CONVENTION

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609058

APPLICATION FOR A STANDARD PATENT

I/We DOUGLAS BRUCE KELL

of Cwmdarren,
Pentbont-Rhydybeddau,
Cwmsylog,
Aberystwyth,
UNITED KINGDOM.

hereby apply for the grant of a standard patent for an invention entitled:

DETERMINATION OF BIOMASS

which is described in the accompanying complete specification.

Details of basic application

Number of basic application: 8622747

Convention country in which basic application was filed: UNITED KINGDOM

Date of basic application : 22 September 1986

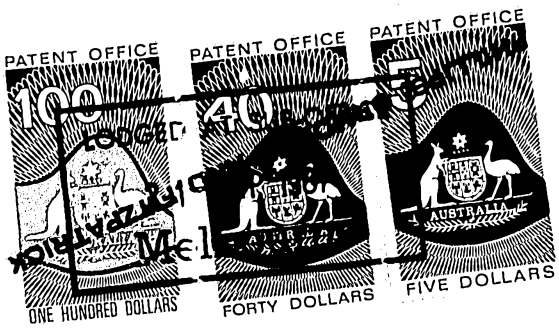
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Dated: 17 September 1987

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By:



Our Ref : 68505
POF Code: 1453/75233

LODGED AT SUB-OFFICE
21 SEP 1987
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APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 29-1-91

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DECLARATION FOR A PATENT APPLICATION

In support of the Convention application made by

DOUGLAS BRUCE KELL

(hereinafter called "applicant") for a patent for an invention entitled

DETERMINATION OF BIOMASS

I, DOUGLAS BRUCE KELL of:

Cwmdarren, Penbont-Rhydybeddau, Cwmsymlog, Aberystwyth, Dyfed, SY23 3HB, United Kingdom,

do solemnly and sincerely declare as follows:

1. I am the applicant.

2. I am the actual inventor of the invention.

The facts upon which I am entitled to make the application are as follows:

I have the consent of IMPERIAL CHEMICAL INDUSTRIES PLC to make this application.

3. The basic application for patent or similar protection on which the application is based is identified by country, filing date and basic applicant as follows:

United Kingdom, 22 September 1986, IMPERIAL CHEMICAL INDUSTRIES PLC.

4. The basic application referred to in paragraph 3 hereof was the first application made in a Convention country in respect of the invention the subject of the application.

Declared at: Aberystwyth

Dated: 31 October 1989

DOUGLAS BRUCE KELL

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To: The Commissioner of Patents

0119x

(12) PATENT ABRIDGMENT (11) Document No. AU-B-78811/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 609058

(54) Title
DETERMINATION OF BIOMASS

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(56) Prior Art Documents
US 4288741
US 4072576

(57) Claim

1. A method for the determination of biomass in a medium comprising a suspending fluid and cells, the method comprising generating a signal dependent on the electrical capacitance, at a predetermined frequency, between electrodes mutually spaced in the medium, and determining from the capacitance dependent signal, the volume fraction of the medium enclosed by the cytoplasmic membranes of the cells.

3. Apparatus for performing a fermentation process utilising a culture comprising a suspending liquid and cells, the apparatus comprising: a fermenter containing said culture, electrodes mutually spaced in the fermenter in contact with the culture so that the capacitance between the electrodes, at a predetermined frequency, depends on the volume fraction of the culture enclosed by the cytoplasmic membranes of the cells; and means for generating a signal dependent on said capacitance between the electrodes at said predetermined frequency.

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COMPLETE SPECIFICATION 609058
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Priority

Related Art:

APPLICANT'S REFERENCE: Z/B 34037/AU

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Complete Specification for the invention entitled:

DETERMINATION OF BIOMASS

Our Ref : 68505
POF Code: 1453/75233

The following statement is a full description of this invention, including the best method of performing it known to applicant(s):

Determination of biomass

This invention relates to a method for the determination of biomass in a medium, to a fermentation process in which the method is used as a control feature, and to apparatus for carrying out both the method and the process.

There is increasing interest in biotechnology, both in the traditional fermentation industry and in the exploitation of living cells in new processes to produce commercially useful products such as antibiotics, vitamins, amino acids and a variety of biologically active proteins.

The productivity of fermentation processes is dependent to a considerable extent upon culture conditions. It is therefore desirable, and has now become conventional, to control those variables such as pH and dissolved oxygen tension for which sensors are available.

