United States Patent [19]

Smith et al.

[11] **3,893,887**

[45] **July 8, 1975**

[54] **METHOD FOR RAPID HARVESTING OF ROLLER CULTURE SUPERNATANT FLUID**

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[73] Assignee: **Duke University Inc.,** Durham, N.C.

[22] Filed: **May 30, 1974**

[21] Appl. No.: **474,369**

Related U.S. Application Data

[62] Division of Ser. No. 377,806, July 9, 1973, Pat. No. 3,847,749.

[52] **U.S. Cl.** **195/1.7;** 195/127

[51] **Int. Cl.²** **C12B 1/00;** C12K 9/00

[58] **Field of Search** 195/127, 1.7, 1.8

[56] **References Cited**

UNITED STATES PATENTS

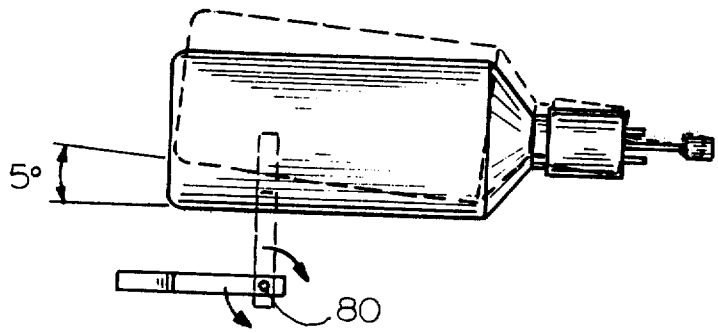
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Primary Examiner—Alvin E. Tanenholtz

[57] **ABSTRACT**

The method of using a conventional perfusion tissue culture apparatus using roller bottles is modified such that the supernatant fluids may be removed automatically by periodic interruption of normal bottle rotation and cell growth, during which the bottles are tilted, are drained of their respective supernatant fluids, are replenished with a predetermined amount of fresh medium, are then lowered and normal rotation and cell growth is resumed until the fluid removing cycle is repeated.

