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## Content

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| 1       | Apparatus for the Cultivation of Microorganisms | 1954           |
| 2       | Methods for the Cultivation of Microorganisms   | 1958           |

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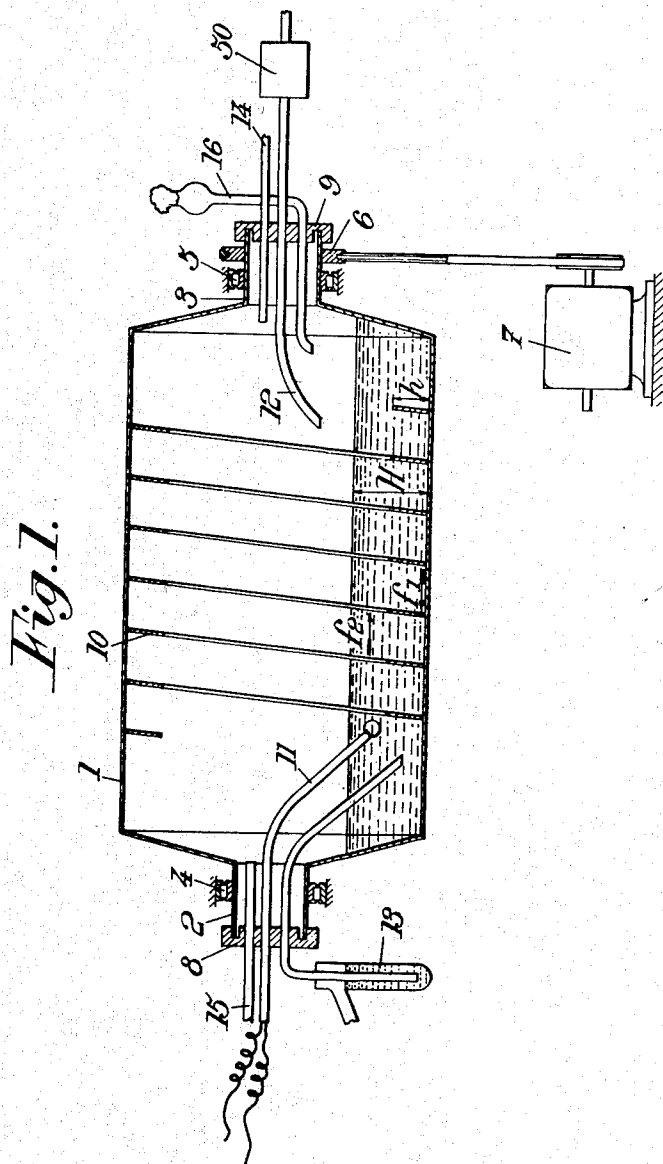
J. MONOD

2,686,754

APPARATUS FOR THE CULTIVATION OF MICROORGANISMS

Original Filed Nov. 28, 1950

2 Sheets-Sheet 1



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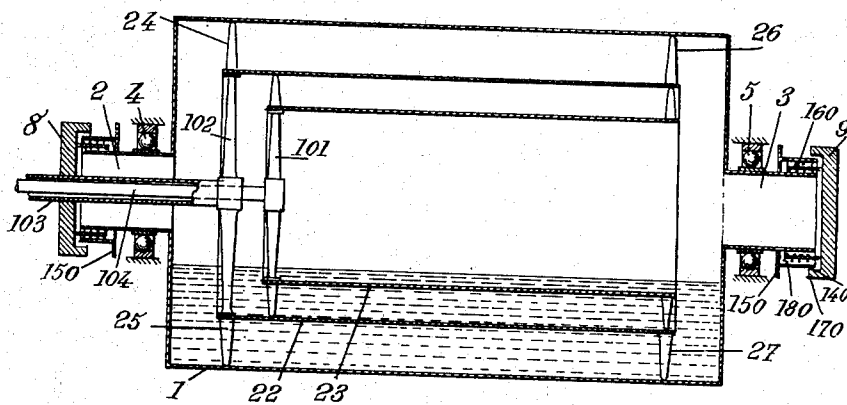
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APPARATUS FOR THE CULTIVATION OF MICROORGANISMS

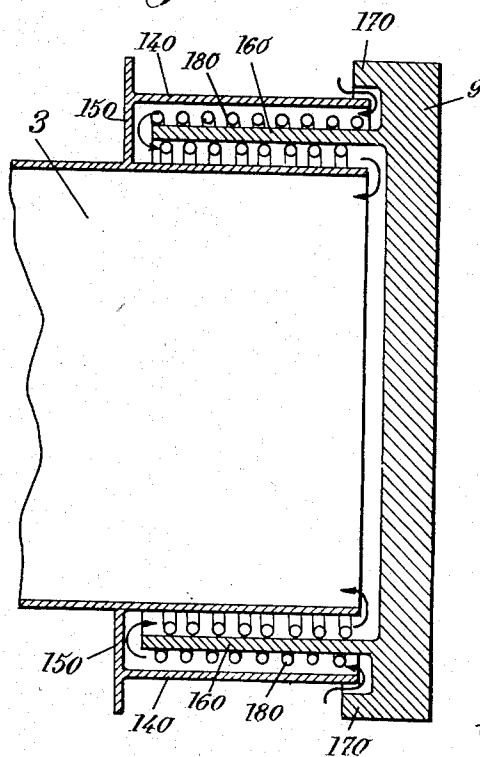
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*Fig. 2.*