One of the most important variables in a fermention process is the reactor biomass concentration, i.e. the concentration of microbial or other cells in the fermenter, since the productivity under a given set of process conditions is directly proportional to this. However, to date no accurate means has been deveoped for measuring the biomass content of a culture in real time, i.e. for measuring the present biomass content rather than the biomass content some time in the past.

The lack of suitable means to measure this important process variable has been commented upon by several writers in recent years, see for example: Pirt, "Principles of Microbe and Cell Cultivation", Blackwell, 1975, p 16; Carleysmith and Fox, "Fermenter Instrumentation and Control, "Adv. Biotechnol. Processes 3, 1-51, 1984; and Harris and Kell, "The estimation of Microbial Biomass," Biosensors J.1, 17-84 (1985).

This last reference notes (1) that the most appropriate measure suitable for estimating biomass in real time is the biovolume, i.e. the volume fraction of a culture enclosed by the cytoplasmic membranes of the microbial or other cells within it, (2) that the only means by which biomass might be estimated in

real time will be by physical as opposed to chemical measurements, and (3) that all presently available physical methods such as light scattering) for estimating biomass, are essentially unusable under the difficult conditions existing in a fermenter.

It is an object of the present invention to provide a measurement of the microbial or other biomass in fermenters in real time and preferably in situ.

According to the present invention we provide a method for the determination of biomass in a medium comprising a suspending fluid and cells, the method comprising generating a signal dependent on the electrical capacitance, at a predetermined frequency, between electrodes mutually spaced in the medium, and determining from the capacitance dependent signal, the volume fraction of the medium enclosed by the cytoplasmic membranes of the cells.

Further in accordance with the invention, we also provide a fermentation process utilising a culture comprising a suspending liquid and cells, the process comprising generating a signal dependent on the electrical capacitance, at a predetermined frequency, between electrodes mutually spaced in the culture of a sample thereof, and providing an indication if the capacitance dependent signal differs from a predetermined value or falls outside a predetermined range, and/or altering the value of a process parameter to return the signal towards the predetermined range, said predetermined frequency being such that the capacitance between the electrodes depends on the volume fraction of the culture enclosed by the cytoplasmic membranes of the cells.

Yet further in accordance with the invention we provide apparatus for performing a fermentation process utilising a culture comprising a suspending liquid and cells, the apparatus comprising: a fermenter containing said culture, electrodes mutually spaced in the fermenter in contact with the culture so that the capacitance between the

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electrodes, at a predetermined frequency, depends on the volume fraction of the culture enclosed by the cytoplasmic membranes of the cells; and means for generating a signal dependent on said capacitance between the electrodes at said predetermined frequency.

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~~frequency.~~

The method of the invention may be used to determine biomass in any medium. The method is most useful for determining biomass in a culture and will generally be described in relation to such use in this specification. However it may also be used to determine biomass suspended in aqueous and in other suspensions and for instance in emulsions.

The apparatus may include means to determine whether the capacitance dependent signal differs from a predetermined value or falls outside a predetermined range of values.

The apparatus may further include means responsive to the means to determine, for altering one or more parameters of the process to return the capacitance dependent signal towards the predetermined value or the predetermined range.

There is an important relationship between the apparent relative permittivity of a suspension of spherical cells measured at a particular frequency, the cell radius, and the cell volume. The relationship can be expressed by the equation:

$$\varepsilon_L = \frac{9, P r C_m}{4 \varepsilon_r} + \varepsilon_{oo}$$

where ε_L is the apparent relative permittivity of the culture

P is the volume fraction of the culture occupied by cells

r is the cell radius

C_m is the capacitance of the cell membrane per unit area, and

ε_{oo} is the permittivity of the culture at a frequency which is high with respect to the measuring frequency

ε_r is the permittivity of free space, approximately 8.85×10^{-12} F/m.

For non-spherical cells, the factor 9/4 is modified. Permittivity is related to capacitance, which can be

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measured, by a factor, referred to as the cell constant, which depends on the electrode number, size and geometry. The permittivity of a sample can thus be determined by measuring the capacitance between electrodes mutually spaced therein. Formerly permittivity was known as dielectric constant.

The relative permittivity of an aqueous solution is dimensionless and depends slightly on the temperature and electrical conductivity of the sample but is always in the range 60 to 85, very often being approximately 78. The permittivity of aqueous solutions, when measured at low voltages, is independent of frequency up to approximately 1GHz, and under the conditions normally pertaining in a fermenter, is essentially unaffected by the presence of dissolved gases and non-cellular particulate matter.

