CONTINUOUS CULTURE APPARATUS WITH MOBILE VESSEL, ALLOWING SELECTION OF FILTER CELL VARIANTS

Field of invention

The described invention provides a method and a device that allow selection of living cells, with increased rates of reproduction and specific metabolic properties, in a liquid or semi-solid medium. For the process of selection (adaptive evolution), genetically variant organisms (mutants) arise in a population and compete with other variants of the same origin. Those with the fastest rate of reproduction increase in relative proportion over time, leading to a population (and individual organisms) with increased reproductive rate. This process can improve the performance of organisms used in industrial processes or academic purpose.

Background of invention

Selection for increased reproductive rate (fitness) requires sustained growth, which is achieved through regular dilution of a growing culture. In the prior art this has been accomplished two ways: serial dilution and continuous culture, which differ primarily in the degree of dilution.

Serial culture involves repetitive transfer of a small volume of grown culture to a much larger vessel containing fresh growth medium. When the cultured organisms have grown to saturation in the new vessel, the process is repeated. This method has been used to achieve the longest demonstrations of sustained culture in the literature (Lenski & Travisano: *Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations*. 1994. Proc Natl Acad Sci U S A. 15:6808-14), in experiments which clearly demonstrated consistent improvement in reproductive rate over period of years. This process is usually done manually, with considerable labor investment, and is subject to contamination through exposure to the outside environment. Serial culture is also inefficient, as described in the following paragraph.

The rate of selection, or the rate of improvement in reproductive rate, is dependant on population size (Fisher: *The Genetical Theory of Natural Selection*.1930. Oxford University Press, London, UK). Furthermore, in a situation like serial transfer where population size fluctuates rapidly, selection is proportional to the harmonic mean (Ñ) of the population

(Wright: *Size of population and breeding structure in relation to evolution.* 1938. Science 87: 430-431), and hence can be approximated by the lowest population during the cycle.

Population size can be sustained, and selection therefore made more efficient, through continuous culture. Continuous culture, as distinguished from serial dilution, involves smaller relative volume such that a small portion of a growing culture is regularly replaced by an equal volume of fresh growth medium. This process maximizes the effective population size by increasing its minimum size during cyclical dilution. Devices allowing continuous culture are termed "chemostats" if dilutions occur at specified time intervals, and "turbidostats" if dilution occur automatically when the culture grows to a specific density.

For the sake of simplicity, both types of devices will hereafter be grouped under the term "chemostat". Chemostats were invented simultaneously by two groups in the 1950's (Novick & Szilard: *Description of the chemostat.* 1950. Science 112: 715-716) and (Monod: *La technique de la culture continue - Théorie et applications.*1950. Ann. Inst. Pasteur 79:390-410). Chemostats have been used to demonstrate short periods of rapid improvement in reproductive rate (Dykhuizen DE. *Chemostats used for studying natural selection and adaptive evolution.*1993. Methods Enzymol. 224:613-31).

Traditional chemostats are unable to sustain long periods of selection for increased reproduction rate, due to the unintended selection of dilution-resistant (static) variants. These variants are able to resist dilution by adhering to the surface of the chemostat, and by doing so, outcompete less sticky individuals including those that have higher reproductive rates, thus obviating the intended purpose of the device (Chao & Ramsdell: *The effects of wall populations on coexistence of bacteria in the liquid phase of chemostat cultures,*. 1985. J. Gen. Microbiol. 131: 1229-36).

One method and chemostatic device (the Genetic Engine) has been invented to avoid dilution resistance in continuous culture (patent US 6,686,194-B1 filed by PASTEUR INSTITUT [FR] & MUTZEL RUPERT [DE]). This method uses valve controlled fluid transfer to periodically move the growing culture between two chemostats, allowing each to be sterilized and rinsed between periods of active culture growth. The regular sterilization cycles prevent selection of dilution-resistant variants by destroying them. This method and device achieves the goal, but requires independent complex manipulations of several fluids within a sterile (sealed) environment, including one (NaOH) which is both very

caustic and potentially very reactive, quickly damaging valves, and posing containment and waste-disposal problems.