5 Claims, 5 Drawing Figures



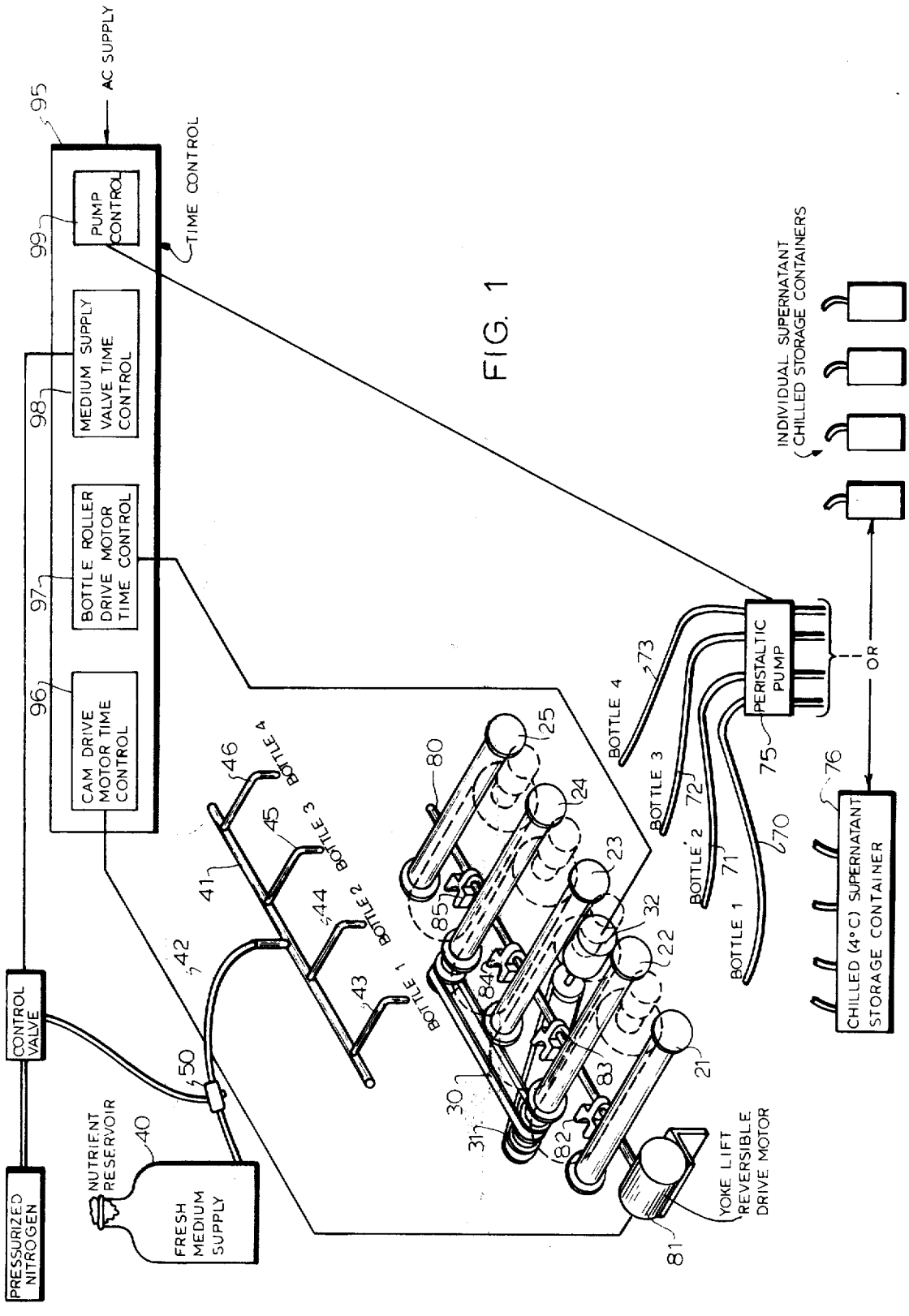

AC SUPPLY
95
TIME CONTROL
99
PUMP CONTROL
98
MEDIUM SUPPLY VALVE TIME CONTROL
97
BOTTLE ROLLER DRIVE MOTOR TIME CONTROL
96
CAM DRIVE MOTOR TIME CONTROL
INDIVIDUAL SUPERNATANT CHILLED STORAGE CONTAINERS
PERISTALTIC PUMP
OR
BOTTLE 4
BOTTLE 3
BOTTLE 2
BOTTLE 1
73
72
71
70
75
76
CHILLED (4°C) SUPERNATANT STORAGE CONTAINER
CONTROL VALVE
PRESSURIZED NITROGEN
NUTRIENT RESERVOIR
FRESH MEDIUM SUPPLY
40
50
42
41
46
45
44
43
80
85
84
83
82
81
25
24
23
32
22
21
30
31
YOKE LIFT REVERSIBLE DRIVE MOTOR