The permittivity of an aqueous solution forms a reference or baseline against which the cell content of a culture may be determined.

The permittivity of a cellular suspension is strongly dependent upon frequency. For example, the permittivity at 100 kHz, of a suspension of bacterial cells of radius 0.5 μm and occupying a volume fraction of 0.1, might be approximately 250. This value will decrease towards a baseline value corresponding essentially to that of the suspending liquid as the frequency is increased.

The frequency dependent increase in permittivity found for cell suspensions over the baseline value for an aqueous solution, is known as a dispersion. Three major dispersions (α, β, and γ dispersions) are generally recognised, with the β-dispersion being particularly important for the invention. The α-dispersion occurs at lower frequencies than the β-dispersion and is mainly caused by the presence of mobile ions at the cell surface. The γ-dispersion occurs at higher frequencies than the β-dispersion and is caused predominantly by the rotation of dipolar species such as water. The α-dispersion is highly dependent on the structure of the cell wall, whilst the γ-

dispersion is not specific for intact cells.

The β-disperion of the dielectric permittivity was named by H P Schwan (Advances in Biological and Medical Physics, Vol. 5, 147-209, 1957). It is caused mainly by the presence of relatively ion-impermiable cellular membranes and has the approximate shape of an inverted sigmoid. Its magnitude for spherical cells, given C_m, can be taken to be dependent only upon cell radius and the volume fraction occupied by the cells. Other influences are, or can be taken to be, constants. Its position on the frequency axis depends upon the cell radius and the internal and external electrical conductivities. The large magnitude of the β-dispersion is due to cells having a conducting interior separated from the exterior by a poorly-conducting membrane providing a large capacitance, for example 1 $\mu F/cm^2$. Its magnitude is therefore directly proportional to the volume fraction occupied by the cells. The magnitude of the β-dispersion is large relative to the effects of non-cellular particles, dissolved gasses or oil droplets in the suspension. This allows the biovolume (related to the biomass by the density of the cell cytoplasm) to be measured without being significantly affected by particles or oil droplets in cell suspensions. The β-dispersion is a property of intact cells (see Pethig "Dielectric and Electronic Properties of Biological materials", Wiley, 1979) and its magnitude is directly proportional to the volume fraction of cells in a suspension up to very high volume fractions.

Suitable frequencies for measurement of biomass include frequencies at which the β-dispersion is substantially complete but at which the α-dispersion is substantially insignificant. Measurements of the whole of the β-dispersion may be made, but optimum measuring frequencies are in the half of the β-dispersion which occurs at lower frequencies before the α-dispersion is reached. Suitable frequencies include radio frequencies from 0.1 to 10 MHz, especially 0.1 MHz to 1 MHz, with a preferred range being 0.2 to 0.3 MHz. For large cells frequencies below 0.1 MHz may be found suitable, whilst for

cultures having a high electrical conductivity, frequencies in excess of 1 MHz may be found suitable.

The method of the invention can be used to determine the biomass content of a wide range of cultures, including plant, animal and microbial cell cultures, and the fermentation process can be used to produce a wide variety of products. Most usefully, however, the method of the invention is applicable to microbial cultures containing bacterial, yeast or fungal cells. Frequently the suspending liquid will be an aqueous culture medium containing nutrients suitable for the cells suspended in it, for example compounds containing carbon, phosphorus, nitrogen and other sources of essential nutrients.

The fermentation process of the invention can be carried out in any type of fermenter. Pervittivity can be determined by measuring the capacitance between electrodes mutually spaced in a sample of the culture. This can be done using a bridge or other circuit. Preferably the measuring electrodes are attached to the fermenter used in the process of the invention to enable direct on-line measurements to be made. Alternatively, samples may be withdrawn from the fermenter.

Examples of the method and process, and an embodiment of the apparatus of the invention, will now be described with reference to the accompanying drawings, in which:

Figure 1 shows typical variations of capacitance against frequency at different concentrations of cells in a culture;

Figure 2 shows the variation with time of capacitance, at fixed frequency, as a culture grows in a gas-lift fermenter;

Figure 3 shows the capacitance plotted against optical density of samples withdrawn from the fermenter; and

Figure 4 is a schematic diagram of apparatus embodying the invention.