*Fig. 3.*



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## UNITED STATES PATENT OFFICE

2,686,754

## APPARATUS FOR THE CULTIVATION OF MICROORGANISMS

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Original application November 28, 1950, Serial No. 197,867. Divided and this application February 6, 1952, Serial No. 270,125

5 Claims. (Cl. 195-143)

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The present invention relates to apparatus for the cultivation of microorganisms (bacteria, yeasts, moulds, protozoa, lower algae, etc.).

Its chief object is to achieve a continuous production of the desired culture with an adjustable output and a high yield while maintaining constancy and homogeneity of the qualities of the product.

Embodiments of my invention will be herein-after described with reference to the accompanying drawings, given merely by way of example, and in which:

Fig. 1 is a diagrammatic sectional view of an apparatus for the obtainment of microorganism cultures according to my invention;

Fig. 2 is a similar view of an apparatus for the same purpose made according to another embodiment;

Fig. 3 is a detail view illustrating a feature of my invention.

For the sake of clarity, it will first be reminded that I start from a culture brought to the desired density in a suitable container, in the presence of a suitable gaseous phase, and I continuously add fresh nutrient medium thereto and continuously remove prepared culture therefrom at the same rate, while subjecting the culture in the course of evolution to an action (for instance a stirring one) capable of keeping it homogeneous, that is to say equally rich in cells in all of its parts.

The rate at which fresh medium is added and culture is removed is equal to or lower than a limit which conditions the maintaining of the density or richness in cells of the culture, at a stable level of equilibrium.

Furthermore, the possibilities of interchange between the gaseous phase and the liquid phase are made as close as possible to the conditions of equilibrium between these two phases and in particular to the conditions for maintaining saturation equilibrium between the oxygen of air and the dissolved oxygen.

Concerning the action of the gaseous phase, it will be achieved or intensified, preferably, by creating in the treatment container large interchange areas constituted by liquid films in movement and quickly renewed on suitable metallic or other surfaces.

As a rule, it will be of interest to have the gaseous phase located above the liquid phase without having to pass therethrough in a continuous manner, so as to avoid the formation of foams. Accordingly, the gases will be preferably introduced without being injected into the liquid. An

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overpressure may eventually be provided, or possibly a partial vacuum.

The gaseous atmosphere may be introduced at the beginning of the operations and remain for the whole duration of the treatment; or it may, on the contrary, be renewed, either in a continuous or in a discontinuous manner.

The treatment container consists of a rotary cylinder or drum, in particular of horizontal or substantially horizontal axis, this arrangement ensuring both the liquid stirring action and the formation of liquid films constituted in this case by the wet surfaces of the container, which are constantly renewed.

At least one of the two above mentioned actions (stirring and film formation) may be completed by suitable means.

For instance, as hereinafter described with reference to Fig. 1, by a spiral rib or piece rotating in the cylinder; and/or (Fig. 2) by one or several cylinders disposed inside the first one; and/or by plates of any suitable shape having relative movements of immersion, then extraction, with respect to the culture liquid; or by static devices including for instance inclined planes on which the liquid is caused to flow.

Finally, means are provided, if necessary, for maintaining a given temperature in the apparatus, which means may consist in heating resistors or include the preliminary heating of the fluids intended to pass through the apparatus, etc.

Fig. 1 shows an apparatus including a cylindrical tank and complying with the above conditions.

It is essentially constituted by a horizontal or substantially horizontal drum, provided at its end with two trunnions 2 and 3 resting in ball bearings 4 and 5. This drum is given a rotary movement through a pulley 6 driven by a motor 7.

The trunnions are closed by discs 8 and 9 fixed to the apparatus frame and removably mounted thereon. Baffle joints ensure bacteriological sterility. The tubes and other parts necessary to the operation of the apparatus enter the cylinder through discs 8 and 9 in which they are fitted with fluidtight joints.

The inner wall of the drum is provided with a spiral-shaped rib 10 the pitch and height of which are determined by the type of operation that is considered. Heating at constant temperature is obtained either by placing the drum in a thermostatic vessel or by disposing electric resistances on the outer surface or even on the inside of the drum (or through any other means).

The thermostatic system, whatever it may be, will be adjusted by a regulator 11 dipping in the culture itself.

