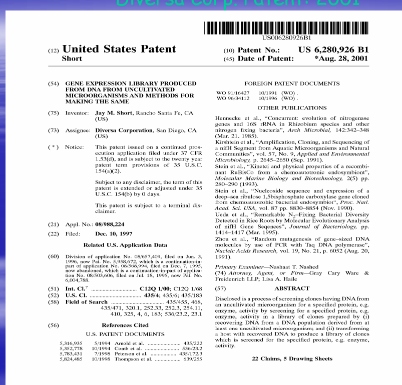
Pharmaceuticals:
1998, 300 billion, 2002 $450 billion world market
focus on anti-microbials, anti-fungals, anti-virals
DirectEvolution technology on proteins, antibodies

Enzymes: Pyrolase 160™ β-mannase from deep-sea thermal vent organism. Reduces viscocity by cleaving long poly-saccharide chains

Pyrolase 200™ hyperthermophilic microbe
activity on glucan and mannan polymers
high temp: textiles, oilfields

ThermalAce™ DNA polymerase, Hi-Fi, long templates
Replicase™

Diversa Corp. Patent: 2001


US006280926B1

(12) United States Patent
Short

(10) Patent No.: US 6,280,926 B1
(45) Date of Patent: *Aug. 28, 2001

(54) GENE EXPRESSION LIBRARY PRODUCED FROM DNA FROM UNCULTIVATED MICROORGANISMS AND METHODS FOR MAKING THE SAME

(75) Inventor: Jay M. Short, Rancho Santa Fe, CA (US)

(73) Assignee: Diversa Corporation, San Diego, CA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

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

This patent is subject to a terminal disclaimer.

(21) Appl. No.: 08/988,224
(22) Filed: Dec. 10, 1997

Related U.S. Application Data

(60) Division of application No. 08/657,409, filed on Jun. 3, 1996, now Pat. No. 5,958,672, which is a continuation-in-part of application No. 08/568,994, filed on Dec. 7, 1995, now abandoned, which is a continuation-in-part of application No. 08/503,606, filed on Jul. 18, 1995, now Pat. No. 6,004,788.

(51) Int. Cl.[7] C12Q 1/00; C12Q 1/68
(52) U.S. Cl. 435/4; 435/6; 435/183
(58) Field of Search 435/455, 468, 435/471, 320.1, 252.33, 252.3, 254.11, 410, 325, 4, 6, 183; 536/23.2, 23.1

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(57) ABSTRACT

Disclosed is a process of screening clones having DNA from an uncultivated microorganism for a specified protein, e.g. enzyme, activity by screening for a specified protein, e.g. enzyme, activity in a library of clones prepared by (i) recovering DNA from a DNA population derived from at least one uncultivated microorganism; and (ii) transforming a host with recovered DNA to produce a library of clones which is screened for the specified protein, e.g. enzyme, activity.

22 Claims, 5 Drawing Sheets

Summary

Technology from Extremophiles

1. Pigments and membrane sensors from halophiles
2. Solutes and "cosmoceuticals" from thermophiles
3. Stable and active enzymes for industrial processes and DNA amplification from hyperthermophiles
4. Manipulate the survival and growth limits of bacteria using hyperstable chaperones
5. Bioprospecting: The new colonialism?

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Supported by NSF, NIH, NOAA, DOE