Method for culturing cells, membrane module, use of a membrane module and reaction system for culturing cells

The present invention relates to a method for culturing cells using a reaction system, in which the reaction system comprises a container for dialysis fluid, a space for culturing cells and a membrane module which connects both spaces with one another and which has at least two spaces which are separated by a membrane and through one of which a dialysis fluid flows and through the second of which a culture fluid containing cells flows, and a first gas is introduced into the culture fluid in the space for culturing the cells. The invention furthermore relates to a membrane module, the use of a membrane module and a reaction system for culturing cells.

The use of dialysis membranes in connection with the culture of microorganisms or animal cells has certain advantages with respect to the obtainable cell densities or concentration of the products obtained. A review of different biotechnological production methods in which membranes are employed is given by R. Pörtner and H. Märkl (Appl. Microbiol. Biotechnol. (1998) 50: 403-414). The essential effect of using dialysis membranes is based on the fact that growth-inhibiting metabolic products of the organisms used, which are generated during growth or during production, are removed via a membrane. The transport is driven here by a difference in the concentrations of said metabolic products. The European patent EP 0 632 827 B1 describes a method and an apparatus, in which the industrial utilization of the concept is illustrated. More precisely, a reaction system is described which comprises at least two chambers which are coupled to one another via at least one membrane, and in which the culture of microorganisms or plant or animal cells is located in one chamber and the growth-inhibiting metabolic products from this chamber reach the solution of the other chamber.

The incorporation of a dialysis membrane directly into the space provided for culture of the organisms, as described therein, has been carried out technically successfully on the laboratory scale (2-l scale) (cf. also R. Pörtner and H. Märkl, ibid.). However, scaling up this technique to the

production scale of up to several m^3 involves difficulties which have not yet been conquered up until now. The root of the problem can be explained easily. Membranes with very low wall thicknesses (from a few µm up to 100 µm) are strived for, since said membranes have a high dialysis capacity. However, the said membranes have only limited mechanical strength. For rapidly growing aerobic organisms, however, the mechanical strain in the culture space is very high, since it is necessary to introduce large amounts of air or oxygen to supply to the said organisms. At the same time, large amounts of carbon dioxide have to be removed. This necessitates high energy input through stirring in the order of 10 kW/m^3 causing great strain on the thin membranes.

Substantially less problematic in this respect is another likewise known arrangement as is depicted in Fig. 1. Here, the dialysis membrane 3 is housed in a separate membrane module 8 outside the culture space 2. The culture fluid is pumped through the membrane module 8 with the aid of a pump 10. Correspondingly, the membrane module 8 is provided with dialysis fluid from container 1 via a second pump 9. The scaling up of said arrangement to the large production scale is unproblematic, since the arrangement comprises essentially three units independently of one another, two containers and one membrane module, whose size can be designed independently of one another. However, as Ogbonna, J. Ch. and Märkl, H., (Biotechnology and Bioengineering, Vol. 41, pp. 1092-1100 (1993)) have shown using the example of dense E.coli cultures, said arrangement has the considerable disadvantage that part of the microorganisms leaves the culture space 2 for a certain period of time and is not provided with oxygen during dialysis in the membrane module 8. In the example cited, the time taken by a particular organism on its way through the dialysis module outside the culture space supplied with gas is stated as 11 seconds. A consequence of this recurring time-limited under-supply is a reduction in the attainable cell density to a value of 110 g of dry weight/liter. In an arrangement which, however, cannot be scaled up and which has a membrane integrated into the culture space, i.e. continuous oxygen supply, an organism density of 160 g of dry weight/liter is achieved under otherwise identical conditions.

It is an object of the invention to develop further the reaction system known from the prior art for culturing cells according to Fig. 1, which comprises at

least one space for culturing cells (2), one container for dialysis fluid (1) and one membrane module (8) inserted in between, through which growth-inhibiting substances from the culture fluid reach the dialysis fluid, such that higher cell densities are obtained.

Another object was to provide a reaction system suitable for carrying out the method of the invention.