Inflow of the fresh medium is ensured through a pipe 12 at one of the ends. The flow rate is adjusted, for instance by a pump, for instance a variable delivery pump 50. Removal of an equivalent amount of liquid is ensured by an adjustable siphon 13 connected to the other end of the drum. Instead of a siphon, I might also use an adjustable delivery pump or any other adequate device. The device further includes a gas inflow tube 14 (for air or any other suitable gaseous mixture, sterilized by heating or filtering) and an outlet tube 15. Other tubes, such as a special pipe 16 for inoculation, may be provided.

I may even, if so desired, provide inside the apparatus a source of radiation (infra-red, ultra-violet, X-rays, etc.).

It should be noted that, in the embodiment shown, the level H of the liquid (culture in treatment) is higher than the height h of the spiral rib 10. It is also visible that the liquid is caused to circulate constantly in a closed circuit along the path indicated by arrows *fifa*. The desired stirring action is thus obtained, which ensures homogeneity of the culture in all of its parts, despite the addition of liquid at 12 and the removal of culture at 13.

In the embodiment of Fig. 2, cylinder 1 is provided with coaxial cylinders, having continuous or perforated surfaces, such as 22, 23, of lengths and diameters smaller than those of the drum, to which they may be secured by braces 24, 25, 26, 27. But they might turn independently of said drum, possibly in the opposite directions, being driven through mechanisms (for instance concentric shafts) such as diagrammatically shown in dotted lines (reference characters 101 to 104). In this case, the above mentioned braces, instead of fixing the cylinders with respect to one another would serve only to keep them centered with respect to one another. They may also be provided with helical or other ribs analogous to rib 10 (Fig. 1) to ensure a thorough stirring. Eventually, drum 1 might be stationary and only cylinders 22, 23, rotary, an arrangement which might facilitate the inflow and outflow of the liquids and gases. Anyway, as they are open at both ends (so as to enable air or gases to circulate freely therethrough) and partly immersed, cylinders 22, 23 increase the areas of interchange between the liquid and gaseous phases.

In some cases, and in particular in that of apparatus of great size, it is of interest to provide as high as possible a bacteriological sterility, through suitable means. These means may be constituted, in particular, by baffles provided in the apparatus bearings and provided with sterilizing devices such as ultra-violet ray tubes receiving a shape adapted to the desired purpose. Thus, although these bearings are not perfectly gastight, sterility is maintained since the gases which may enter through the joints are sterilized.

On Fig. 3, which illustrates one embodiment of such means, the end of trunnion 3 carries a coaxial cylindrical element 140, provided with a hole in its base 150. Between cylinders 3 and 140 there is provided a cylindrical element 160 rigid with closure plate 9, which is provided with a flange 170. On either side of cylinder 150 are provided mercury vapour tubes 180, for instance spiral wound, which are fed with current from 75

an external source by means of terminals, not shown on the drawing, fixed to the closure plate. Any air which may enter along the path indicated by the arrows is therefore necessarily subjected to the ultra-violet radiation and therefore sterilized.

Closure plate 9 is fixed. It is, for instance, rigid with the apparatus frame or bearing 5 on which rest the rotary drums. No mechanical contact exists between the elements of the trunnion and those of the closure plate.

In an apparatus such as those shown by way of example for carrying out the invention, a slight overpressure may be provided in the container, which makes it possible to prevent ozone formed by the ultra-violet rays for instance, to penetrate to the inside. The atmosphere might then become toxic. It suffices, in order to obtain this overpressure, suitably to adjust the flow rates through conduits such as 14 and 15 (Fig. 1).

The operation of such an apparatus, for instance that of Fig. 1, takes place as follows:

Drum 1 is first filled with fresh nutrient medium up to a level corresponding to the volume V which it is desired to maintain permanently in the apparatus, and therefore to the desired output per hour. A sterilization is performed by steam or other suitable means, or by making use of the thermostat heating device.

Once the respective tubes have been connected and the temperature is correct, the medium is inoculated in a sterile manner through tube 16 and the motor is started. The speed of this motor will be chosen so as to obtain an efficient and quick stirring. Stirring is achieved in the plane perpendicular to the axis through rotation itself and in the plane of the generatrices by spiral rib 10. Equilibrium between the gaseous and liquid phases will be further ensured by the large area of interchange constituted by the liquid surface and the wet surfaces of the drum and spiral rib. Growth of the culture is then studied by collecting samples at time intervals until the desired density is obtained.

From this time on, production goes on in a continuous manner by feeding fresh liquid at 12 according to the desired output rate. In the embodiment shown by the drawing, siphon 13 supplies an output of culture at the rate of the feed of fresh medium.

If ratio D is lower than the above mentioned limit or nearly equal to this limit, the apparatus supplies a culture which is perfectly homogeneous and the density of which remains constant both with relation to space and to time. This apparatus therefore works at the most favorable density and the yield is optimum.