FIG. 1

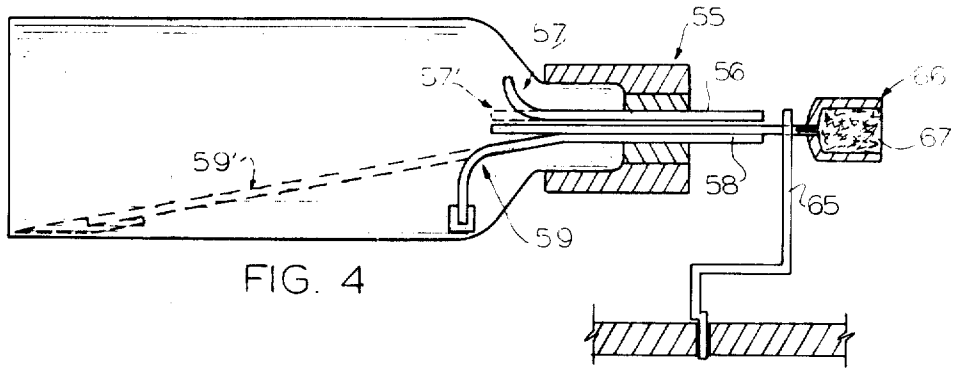


FIG. 4

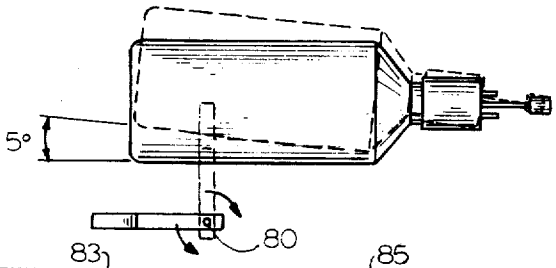


FIG. 5

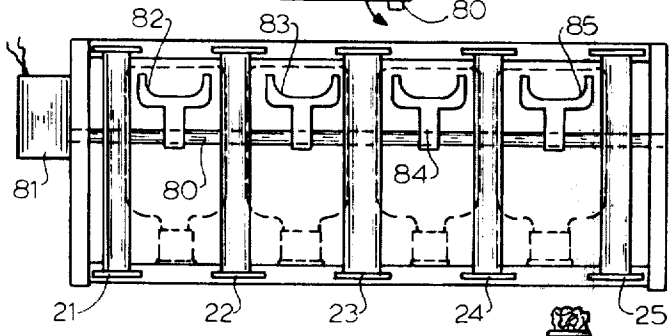


FIG. 3

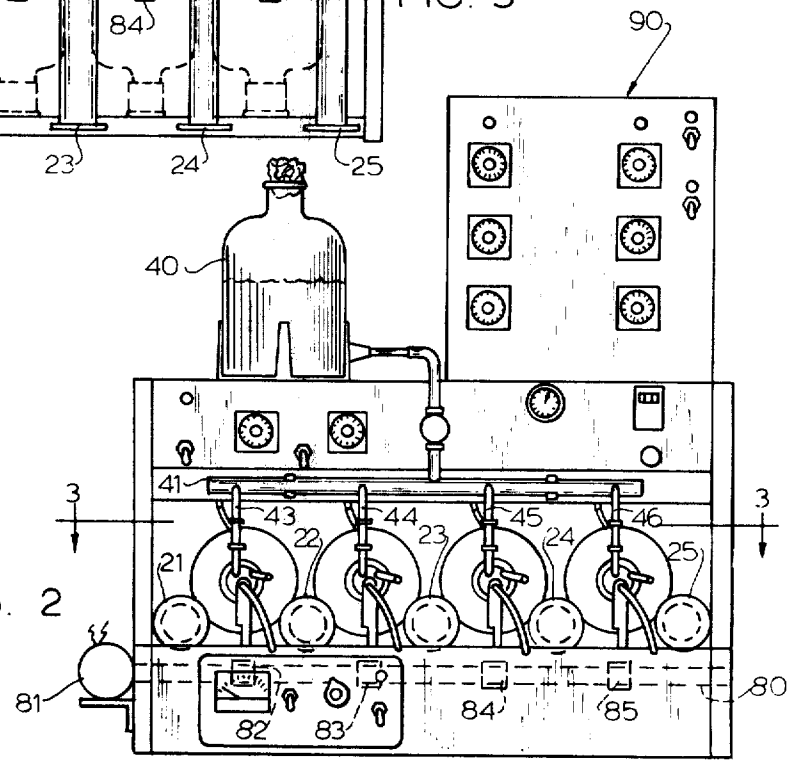


FIG. 2

METHOD FOR RAPID HARVESTING OF ROLLER CULTURE SUPERNATANT FLUID

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education, and Welfare.

CROSS REFERENCE TO RELATED APPLICATION

This application is a division of copending application Ser. No. 377,806, filed July 9, 1973, now U.S. Pat. No. 3,847,749, entitled "Apparatus and Method for Rapid Harvesting of Roller Culture Supernatant Fluid."

BACKGROUND OF THE INVENTION

1. Field of the Invention:

This invention relates to methods used in perfusion tissue culture wherein the cells are grown in bottles which rotate during the growth of the cells and are therefore referred to as roller bottles.