Summary of invention

It is therefore an object of the present invention to provide an improved (and completely independent) method and device for continuous culture of organisms (including bacteria, archaea, eukaryotes and viruses) without interference from dilution-resistant variants. Like other chemostats, the device provides a means for regular dilution of a grown culture with fresh growth medium, a means for gas exchange between the culture and the outside environment, sterility, and automatic operation as either a chemostat or a turbidostat.

The present invention is designed to achieve this goal without any fluid transfer, including sterilization or rinsing functions. This represents a specific advantage of the present invention with respect to prior art in so far as it avoids the hazards and difficulties associated with sterilization and rinsing, including containment and complex fluid transfers involving caustic solvents.

Continuous culture is achieved inside a flexible sterile tube filled with growth medium. The medium and the chamber surface are static with respect to each other, and both are regularly and simultaneously replaced by peristaltic movement of the tubing through "gates", or points at which the tube is sterilely subdivided by clamps that prevent the cultured organisms from moving between regions of the tube. UV gates can also (optionally) be added upstream and downstream of the culture vessel for additional security.

The present method and device are also an improvement over prior art insofar as they continually, rather than periodically, select against adherence of dilution-resistant variants to the chemostat surfaces, as replacement of the affected surfaces occurs in tandem with the process of dilution.

The tube is subdivided in a transient way such that there are regions that contain saturated (fully grown) culture, regions that contain fresh medium, and a region between these two, termed the growth chamber, in which grown culture is mixed with fresh medium to achieve dilution. The gates are periodically released from one point on the tube and replaced at another point, such that grown culture along with its associated growth chamber surface and attached static organisms, is removed by isolation from the growth

chamber and replaced by both fresh medium and fresh chamber surface. By this method, static variants are specifically counter-selected by removal from the region in which selection is occurring (the growth chamber).

Brief description of drawings

Without being exhaustive and limiting, one possible general configuration will include several components as described hereafter. In the following the present invention is exemplarily explained on the basis of a preferred embodiment, thereby referring to the drawings in which:

Figure 1 displays an overall view of a possible configuration of the device in which:

(1) represents the flexible tubing containing the different regions of the device which are: upstream fresh medium (7), growth chamber (10), sampling chamber (11) and disposed grown culture region (15)

(2) represents the thermostatically controlled box allowing regulation of temperature according to conditions determined by user, and in which may be located:

a. said growth chamber (10),
b. said sampling chamber (11),
c. upstream gate (3) defining the beginning of said growth chamber (10),
d. downstream gate (4) defining the end of said growth chamber (10) and the beginning of said sampling chamber (11)
e. second downstream gate (5) defining the end of said sampling chamber (11),
f. turbidimeter (6) allowing the user or automated control system to monitor optical density of growing culture and to operate a feedback control system (13), allowing controlled movement of the tubing (1) on the basis of culture density (turbidostat function),
g. one or several agitators (9).

It should be noted that the device elements listed in a-g may also be located outside of, or in the absence of, a thermostatically controlled box.

(7) represents the fresh medium in unused flexible tubing,

(8) represents a barrel loaded with fresh medium filled tubing, in order to dispense said fresh medium and tubing during operations.

(12) represents optional ultra-violet radiation gates,

(13) represents the control system that can consist of a computer connected with means of communication to different monitoring or operating interfaces, like optical

density turbidimeters, temperature measurement and regulation devices, agitators and tilting motors, etc, that allow automation and control of operations,

(14) represents the optional disposal barrel on which to wind up tubing containing disposed grown culture filled tubing,

(15) represents disposed grown culture located downstream of said sampling chamber.

Figure 2 displays two possible positions of the device, exemplifying the fact that said thermostatically controlled box (2) and other pieces of said device associated with said culture chamber can be tilted to various degrees for agitation purposes, gas circulation and removal purposes, and purposes of guaranteeing the removal of granulated (aggregated) cells that might escape dilution by settling to the bottom.