The production can be stopped instantaneously by stopping the delivery and again started very quickly without requiring a new sterilizing or inoculating as long as sufficient portion of the cells is still living.

Furthermore, inside limits determined by the capacity of the apparatus and the properties of the microorganism that is used, the rate of production per hour may be modified at any time and given the desired value by modifying volume V, that is to say the level of the liquid in the apparatus and modifying the output rate accordingly, the ratio D remaining the same or still complying with the same condition. I might also modify the output rate without modifying volume V.

In a general manner, the rate of growth will

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automatically become adjusted in accordance with the output rate

$$\left(\mu = \frac{D}{0.69}\right)$$

whereby my method makes it possible to adjust the rate of growth of the culture and to give it any value lower than or nearly equal to the maximum rate of growth of the micro-organism that is considered in the medium used, i. e. to fix and determine one of the essential characteristics of growth, upon which most of the qualities and properties of the product depend. My method therefore truly makes it possible to obtain adjustment at will of the rate of growth.

Of course, while operating in a continuous manner, I might modify at will, for given periods of time, the rates of output.

In a general manner, while I have, in the above description, disclosed what I deem to be practical and efficient embodiments of my invention, it should be well understood that I do not wish to be limited thereto as there might be changes made in the arrangement, disposition and form of the parts without departing from the principle of the present invention as comprehended within the scope of the accompanying claims.

What I claim is:

1. An apparatus for the obtainment of a micro-organism culture in liquid form which comprises, in combination, a cylindrical drum of substantially horizontal axis to contain the liquid culture in treatment, at least one other drum open at both ends mounted in said first mentioned drum coaxially therewith, the first mentioned drum having end walls extending continuously, in the vertical plane of said axis, from the lowest generatrix of said first mentioned drum to a level higher than the level of the lowest generatrix of the second mentioned drum, whereby liquid may be kept in the first mentioned drum at a level such that the second mentioned drum constantly dips in this liquid, means for rotating at least one of said drums, means for continuously feeding fresh nutrient medium into said first mentioned drum at one end thereof, and means for continuously removing culture from said first mentioned drum at the other end thereof.

2. An apparatus for the obtainment of a micro-organism culture in liquid form which comprises, in combination, a cylindrical drum of substantially horizontal axis to contain the liquid culture in treatment, at least one other drum open at both ends mounted in said first mentioned drum coaxially therewith, the first mentioned drum having end walls extending continuously, in the vertical plane of said axis, from the lowest generatrix of said first mentioned drum to a level higher than the level of the lowest generatrix of the second mentioned drum, whereby liquid may be kept in the first mentioned drum at a level such that the second mentioned drum constantly dips in this liquid, means for rotating at least one of said drums, means for continuously feeding fresh nutrient medium into said first mentioned drum at one end thereof, means for continuously removing culture from said first mentioned drum at the other end thereof, and a helical rib carried by said rotating drum for stirring said liquid culture.

3. An apparatus for the obtainment of a micro-organism culture in liquid form which comprises, in combination, a cylindrical drum of substantially horizontal axis to contain the liquid structure in treatment, at least one other drum, open

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at both ends, mounted in said first mentioned drum coaxially therewith, the first mentioned drum having end walls constituted by annular inward flanges of an inner diameter smaller than the diameter of the second mentioned drum, whereby liquid may be kept in the first mentioned drum at a level such that the second mentioned drum constantly dips in said liquid, cylindrical hollow trunnions rigid with said first mentioned drum extending outwardly from the inner edges of the end walls of said first mentioned drum, bearings for supporting said trunnions, fixed plates located opposite the outer ends of said trunnions, cooperating baffle means carried by said plates and said trunnions to restrict communication between the inside of said first mentioned drum and the external atmosphere, conduit means extending through one of said fixed plates for continuously adding fresh nutrient medium into said first mentioned drum, conduit means extending through the other of said plates for continuously removing culture from said first mentioned drum, a helical rib carried by the inner wall of said first mentioned drum for stirring said liquid culture therein, and means for rotating said first mentioned drum about its axis.

4. An apparatus according to claim 3 further including a mercury vapour tube wound helically in the space between said baffle means for sterilizing gas circulating through said space.