Referring to Figure 4, a gas-lift fermenter 2 has a pair of spaced wire electrodes 4 fitted in the base. The electrodes 4 are connected to respective terminals of a Hewlett Packard low frequency impedance analyser 6 type No. 4192A. In order to reduce

the effects of the capacitance inherent in the connecting leads, the impedance analyser 6 is placed as close as possible to the connections with the electrodes. Indeed, the impedance analyser may be beneath the fermenter. In order to reduce the effects of polarisation of the culture medium at the electrodes, these are platinum black. Air is supplied to the culture via an inlet 8.

Saccharomyces cerevisiae is grown in the gas-lift fermenter. The culture medium contains 5% (w/v) malt extract and 0.5% (w/v) yeast extract. The initial pH is adjusted to 4.5 and the temperature is 30 degrees centrigrade. The conductance of this medium is ca. 1.57 mS/cm. Typical frequency dependent capacitance is shown in Figure 1 at a number of different cell concentrations. It is evident that there is a sizable dielectric dispersion which depends on cell concentration. The dispersion corresponds in magnitude and relaxation time to the β-dispersion. At a fixed frequency of 0.3 MHz, Figures 2 and 3 show respectively, the change in capacitance with time and against the optical density of samples taken from the fermenter and appropriately diluted.

The productivity of a fermentation process is dependent on culture conditions. One of the important variables in the fermentation process which it is advantageous to measure, is the biomass concentration. It may also be advantageous to run the fermentation at a particular biomass concentration, or with the concentration within a predetermined range.

The apparatus described above can be calibrated to determine the capacitance at a fixed frequency, say 0.3 MHz, corresponding to particular biomass concentrations. The apparatus may be calibrated to indicate biomass directly.

The on line measurement of capacitance, or biomass, provides a capacitance dependent signal which is fed to a process controller 10 where it is compared with a reference signal. Alternatively the process controller may determine whether the capacitance dependent signal lies within a predetermined range. The process controller is arranged to adjust one or more

parameters of the fermentation process to return the capacitance dependent signal towards the reference signal value or towards the predetermined range. Parameters which may be adjusted include the oxygen rate, i.e. the air supply rate, the dilution rate, the rate of addition of nutrients or new culture in a continuous process, the temperature, and so on.

A preferred specific embodiment of an apparatus for use in the invention is described in our co-pending UK Application No. 8622748 (corresponding to Australian Patent Application No. 78810/87), the contents of which are incorporated herein by reference.

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The claims defining the invention are as follows:

1. A method for the determination of biomass in a medium comprising a suspending fluid and cells, the method comprising generating a signal dependent on the electrical capacitance, at a predetermined frequency, between electrodes mutually spaced in the medium, and determining from the capacitance dependent signal, the volume fraction of the medium enclosed by the cytoplasmic membranes of the cells.

2. A fermentation process utilising a culture comprising a suspending liquid and cells, the process comprising generating a signal dependent on the electrical capacitance, at a predetermined frequency, between electrodes mutually spaced in the culture of a sample thereof, and providing an indication if the capacitance dependent signal differs from a predetermined value or falls outside a predetermined range, and/or altering the value of a process parameter to return the signal towards the predetermined range, said predetermined frequency being such that the capacitance between the electrodes depends on the volume fraction of the culture enclosed by the cytoplasmic membranes of the cells.

3. Apparatus for performing a fermentation process utilising a culture comprising a suspending liquid and cells, the apparatus comprising: a fermenter containing said culture, electrodes mutually spaced in the fermenter in contact with the culture so that the capacitance between the electrodes, at a predetermined frequency, depends on the volume fraction of the culture enclosed by the cytoplasmic membranes of the cells; and means for generating a signal dependent on said capacitance between the electrodes at said predetermined frequency.

4. Apparatus as claimed in claim 3, including means to determine whether the capacitance dependent signal differs

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from a predetermined value or falls outside a predetermined range of values.

5. Apparatus as claimed in claim 4, including means responsive to the means to determine, for altering one or more parameters of the fermentation process to return the capacitance dependent signal towards the predetermined value or the predetermined range.

6. Apparatus as claimed in any one of claims 3 to 5, wherein the predetermined frequency is in the range 0.1 mHz to 10 MHz.

7. A method as claimed in claim 1 substantially as hereinbefore described with reference to any one of the drawings or examples.

8. A process as claimed in claim 2 substantially as hereinbefore described with reference to any one of the drawings or examples.

9. Apparatus as claimed in claim 3 substantially as hereinbefore described with reference to any one of the drawings or examples.