Figures 3 to 9 represents said flexible tubing (1) in place in said thermostatically controlled box (2) and introduced through gates (3), (4) and (5) through which said tubing will stay during all steps of process and through which said tubing will move according to its peristaltic movement.

Figure 3 symbolizes status T0 of the device in which all regions of said flexible tubing are filled with fresh medium before injection of the organism intended for continuous culture.

Figure 4 symbolizes status T1 of said flexible tubing just after injection of organism strain.

Figure 5 symbolizes status T2 of the device which is a growing period during which the culture grows in the region defined as the growth chamber (10) limited by said gates (3) and (4).

Figure 6 symbolizes status T3 of device, just after the first peristaltic movement of tubing and associated medium, which determines the beginning of the second growing cycle, introducing fresh tubing and medium through movement of gate 3 simultaneous with a transfer of equivalent volume of tubing, medium, and grown culture out of the growth chamber region (10) and into the sampling chamber region (11) by movement of gate 4. It is critical to recognize that the tubing, the medium that is within the tubing, and any culture that has grown in that medium, all move together. Fluid transfer only occurs insofar as fresh medium and grown culture mix together through agitation within the growth chamber region.

Figure 7 symbolizes status T4 of the device which is the second growing cycle; during this cycle organisms that remain in the growth chamber after peristaltic movement of the tubing can now grow using nutrients provided in the fresh medium that is mixed with the remaining culture during this step.

Figure 8 symbolizes status T5 of device, just after the second peristaltic movement of the tubing and the contained medium, which determines the beginning of the third growing cycle, introducing fresh tubing and medium through movement of gate 3 simultaneous

with a transfer of equivalent volume of tubing, medium, and grown culture out of the growth chamber region (10) and into the sampling chamber region (11) by movement of gate 4.

Figure 9 symbolizes status T6 of device which is the third growing cycle; this step is equivalent to status T4 and indicates the repetitive nature of further operations. Samples of selected organisms may be removed at any time from the sampling chamber region (11) using a syringe or other retrieval device.

Figure 10 displays a possible profile of teeth determining a gate in the configuration which consists of two stacking teeth pinching flexible tubing. Gates could also be determined by single teeth pressing against a moveable belt, removable clamps, or other mechanisms that prevent movement of organisms through the gate and which can be alternately placed and removed in variable positions along the tubing.

Detailed description of invention

The basic operation of the device is depicted in figures 3 through 9.

One potential configuration for the present device is shown in figure 1, as it appears after having been loaded with a fresh tube of sterile medium (shown divided into regions A-H by said gates (3), (4) and (5)).

Inoculation of the device with the chosen organism could be achieved by introduction of the organism into the growth chamber (fig 3), through injection (figure 4, region B). The culture would then be allowed to grow to the desired density and continuous culture would begin (fig 5).

Continuous culture would proceed by repetitive movements of the gated regions of tubing. This involves simultaneous movements of the gates, the tubing, the medium, and any culture within the tubing. The tubing will always move in the same direction; unused tubing containing fresh medium (and hereafter said to be 'upstream' of the growth chamber (7)) will move into the growth chamber and mix with the culture remaining there, providing the substrate for further growth of the organisms contained therein. Before introduction into the growth chamber region, this medium and its associated tubing will be maintained in a sterile condition by separation from the growth chamber by the upstream gates (3). Used tubing containing grown culture will simultaneously be moved 'downstream' and separated from the growth chamber by the downstream gates (4).

Gate configuration is not a specific point of the present patent application. For example, in a given configuration, gates can be designed through one chain of multiple teeth simultaneously moved or in another configuration separated in distinct synchronized chains as depicted in figure 1. Gates can consist of a system made of two teeth pinching

the tubing in a stacking manner as described in figure 10, avoiding contamination between regions G and H of the tubing through the precision of the interface between the teeth. In another configuration, sterile gates can be obtained by pressing one tooth against one side of the tubing and thereby pressing the tubing tightly against a fixed chassis along which tubing is slid during its peristaltic movement, as sketched in figure 3 to 9, marks 3, 4 and 5.

Said thermostatically controlled box (2) is obtained by already known means such as a thermometer coupled with a heating and cooling device.