5. An apparatus for the obtainment of a micro-organism culture in liquid form which comprises, in combination, a cylindrical drum of substantially horizontal axis to contain the liquid structure in treatment, at least one other drum, open at both ends, mounted in said first mentioned drum coaxially therewith, the first mentioned drum having end walls constituted by annular inward flanges of an inner diameter smaller than the diameter of the second mentioned drum, whereby liquid may be kept in the first mentioned drum at a level such that the second mentioned drum constantly dips in said liquid, cylindrical hollow trunnions rigid with said first mentioned drum extending outwardly from the inner edges of the end walls of said first mentioned drum, bearings for supporting said trunnions, fixed plates located opposite the outer ends of said trunnions, cooperating baffle means carried by said plates and said trunnions to restrict communication between the inside of said first mentioned drum and the external atmosphere, conduit means extending through one of said fixed plates for continuously adding fresh nutrient medium into said first mentioned drum, conduit means extending through the other of said plates for continuously removing culture from said first mentioned drum, conduit means extending through one of said fixed plates for feeding gas under pressure into said first mentioned drum, to maintain therein a pressure slightly above the pressure in the external atmosphere, a helical rib carried by the inner wall of said first mentioned drum for stirring said liquid culture therein, and means for rotating said first mentioned drum about its axis.

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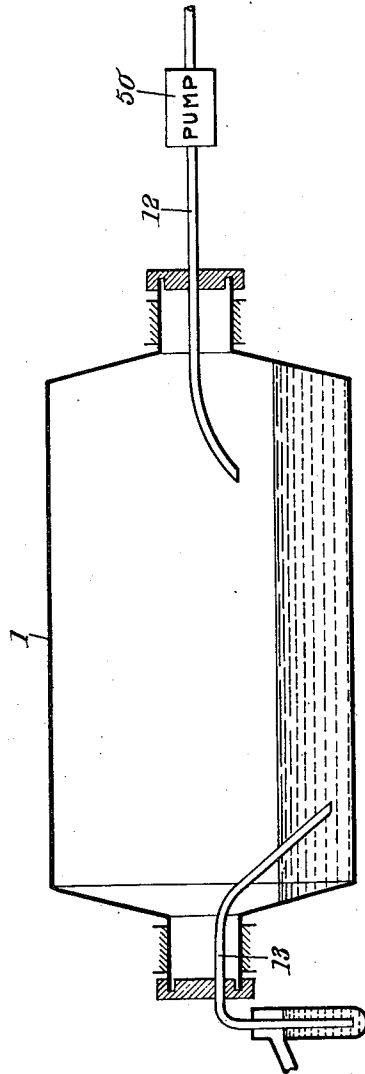
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2,822,319

METHODS FOR THE CULTIVATION OF MICRO-ORGANISMS

Filed Aug. 17, 1954





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2,822,319

METHODS FOR THE CULTIVATION OF  
MICRO-ORGANISMS

Jacques Monod, Paris, France

Application August 17, 1954, Serial No. 450,308

2. Claims. (Cl. 195-115)

The present invention relates to methods for the cultivation of microorganisms, its object being to achieve a self-stabilizing continuous production of the desired organism, with an adjustable output and a high yield, while maintaining constancy and homogeneity of the characteristics of the product, and providing the possibility of influencing and controlling the composition and properties of the cellular and other material, formed as a result of growth.

The essential idea of my method (and that which distinguishes it from any method previously employed in the laboratory or in industry for the cultivation of microorganisms) is to set up the culture as a permanent steady-state system in which the growth rate of the organisms is determined, controlled and permanently maintained at a chosen value, through the agency of a specific, chosen, limiting condition.

Figure 1 diagrammatically illustrates a fermenter for carrying out my method. The organisms are grown in fermenter 1 maintained at the desired temperature by appropriate means. New medium is continuously flown into fermenter 1 through pipe 12, at a constant rate, determined by, and adjustable through, any convenient means of pumping or control such as 50. A volume of culture exactly equivalent to the inflow of new medium, is constantly removed from fermenter 1 through evacuating pipe 13. The volume of culture in fermenter 1 therefore remains constant. It can, however, through proper means, be adjusted to any desired value within the limits of the capacity of the fermenter. Means of efficient stirring are provided in fermenter 1 in order that the culture should remain completely homogeneous, i. e. substantially identical in composition throughout its volume, in spite of the continuous addition of new medium. In other words, the formation of a gradient of any sort within the liquid in treatment is avoided, so that any small element of volume within the culture may be considered equivalent to any other element of the same size.

To sum up: the set up may be described as allowing continuous, homogeneous and rate-controlled dilution of the culture by the new medium, with simultaneous collection of spent culture.

In order now to understand the principle involved in the steady-state control of the growth rate with such a system, I will first consider the growth of a population of microorganisms in fermenter 1 when the dilution system is not working.