2. Description of the Prior Art:

The technique of growing cells in a medium which is placed in a bottle which is rotated during the growth of the cells has been recognized. The perfusion tissue culture technique has been widely practiced. References which in turn cite a multitude of other references include the following:

"Production and Purification of Large Amounts of Rous Sarcoma Virus," Ralph E. Smith and Eugene H. Bernstein, *Applied Microbiology*, March 1973, pp. 346–353.

"Comparison of Immature (Rapid Harvest) and Mature Rous Sarcoma Virus Particles," Kwok-Sing Cheung, Ralph E. Smith, Marie P. Stone, and Wolfgang K. Joklik, *Virology*, December 1972, pp. 851–864.

"Multi-Layer Perfusion Tissue Culture," R. Elsworth, *Process Biochemistry*, March 1970.

The commercial tissue culture roller bottle apparatus which has thus far been available has not provided a satisfactory means or method for removing the supernatant fluids automatically and at frequent intervals to enable rapid harvesting of the cells. As an example, the New Brunswick perfusion control apparatus which is referred to in the above references has been provided with a so-called perfusion swivel cap which allows fluids to be admitted to and removed from the bottles while the bottles are rotated but the conventional swivel cap arrangement does not allow for the complete removal of the supernatant fluids. Further, this type of conventional roller bottle tissue culture apparatus does not provide an overall system and method for operation on a programmed basis whereby the supernatant fluids from a plurality of roller bottles may be automatically and periodically collected in chilled flasks to arrest viral maturation, the medium automatically replaced and the virus cells maintained at physiological temperatures.

SUMMARY OF THE INVENTION

According to the method and apparatus of the invention the plurality of roller bottles conventionally employed in perfusion culture apparatus are mounted on and rotated by conventional bottle roller apparatus. Through use of a timing control the bottle rotating apparatus is periodically stopped and the bottles are tilted slightly by means of a plurality of fork or yoke like arms which are mounted on a common shaft turned by a reversible motor. In one direction of movement of the fork or yoke arms, the rear of each bottle is engaged by a fork arm which slides on the bottle surface and gradually tilts the bottle in some predetermined amount at which point the forks are brought to a rest with all of the bottles in a tilted position. Each bottle connects through an outlet tube to a suitable storage container. A pump is activated after the forks bring the bottles to a full tilt and the supernatant fluid in the bottles is then drained through the respective outlet tubes and collected either in a common or individual chilled storage flasks. To insure essentially complete emptying of each bottle the conventional straight entrance end of the outlet tube is given a relatively sharp bend within the swivel cap so that when the bottle is tilted essentially all of the fluid can be drained. The timing control now causes a predetermined amount of fresh medium to be added through a commercially available manifold-pinch valve arrangement and through inlet tubes passing through the swivel cap of each respective bottle. The reversible motor is then energized to cause the shaft on which the fork arms are mounted to rotate in a direction to allow the bottles to return to their normal horizontal position. The described cycle is repeated whenever it is desired to drain the supernatant fluid and add fresh medium.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a somewhat generalized schematic view of a roller tissue culture system according to the invention.

FIG. 2 is a front view of a modified commercially available roller tissue culture apparatus incorporating the invention.

FIG. 3 is a plan section view taken in the direction of line 3—3 of FIG. 2 and showing the yoke lifting arrangement.

FIG. 4 is a partial sectional, partial schematic view illustrating the modified swivel cap arrangement used in the invention apparatus.

FIG. 5 is a partial side elevation view of one bottle and one yoke lifter showing how the bottle is tilted for draining purposes.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The invention apparatus has been arrived at by modifying a conventional perfusion control apparatus as sold by New Brunswick Scientific Co., Inc. of New Brunswick, N.J. Such an apparatus is fully described in the company catalog entitled "Perfusion Control Apparatus, Models PF–4 and PF–8" dated Oct. 22, 1971 and labeled M1006–5050. The operation and construction of this apparatus is fully described in that catalog. Therefore, the present description will go only into enough detail for those skilled in the art to understand how the method and apparatus of the invention are related to the conventional method and apparatus and the mentioned New Brunswick apparatus will be used as a basis of comparison.