Aeration (gas exchange), when required for growth of the cultured organism or by the design of the experiment, is achieved directly and without mechanical assistance by the use of gas permeable tubing. For example and without being limiting, flexible gas permeable tubing can be made of silicone. Aeration could be achieved through exchange with the ambient atmosphere or through exchange with an artificially defined atmosphere (liquid or gas) that contacts the growth chamber or the entire chemostat. When an experiment demands anaerobiosis the flexible tubing can be gas impermeable. For example and without being limiting, flexible gas impermeable tubing can be made of coated or treated silicone.

For anaerobic evolution conditions, regions of the tubing can also be confined in a specific and controlled atmospheric area to control gas exchange dynamics. This can be achieved either by making said thermostatically controlled box gastight and then injecting neutral gas into it or by placing the complete device in an atmosphere controlled room.

Counter-selection of static variants is achieved by replacement of the growth chamber surface along with growth medium.

The device is further designed to be operable in a variety of orientations with respect to gravity, that is, to be tilted as shown by figure 2, along a range of up to 360°.

Dilution-resistant variants may avoid dilution by sticking to one another, rather than to the chamber wall if aggregated cells can fall upstream and thereby avoid removal from the chamber. Hence it is desirable that the tubing generally be tilted downward, such that aggregated cells will fall toward the region that will be removed from the growth chamber during a cycle of tube movement. This configuration involves tilting the device so that the downstream gates are below the upstream gates with respect to gravity.

The growing chamber can be depressurized or over pressurized according to conditions chosen by the experimenter. Different ways of adjusting pressure can be used, For instance applying vacuum or pressurized air to the fresh medium and tubing through its upstream extremity and across the growth chamber; another way of depressurizing or overpressurizing tubing can be done by alternate pinching and locking tubing upstream of the growth chamber.

When the medium is contained in gas permeable tubing, air bubbles may form within the medium. These will rise to the top of a sealed region of tubing and become trapped there until the movement of the region (and the gates defining it) releases the region into either the growth chamber, the sampling chamber or the endpoint of the chemostat (figures 6, regions D-C, B or A, respectively). If the device is tilted downward such bubbles will accumulate in the growth chamber or sampling chamber and displace the culture. The device is designed to periodically tilt upward for a cycle of the tube movement, allowing for the removal of accumulated gas from said chambers.

Tilting movements of the device, and/or shaking of the growth chamber by an external device (9) can be used to decrease aggregation of cells within the growth chamber. Alternatively, one or several stirring bars can be included in the tubing filled with fresh medium before sterilization and magnetically agitated during culture operations.

The proportional length of the regions of fresh medium defined by the upstream gates as compared to the length of the culture chamber will define the degree of dilution achieved during a cycle.

The frequency of dilution can be determined either by timing (chemostat function) or by feedback regulation whereby the density of the culture in the growth chamber is measured by a turbidimeter (figure 1- mark 6) and the dilution cycle occurs when the turbidity reaches a threshold value (turbidostat function).

The sampling chamber allows withdrawing grown culture in order to analyze the outcome of an experiment, collect organisms with improved growth rate for further culture, storage, or functional implementation, or other purposes such as counting the population, checking the chemical composition of the medium, or testing the pH of grown culture. In order to achieve permanent monitoring of pH inside growth chamber, tubing can include by construction a pH indicator line embedded/encrusted in the wall of the tubing.

Any form of liquid or semi-solid material can be used as a growth medium in the present device. The ability to utilize semi-solid growth substrates is a notable advancement over prior art. The growth medium, which will define the metabolic processes improved by the selection process, can be chosen and defined by the user.

If needed, this device can contain multiple growth chambers, such that the downstream gates of one growth chamber become the upstream gates of another. This could, for example, allow one organism to grow alone in the first chamber, and then act as the source of nutrition for a second organism (or virus) in the second chamber.