As it is well known the growth of a population of microorganisms in an adequate nutrient medium, follows an exponential law, expressing the fact that the mean generation time, or its inverse the growth-rate, remains constant, as long as conditions in the medium remain compatible with such constancy. However, as a result of the growth and metabolism of the organisms, the composition and properties of the medium (if it is not renewed) become altered in such a manner that the growth rate must gradually decrease and eventually fall to zero,

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when the population becomes stationary. The nature of the factor in the medium which thus becomes limiting depends on the composition of the medium and on the properties of the organism. Two classes of limiting factors are to be considered. (1) The decrease in concentration and eventually the depletion of one essential nutrient source. (2) The increase of concentration of a toxic product of the metabolism. If the composition of a medium could be permanently stabilized, in particular with respect to the limiting factor, growth of the organisms should continue indefinitely, at a rate determined by the constant concentration of the limiting factor. If moreover, the concentration of the limiting factor could be adjusted at will to any value corresponding to a given growth rate, it would be possible to control the growth rate of the organisms. The principle of my method consists in choosing the composition of the medium and the rate of dilution of the culture in fermenter 1 in such a manner (given the requirements and properties of the organisms) that a steady state is reached with respect to a given limiting factor, the concentration of which determines and controls the growth rate of the organisms.

How this is achieved will become clear when considering a number of concrete examples.

I will take as example the case of the bacterium *Escherichia coli* growing at 37° in a medium of the following approximate composition:

|   | Gr. per l. |
|---|------------|
| 30 Glucose -----                        | 5          |
| Ammonium sulfate -----                  | 2          |
| Acid potassium phosphate -----          | 10         |
| Magnesium sulfate -----                 | 0.2        |
| Ferrous sulfate -----                   | 0.002      |
| 35 Potassium hydroxide, g. s. for pH 7. |            |

In this medium and with this organism, the limiting factor is glucose, because all other essential nutrients are in relative excess with respect to the requirements of the organism, while the strong buffering capacity of the potassium phosphate prevents any significant change in pH. The organisms accordingly grow at a rate corresponding to about 1.2 doublings per hour, as long as the concentration of glucose in the medium does not become very small (below 0.1 gram per liter). If the medium is, not renewed, growth stops when all the glucose is exhausted.

It will be supposed that, in a culture growing under these conditions in this medium, the growth is nearing its completion, when already above 95% glucose has been consumed, and the culture approaches the point where the growth rate shall begin to decline, due to the decrease in glucose concentration. The flow system is now started as above set forth, and the rate of dilution is set at a value either slightly or more or less significantly below the rate that would exactly equilibrate the maximal growth rate of 1.2. The concentration of glucose in fermenter 1 will tend to increase, due to the inflow of new medium. In the meantime, the density of organisms in the culture will also increase, since the maximal rate of growth exceeds the rate of dilution of the culture. However, as the mass of the organisms increases in the fermenter, the rate at which they consume glucose also increases, and tends to decrease the concentration of glucose, until this in turn begins to limit the growth rate of the organisms, and therefore to counteract the increased consumption of glucose.

It can be predicted, on the basis of a simple mathematical theory, and it was experimentally verified that, under these conditions, the system tends toward a steady-state where the concentration of glucose is just that which limits the growth rate of the organisms to the value corresponding to the dilution rate that has been set. Such

a steady-state can be indefinitely maintained. Under such steady-state conditions, therefore, the rate of growth of the organisms is in fact controlled and determined by the dilution rate, through the intermediary of the glucose concentration.

The working of such a system and in particular the steady-state condition, can be described in mathematical terms as follows.

Let  $x_f$  be the mass of organisms in the fermenter,  $x_p$  be the mass of organisms collected as product,  $x_t$  the total mass of organisms in the system, such that

$$x_t = x_f + x_p$$

deriving with respect to time, I have

$$\frac{dx_t}{dt} = \frac{dx_f}{dt} + \frac{dx_p}{dt} \quad (1)$$

The output of the system, i. e. the rate of product collection, is evidently

$$\frac{dx_p}{dt} = Dx_f \quad (2)$$

where  $D$  is the rate of dilution defined as the ratio of the amount of liquid flown through the fermenter per unit of time to the volume of liquid in the fermenter.

The rate of total increase is proportional at any time to the mass of organisms in the culture  $x_t$ , and to their growth rate.

$$\frac{dx_t}{dt} = \mu x_t \quad (3)$$

where the growth rate,  $\mu$ , is defined, for convenience, as the number of times the mass of organisms is multiplied by  $e$  ( $=2.71828$ ) per unit of time; combining Equations 1, 2 and 3 gives

$$\frac{dx_f}{dt} = x_f(\mu - D)$$

The condition for a steady-state equilibrium to be reached is that  $D$  be set at any value which does not exceed the maximal growth rate of the organism considered in the medium considered, at saturating concentrations of the limiting factor. When the equilibrium is reached,

$$\frac{dx_f}{dt} = 0 \text{ and } \mu = D$$

With respect to the concentration and consumption of the limiting nutrient factor, I may write (see J. Monod.—Ann. Inst. Pasteur, 79, 1950, 390):

$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{R} \frac{dx_t}{dt}$$

where:

$S$  = concentration of the limiting factor in the fermenter,  
 $S_0$  = concentration of the limiting factor in the new medium,

$R$  = the yield constant expressing the amount of limiting nutrient consumed in the formation of a unit mass of cells.