Referring to FIG. 1, there is shown a schematic diagram of the invention apparatus as well as the conventional apparatus with which it most closely relates. The conventional perfusion control apparatus includes five rollers, 21, 22, 23, 24 and 25. Rollers 22 and 24 are driven through belts 30 and 31 by a roller driven motor 32. Rollers 21, 23 and 25 are thus idler rollers and four bottles are received as indicated in dashed lines in FIG.

1 between each pair of rollers. When this roller driven motor **32** is energized, each of the four bottles tends to rotate in a conventional manner.

The nutrient is retained in a reservoir **40** which is connected to a conventional manifold **41** through a supply tube **42** and each of the four bottles are fed through individual supply tubes **43, 44, 45** and **46.**

A pinch cock valve **50** controls the gravity flow between reservoir **40** and manifold **41.** As best seen in FIG. **4,** each bottle is provided with a modified swivel cap **55.** The modified swivel cap includes an individual bottle inlet tube **56** which has a sharp upward bend **57** at the fluid discharge end as compared to the normally straight configuration, **57'**, shown in dashed lines. The manifold swivel cap also includes a fluid drain fluid **58** whose entrance end **59** is bent sharply downwardly as compared to the normally longer and straight configuration **59'**. The so called innoculation or gas exhaust line is stuffed with a suitable cotton wadding or other air filtering material **67.** A thin metal bracket **65** is conventionally used on each bottle and supports the front of the bottle during rotation as shown.

The fluid drain line **58** from each respective bottle connects to respective storage drain lines **70, 71, 72** and **73.** Lines **70, 71, 72** and **73** in turn pass through a peristaltic pump **75** and then to a suitable chilled supernatant storage container **76.**

A horizontal shaft **80** extends below and perpendicular to and rearwardly of the rollers **21, 22, 23, 24** and **25.** Shaft **80** is in turn connected through a suitable reversible motor gear drive **81** such that shaft **80** can be rotated back and forth upon suitable energization of motor drive **81.** Between each pair of rollers there are mounted on shaft **80** lifting yokes **82, 83, 84** and **85.** Referring particularly to FIGS. **1, 3** and **5** it can be seen that when shaft **80** rotates in a clockwise direction, as seen in FIG. **5,** each respective yoke lifts the rear portion of each respective bottle and causes the bottle itself to be tilted in some predetermined amount, e.g. 5°. Then when shaft **80** is rotated in a counterclockwise direction as seen in FIG. **5** the bottle is lowered back to a horizontal position. Motor drive **81** is purposely chosen so that this movement is slow and gradual and the timing of motor drive **81** is such that shaft **80** stops turning when the respective bottles have reached a maximum tilt. Further, each yoke is formed with its bottle engaging surface relatively smooth so that the yoke tends to slide along the length of the bottle during the tilting process. Since the rollers **21–25** are normally surfaced with a friction material the bottles do not tend to slide axially of the rollers **21–25.** Also the previously mentioned brackets **65** tend to maintain the bottles in their proper position. Thus, the bottles can be tilted, drained, replenished with medium, and lowered back many times without changing the relative position of the bottles on the rollers.

Since those skilled in the art will readily see the general type of timing controls called for by the invention, the same are only generally represented in FIG. **1.** In FIG. **2** the auxiliary timing controls are also shown at **90** as they might appear in an actual apparatus embodying the invention. Also in FIG. **2,** since many of the parts shown are conventional, only those parts of particular interest to the invention have been identified by numbers since those skilled in the art will readily see how the invention is incorporated into a conventional apparatus.