DATED: 3 January 1991
PHILLIPS ORMONDE & FITZPATRICK
Attorneys For:
DOUGLAS BRUCE KELL

David B Fitzpatrick

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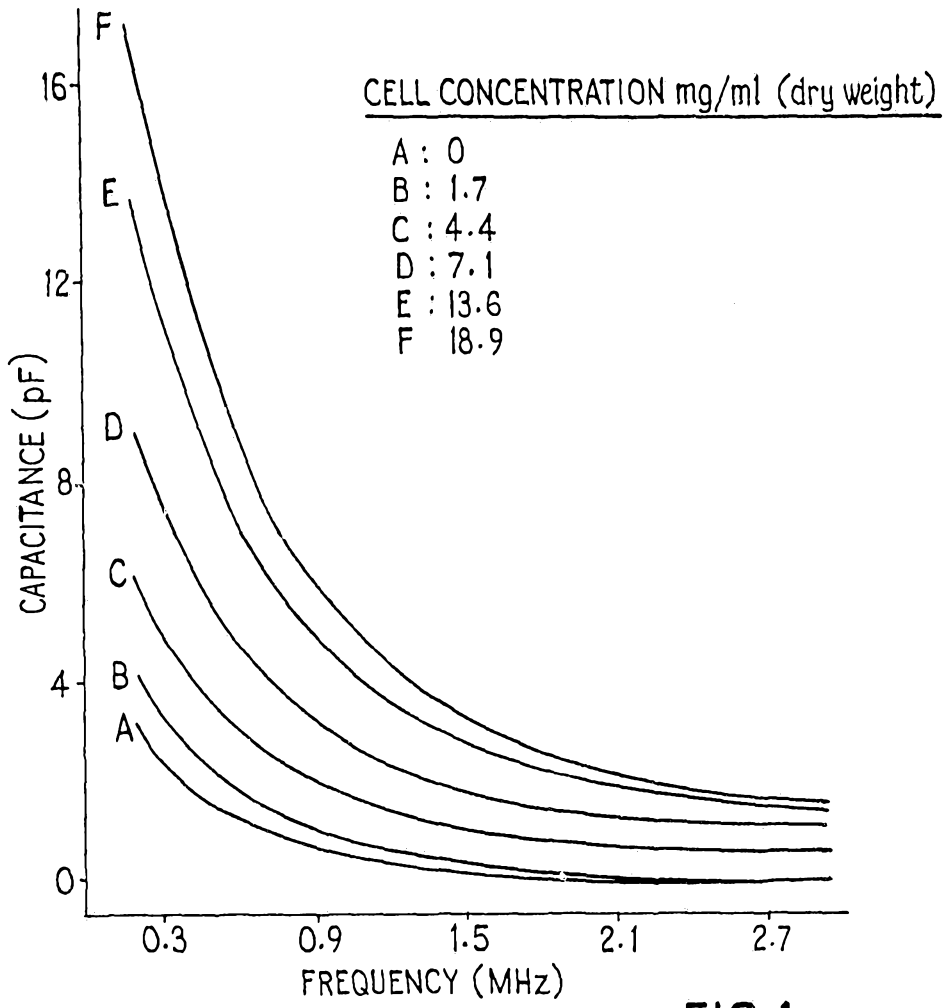

CELL CONCENTRATION mg/ml (dry weight)
A : 0
B : 1.7
C : 4.4
D : 7.1
E : 13.6
F 18.9
CAPACITANCE (pF)
16
12
8
4
0
F
E
D
C
B
A
0.3
0.9
1.5
2.1
2.7
FREQUENCY (MHz)