In the steady-state, the consumption of limiting nutrient, and the production of the system is given by the formula:

$$\frac{dx_p}{dt} = RD(S_0 - S)$$

Moreover, it is an experimental fact that in general  $S$  is negligible by comparison with  $S_0$ . In other words, the limiting nutrient is, for all practical purposes, entirely consumed.

For instance, in the case of *E. coli* growing in a medium where glucose (5 gr./liter) is the limiting factor, it is found that the system can easily be stabilized and indefinitely maintained at any dilution rate from zero to 0.8 or 0.9 with a steady production of about 0.3 gram dry

weight of organisms per gram of glucose flown into the fermenter.

I will now describe a detailed example of an actual experimental run made by me and in which the organism used was *E. coli*, strain "ML," the medium that above described, the fermenter of the type described in my U. S. patent application Ser. No. 270,125 for "Apparatus for the Cultivation of Microorganisms," filed February 6, 1952.

The sterilized fermenter was filled with 50 liters of sterile medium, and the temperature brought to 37° C. The fermenter was inoculated with 500 milliliters of a batch culture of *E. coli* (strain ML), grown on the same medium. The density of the culture was followed turbidimetrically, using a photoelectric densitometer. In addition, viable counts and determinations of dry bacterial mass were run at intervals on small samples of the culture. Glucose determinations were also run at intervals on supernatants of the centrifuged culture samples.

The culture was first allowed to grow without dilution of the medium. After a short lag period of about ½ hour (inoculation time being taken as zero time), the density of the culture increased exponentially at a rate corresponding to a mean generation time of about 50 minutes. Five and a half hours after inoculation, the culture had reached a density of about  $5 \times 10^9$  cells per milliliter and contained about 1.4 grams dry weight bacterial substance per liter.

At this time, the continuous dilution system was started at a rate of 40 liters per hour. The dilution rate ( $D$ ) as defined above was therefore

$$\frac{40}{50} = 0.8$$

The density of the culture thereafter continued to increase very slowly in the fermenter for about three hours. It stopped increasing at the ninth hour after inoculation and thereafter remained completely stable (within the limits of sensitivity of the optical density measurement, i. e.  $\pm 2$  or 3 percent), through the run, which was continued for one week. Repeated determinations of bacterial mass, and of glucose were made during this period on samples taken from the culture. The bacterial mass per unit of volume remained stable at 1.5 gram dry weight per liter. Only a trace of glucose (less than 100  $\mu$ g. per milliliter) was present, showing that virtually all the glucose was effectively used for growth. Moreover, the size and shape of the cells, as examined with the microscope remained quite constant, and characteristic of so-called "young" viable cells, while the pH of the culture remained also perfectly constant (although it was not independently or especially controlled). The production amounted to about 1.000 liter of fully-grown culture liquid and to 1.5 kg. of dry bacterial mass per day.

It will be readily understood that the same functional principles apply when the limiting factor is not the source of carbon, but another essential nutrient source. As a further example I may state the case of *E. coli* growing in a medium of the same qualitative composition as above, but in which the ammonium sulfate concentration is only 1 gr. per liter while the glucose concentration is raised above 6 gr. per liter. In this medium, it is the nitrogen supply which becomes limiting, and it is the concentration of  $(\text{NH}_4)$  which controls the growth-rate in the steady-state condition. The production can be stabilized in the steady state up to dilution rates of 0.8 or 0.9 and the production amounts to 2 grams dry weight of organisms, per gram of ammonium sulfate flown into the fermenter.

Similarly, by adequate alterations of the composition of the medium, any essential nutrient may be used as limiting factor, and in the case of organisms which require special growth-factors or vitamins, such special requirement may be used for the establishment of a steady state and for the control of the growth rate.

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For instance a strain of *Bacillus subtilis* which requires methionine for growth can be grown and stabilized in a continuous dilution system in a medium of the same composition as the one given above and containing in addition 10 mg. of methionine per liter. The limiting factor here is methionine, and when the dilution rate is set at a value not exceeding the maximal growth rate, a steady state is eventually established where the concentration of methionine in the fermenter controls the growth rate at the value corresponding to the chosen dilution rate.

As stated above, the principle involved in the establishment of a steady-state continuous dilution culture system where the growth rate is controlled by the dilution rate, is also applicable when the factor controlling growth is not the exhaustion of an essential nutrient, but the accumulation of a product of metabolism. Such a product may for instance be the acid or alternatively the alkali equivalents liberated as a result of metabolism.