Referring back to FIG. **1,** the overall timing control generally designated **95** includes a time control for the yoke drive motor designated **96,** a time control for the bottle roller drive motor designated **97,** a time control for the nutrient supply control valve **98** and a time control designated **99** for the peristaltic pump designated **75.** The necessary timing circuitry to coordinate, interlock, etc. the individual timers is not shown, since those skilled in the art will readily see a great variety of timing and programming circuits suitable to the invention. In one embodiment, eight timers (Industrial Timers, Inc., Parsippany, N.J.) were required to allow the necessary operations. The main timer regulated the length of time between medium/add cycles. Two timers were provided: a three-hour timer was used for intervals of from 5 minutes to 3 hours, and a five-minute timer was used for intervals of 5 minutes or less. The minimum collection time was 30 seconds, in which bottles were rotated for 12 seconds, and the balance of time was required to complete the collection cycle. The bottle up timer regulated the length of time that the motor driving the cam shaft was in operation. This timer was regulated to stop the cams at their maximum vertical elevation. The delay timer provided a 5 second delay to allow medium drainage to the front of the bottles after they were tilted forward, and reversed the circuit to the motor driving the cam device. The pump-on timer activated a Kraft automatic pipettor (Model P300, Kraft Apparatus, Inc., Queens, N.Y.).

In operation, medium was pumped into prechilled collection vessels maintained either in an ice bath in the same room as the perfusion apparatus, or in an adjacent cold room maintained at 4° C (a hole was cut in the wall separating the rooms). The medium/add timer served the same function as in the unmodified perfusion system, namely, to activate a solenoid, which released pressure (provided by bottled nitrogen) on the automatic pinchcocks and allowed the addition of fresh medium by gravity flow. The amount of medium added to each bottle varied according to the frequency of collection. For example, 10 ml of medium was added per bottle during collection at five minute intervals, and 25 ml of medium was added during collection at 2 hour intervals. During extended perfusion cultures, the pH of the medium in the reservoir was readjusted daily by bubbling with carbon dioxide passed through a sterile cotton-plugged pipette. The bottle down timer lowered the bottles, and the reset timer prepared the electronic circuitry for the next cycle and started rotation of the apparatus of the invention.

A prototype apparatus has performed satisfactorily during numerous experiments for long-term collections of virus at frequent intervals. For example, roller culture bottles containing chick embryo fibroblasts transformed with Rous sarcoma virus (RSV) were maintained on the apparatus for over three weeks, with collection of supernatant fluid every 2 hours. The bottles were free of contamination during the entire period of collection, and no malfunction was encountered. Cell growth was vigorous under the perfusion conditions outlined. For example, a roller culture bottle maintained by perfusion culture yielded more than twice the number of cells obtained from a roller culture bottle maintained by daily medium changes.

The device was designed to study virus particles recently budded from the cell surface. During the course of an investigation reported elsewhere it was found that

ribonucleic acid (RNA) purified from RSV harvested at intervals of 2 hours or shorter was more uniform in size than the virus harvested at longer intervals (12 to 24 hours). The manual harvesting of supernatant fluids every 2 hours is tedious, and can be done for only a portion of the day. Since the modified perfusion allows automatic harvesting during the entire day, including weekends, the apparatus is now proving useful for obtaining large quantities of high quality RNA with relative ease. The perfusion culture technique outlined could also be used whenever sequential harvests of a virus, cell metabolite, or other extracellular material is desired.

A number of advantages can be seen in the method and apparatus just described. A principal advantage is that rapid harvesting can be accomplished automatically and with essentially complete removal of the supernatant fluids. The use of the peristaltic pump provides positive removal of the supernatant fluids and allows the various drain lines to be purged as required. Since the bottles are not shifted axially and lifted, and since the tilting is accomplished slowly and gradually, the cell growth is not substantially disturbed during the tilting and draining cycles. A typical work schedule according to the method of the invention would include the following steps:

1. Fill the fresh nutrient reservoir;
2. Admit to the bottles a predetermined amount of fresh nutrient and, if if not already accomplished, simultaneously pre-chill the storage container or containers;
3. Warm the bottle surroundings if not already done;
4. Start the bottle rollers;
5. Rotate the rollers for a predetermined time;
6. Stop the bottle rollers;
7. Tilt the bottles and hold tilted for a predetermined time;
8. Drain the supernatant fluids by positive pump action from the bottles while they are tilted;
9. Replenish the bottles with a predetermined amount of fresh nutrient while the bottles are tilted;
10. Lower bottles;
11. Resume rotation.