FIG. 1

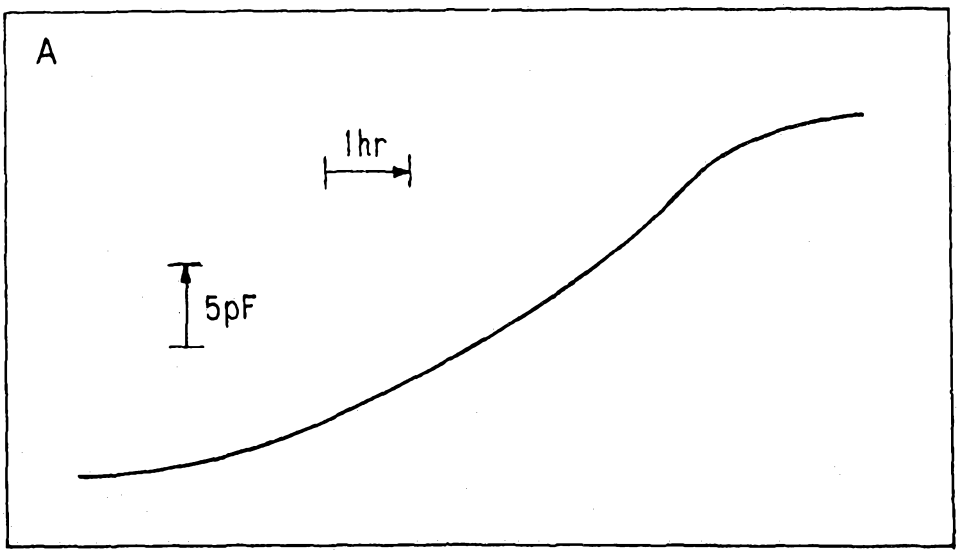

A
1hr
5pF

FIG. 2

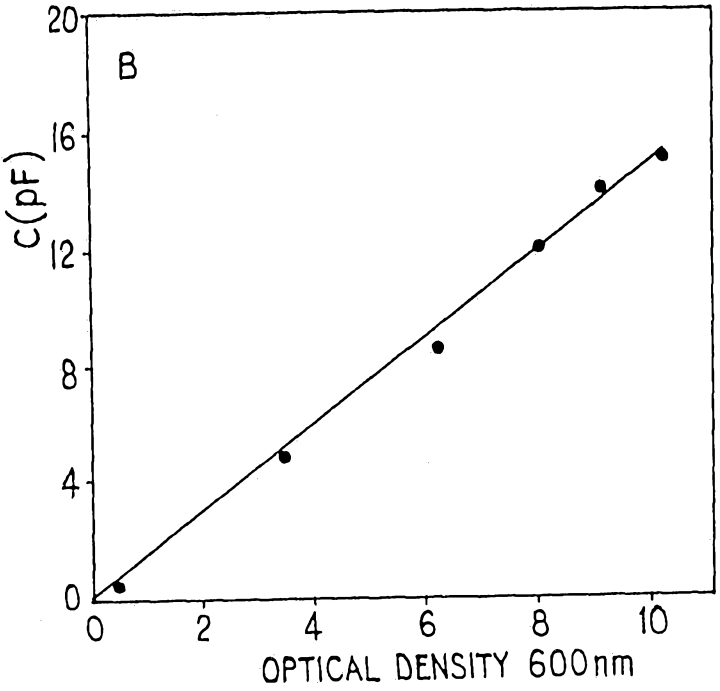


FIG.3

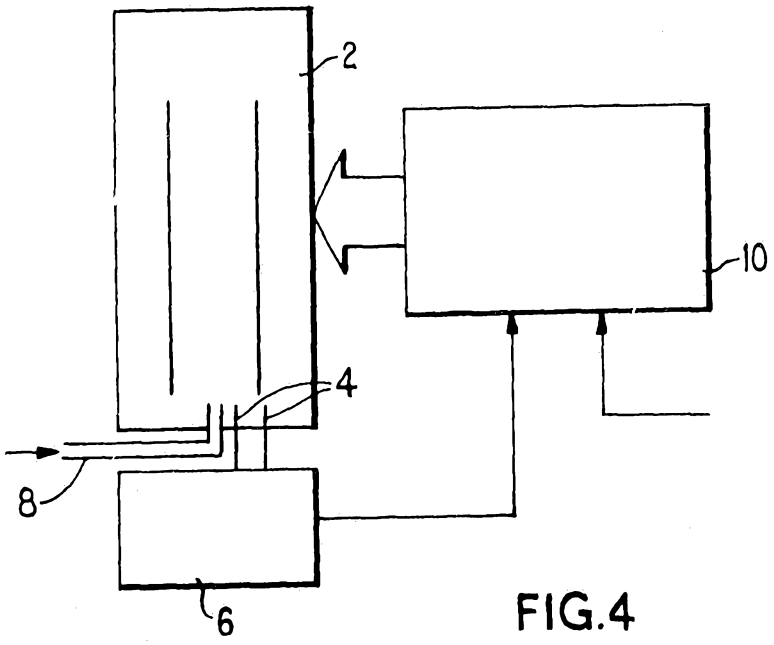


FIG.4