As an example, suppose the first medium given above is modified by cutting the phosphate concentration down to 1 gr. per liter. The metabolism of glucose on the one hand, and the consumption of  $(\text{NH}_4)^+$  ions on the other, liberate acid equivalents and tend to acidify this relatively unbuffered medium. Under these conditions the growth of *E. coli* in this medium is not limited by the exhaustion of a nutrient source, but by the acidification of the medium. This limiting factor can then be used to establish a steady state and control the growth rate, by setting the rate of dilution at any convenient value not exceeding the maximal rate of growth of the organism at the pH of the new medium. Similarly by growing microorganisms in a medium where their metabolism tends to accumulate alkali equivalents, steady-state systems can be established where alkali is the controlling limiting factor. Such is the case for instance when e. g. yeast is grown in a medium where an organic acid e. g. succinic acid is the main or only source of carbon. Other products of metabolism can similarly be used as controlling factors in steady-state culture systems established in accordance with the principle of my method. For instance again, in anaerobic cultures of yeast, alcohol can be used as the controlling factor, allowing the establishment of permanent, steady-state anaerobic cultures, growing at constant rate, in a constant concentration of alcohol.

The range of applicability of my method is therefore extremely wide, since it can be used with any micro-organism which can be grown as a homogeneous suspension, and since a wide variety of limiting conditions can be used for control of the steady state, depending on the properties of the organism, and on the type of production required.

Actually, and only as examples, the following organisms have been grown according to my method.

Bacteria: *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium diphtheriae*, *Clostridium welchii*, *Clostridium botulinum*, *Streptococcus lactis*, etc., etc.

Yeasts: *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, *Torulopsis utilis*, etc.

Moulds: *Ophiostoma*.

Protozoa: *Chlamydomonas* sp., *Euglena* sp., *Polytomella* sp.

One of the main advantages of my method is the continuity and stability of the production. It is obvious that in the steady-state not only is the concentration of the limiting factor constant, but also that of all other factors, components or nutrients in the medium. Complete stability in all respects is therefore obtained without any controls and adjustments as usually performed in the conventional methods. This in turn insures stability of the properties of the product, one of the most desirable and also one of the most difficult results to be obtained in biological productions.

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Another conspicuous advantage is the maximal utilization of fermenter capacity. In all other methods of cultivation, whether continuous or not, the density of organisms in the culture varies, either in space, or time, or both. With the present system, where growth takes place in a single homogeneous phase, the density of organisms throughout the fermenting volume remains constant and maximal, insuring therefore a maximal output, since the instantaneous output of any system is always proportional to the mass of organisms in treatment.

Another particularly significant and quite unique advantage of the method, is the fact that by an adequate choice of the limiting factor and of the growth rate, the physiology and biochemistry of the organisms can be influenced to a very remarkable extent. As an example, bacteria or yeasts grown according to my method, with glucose (or other carbohydrate) as limiting factor in the steady state, tend to synthesize relatively less polysaccharide and more protein than the same organisms grown in a medium where the source of nitrogen is the controlling factor. Moreover, the relative composition of the organisms depends to a very significant extent on the growth-rate which is imposed upon them.

Similarly, it is well known that the physiology and biochemistry of microorganisms, and the type of products which they synthesize as they grow depends on the pH of the medium. My method affords the possibility of setting up cultures in which the pH will spontaneously be stabilized at a desired value, determined once and for all as corresponding to a given dilution-rate in a given medium.

It should be noted that in all that precedes, it has been assumed, for the sake of simplicity and clarity, that the various components of the medium were always added as a single mixture, through a single pipe, into fermenter 1. In actual practice, it may be convenient to add separately, through separate pipes, certain of the components. This does not in any way alter the principle of the method. It should also be pointed out that the method is equally applicable to aerobic and anaerobic organisms, provided the culture in fermenter 1 is maintained in proper equilibrium with the proper gas phase.

The present application is a continuation-in-part of my application Ser. No. 197,867, filed November 28, 1950.

What I claim is:

1. The method of growing microorganisms in continuous culture and of controlling their growth rate, which comprises continuously and simultaneously adding fresh medium at a constant volumetric rate to, and removing culture in liquid state at the same rate from, a fermenter, while mixing and stirring said culture to keep it homogeneous with respect to all biological and chemical characteristics in the totality of its volume, the rate at which components of the medium are added being chosen such that the opposed effects of the addition of medium components on the one hand and the influence on a growth controlling factor by the consumption of said components by the microorganisms on the other hand create a steady-state where the rate of multiplication of the microorganisms is actually limited and controlled to remain constant, in all parts of the culture simultaneously and at all times, by said rate of addition of medium components.

2. The method of controlling the growth in a micro-organism culture which comprises actively stirring and mixing the culture to maintain it substantially homogeneous whereby each portion of the total volume thereof has substantially the same chemical and biological composition, and simultaneously adding nutrient to the culture and removing culture therefrom at the same constant volumetric rate, the ratio of nutrient medium added per unit of time to total volume of culture being chosen such that the growth rate of microorganisms throughout the culture is maintained at a constant value due to the quantity of a given ingredient contained in the added

nutrient medium being such that it is supplied at a rate less than the maximum potential consumption by the microorganisms.

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