Those skilled in the art will readily appreciate that variations on the above method of using the invention can be adopted. For example, the fresh nutrient can be restored either with the bottles in a filted position or in a lowered position. It is preferred to do this when tilted to avoid a sudden rush of nutrient and a damaging action were the nutrient to suddenly flow as a wave from one end of the bottle to the other. Also the bottle warming and storage chilling steps can be accomplished at any suitable time. Considering that the prior art method required manual operations for all of the filling and removing steps indicated, it can be seen that the researcher is given a new tool and an extremely valuable apparatus and method for rapid harvesting.

What is claimed is:

1. The method of operating a roller tissue culture apparatus of the type having a plurality of parallel drive and idler rollers on which a plurality of roller culture bottles are rotated during cell growth and which permits supernatant fluids to be removed from the bottles and fresh nutrient fluid added on an intermittent basis, comprising the steps, under automatic programmed control of:

a. periodically interrupting the normal rotation of the bottles on the rollers;
b. during such interruptions and while leaving all of the mouth ends of the bottles positioned on the rollers, engaging all of the rear ends of the bottles with mechanical positioning means and gradually tilting all of the bottles with such positioning means to an acute angle of tilt sufficient to allow the supernatant fluid in each bottle to gradually flow to the respective lowest point within the mouth end of the tilted bottle and there collect proximate an open end of an effluent line proximate such point;
c. while holding the bottles so tilted, removing the supernatant fluid essentially completely from each of the bottles through such effluent lines by pumping;
d. gradually lowering all of the bottles with said positioning means until they assume the same respective positions on said rollers as were occupied prior to being tilted;
e. disengaging said positioning means from contact with said bottle rear ends;
f. at a selected time after the supernatant fluid has been removed from the bottles and prior to resuming their normal rotation on the rollers feeding a new supply of nutrient fluid into the bottles through an influent line; and
g. resuming the normal rotation of the bottles until the bottle rotation is again interrupted for the purpose of repeating the cycle.

2. The method of claim 1 wherein said new supply of nutrient fluid is fed to said bottles while they are tilted.

3. The method of claim 1 wherein said acute angle of tilt is 5°.

4. The method of claim 1 wherein during each interruption the rotation of all the bottles on the rollers is completely stopped.

5. The method of operating a roller tissue culture apparatus of the type having a plurality of parallel drive and idler rollers on which a plurality of roller culture bottles are rotated during cell growth and which permits supernatant fluids to be removed from the bottles and fresh nutrient fluid added on an intermittent basis, comprising the steps, under automatic programmed control of:

a. periodically interrupting the normal rotation of the bottles on the rollers by completely stopping such rotation;
b. during such interruptions and while leaving all of the mouth ends of the bottles positioned on the rollers, engaging all of the rear ends of the bottles with mechanical positioning means and gradually tilting all of the bottles with such positioning means to an acute angle of tilt sufficient to allow the supernatant fluid in each bottle to gradually flow to the respective lowest point within the mouth end of the tilted bottle and there collect proximate an open end of an effluent line proximate such point;
c. while holding the bottles so tilted, removing the supernatant fluid essentially completely from each of the bottles through such effluent lines by pumping;
d. at a selected time after the supernatant fluid has been removed from the bottles and while in said tilted position feeding a new supply of nutrient fluid into the bottles through an influent line;
e. gradually lowering all of the bottles with said positioning means until they assume the same respec-

tive positions on said rollers as were occupied prior to being tilted;

f. disengaging said positioning means from contact with said bottle rear ends; and

g. resuming the normal rotation of the bottles until the bottle rotation is again interrupted for the purpose of repeating the cycle.

* * * * *

UNITED STATES PATENT OFFICE

CERTIFICATE OF CORRECTION

Patent No. 3,893,887 Dated July 8, 1975

Inventor(s) Ralph E. Smith; Frank E. Kozoman

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

Column 3, line 15, the second appearance of "fluid" should be -line-.

Signed and Sealed this

fourteenth Day of *October 1975*

[SEAL]

Attest:

RUTH C. MASON
Attesting Officer

C. MARSHALL DANN
Commissioner of Patents and Trademarks