This device and method allows researchers and product developers to evolve any strain of culturable living cells in suspension through sustained growth (continuous culture); the resulting improved organism can constitute a new strain or species. These new organisms can be identified by mutations acquired during the course of culture, and these mutations may allow the new organisms to be distinguished from their ancestors genotype characteristics. This device and method allow the researcher to select new strains of any living organism by segregating individuals with improved rates of reproduction through the process of natural selection.

Claims

What is claimed is:

1. A device that increases the rate of reproduction (through increased speed of reproduction and/or increased reproductive yield) of living cells in suspension or of any culturable organisms through the process of natural selection, said device comprising: a) a flexible, sterile tube containing culture medium, b) a system of movable gates (clamps) that divide the tube into separate regions containing spent culture (downstream region), growing culture (growth chamber), and fresh growth medium (upstream region), c) a means of moving the gates and the tubing such that a portion of the growth chamber and the associated culture can be clamped off and separated from the growth chamber, and such that a portion of fresh tubing containing unused medium can be joined with a portion of the culture and associated medium already present in the growth chamber.

2. The device according to claim 1, wherein the tubing is flexible to allow clamping and segregation into separated chambers.

3. The device according to claim 1, wherein the tubing is gas permeable, for example comprised primarily of silicon, to allow gas exchange between the cultured organism and the outside environment, according to the type of experiment.

4. The device according to claim 1, wherein the tubing is gas impermeable, to prevent gas exchange between the tubing and the outside environment, if the experiment demands anaerobiosis.

5. The device according to claim 1, wherein the tubing is transparent or translucent, to allow the measurement of turbidity.

6. The device according to claim 1, wherein the growth chamber tubing and associated media and culture can be depressurized or over pressurized relative to ambient atmosphere as necessitated by experimental requirements.

7. The device according to claim 1, wherein the tubing allows the measure of pH of medium by inclusion of a pH indicator in the tubing composition or lining.

8. The device according to claim 1, wherein the growth chamber tubing and associated media and culture can be heated or cooled as appropriate for experiment conditions.

9. The device according to claim 1, wherein the growth chamber tubing and associated media and culture can be kept motionless or agitated by any already known method.

10. The device according to claim 9 wherein the tubing can include one or several stirring bars for agitation purpose.

11. The device according to claim 1 wherein regions of the tubing can be confined in a specific and controlled atmospheric area to control gas exchange dynamics.

12. The device according to claim 1 wherein the growth chamber tubing and associated media and culture can be tilted either downward to remove aggregated cells, or upward to remove air through the functions described in claim 1-c.

13. A method that increases the rate of reproduction (through increased speed of reproduction and/or increased reproductive yield) of living cells in suspension or of any culturable organisms through the process of natural selection, comprising: a) providing an initial culture in the described growth chamber through sterile injection of a starter culture into a sterile tube containing sterile growth medium; b) maintaining growth conditions according to experimental requisites ; c) after a certain period of time and associated growth of the culture, adjusting the position of the described gates so as to move equal portions of fresh medium and of grown culture (respectively) into and out of the region defined as the growth chamber, allowing the remaining portion of grown culture to mix with the introduced portion of fresh medium and continue to grow; d) reproducing steps b) and c) until the end of experiment to achieve continuous culture and selection of variants with increased reproductive rates; e) withdrawing on demand a sample of grown culture from sampling chamber.

14. A method according to claim 13 wherein applying a simultaneous peristaltic movement of the gates, the tubing, and the medium and culture within the tubing, allows provision of a certain quantity of fresh medium to the growth chamber while an equal quantity of culture is isolated and removed through the other extremity of said growth chamber, terminating a growth cycle and starting a new one.

15. A method according to claim 13 wherein an experiment can include as many growth cycles as required by the experimenter without possible contamination of isolated growing chamber and without possible proliferation of a dilution-resistant population.

16. A method according to claim 13 such that during the operations the experimenter can maintain growth conditions according to experimental requisites which may include temperature, pressure, optical density, chemical acidity, agitation and aeration with various gases.

17. A method according to claim 13 wherein a combination of tilting the device and operating agitators leads to an appropriate agitation for mixing the growing culture in order to prevent or repress aggregation of living organisms.