According to the invention, this object is achieved by a method for culturing cells using a reaction system, in which the reaction system scomprises a container for dialysis fluid, a space for culturing cells and a membrane module which connects both spaces with one another and which has at least two spaces which are separated by a membrane and through one of which a dialysis fluid flows and through the second of which a culture fluid containing cells flows, and a first gas is introduced into the culture fluid in the space for culturing the cells and a second gas is introduced into the culture fluid in the membrane module.

The object is achieved in particular by a method for culturing cells using a reaction system, in which the reaction system comprises a container for dialysis fluid, a space for culturing cells and a membrane module which connects both spaces with one another and which has at least two spaces which are separated by a membrane and through one of which a dialysis fluid flows and through the second of which a culture fluid containing cells flows, a first gas is introduced into the culture fluid in the space for culturing the cells and a second gas is introduced into the culture fluid in the membrane module, and the membrane is functionally a dialysis membrane.

One embodiment of the method of the invention provides for gas to be passed directly into the culture fluid present in the membrane module by introducing the second gas directly into the culture fluid in the membrane module.

An alternative embodiment of the method of the invention provides for gas to be passed indirectly into the culture fluid present in the membrane module by introducing the second gas into the dialysis fluid in the container for dialysis fluid and the gas reaching from there the culture fluid present in the membrane module via the membrane of the membrane module.

In a preferred embodiment of the methods of the invention gas is introduced both directly and indirectly at the same time.

In one embodiment the membrane module comprises at least one gas supplying means and the gas supplying means supplies at least one of the spaces separated by the membrane and carrying a liquid with the second gas.

In this connection, preference is given to the gas supplying means having an outlet which is located in particular in the membrane module space carrying the culture fluid.

In a particularly preferred embodiment the gas supplying means is a tube.

In a very particularly preferred embodiment the gas supplying means has the shape of a nozzle.

One embodiment provides for the membrane of the membrane module to be composed of a material selected from the group comprising regenerated cellulose, polyamide, polypropylene and polysulfone.

Another embodiment provides for the membrane module to be a plate module.

One embodiment of the method of the invention and also of the membrane module of the invention provides for the membrane of the membrane module to be a dialysis membrane.

In this connection, particular preference is given to the dialysis membrane material being Cuprophan.

In a preferred embodiment of the methods of the invention the membrane module, due to its area/volume ratio and/or its gas permeability coefficient, provides for a gas exchange sufficient for the cells.

One embodiment provides in this connection for the area/volume ratio to be at least about 5 m^2 per liter, in particular at least 10 m^2 per liter.

In a preferred embodiment the area/volume ratio is about 13 m^2 per liter.

In a further embodiment the oxygen permeability coefficient is about 0.066 cm per minute or higher.

One embodiment may provide for the container for dialysis fluid to have a means for supplying gas and for removing gas.

Furthermore, the methods of the invention may provide for the membrane module, the space for culturing the cells and/or the container for dialysis fluid to have increased pressure.

The methods of the invention may provide for the first and second gas to be individually and independently of one another selected from the group comprising air, oxygen, nitrogen, carbon dioxide and mixtures thereof.

In a preferred embodiment the second gas is oxygen.

In an alternative embodiment the second gas is carbon dioxide.

The methods of the invention may provide for the cells to be selected from the group comprising microbial cells, fungal cells, animal cells and plant cells.

Very particular preference is given to cells which are Escherichia coli cells.

Furthermore, the object is achieved by a membrane module which comprises at least two spaces separated by a membrane and through each space a liquid flows, the liquid in one space is dialysis fluid and in the other space culture fluid, and in which the membrane module comprises at least one gas supplying means and the gas supplying means in at least one of the spaces separated by the membrane has an outlet.

One embodiment provides for the membrane to have the shape of a tube and for its volume to form one of the two spaces.

In this connection, it may furthermore be provided for the diameter of the space formed by the tube-shaped membrane to be about 3-10 mm, preferably 6-8 mm.

In one embodiment the membrane module of the invention comprises a plurality of spaces formed by a tube-shaped membrane.

In a further embodiment an outlet of the gas supplying means is located in at least one of the spaces formed by the tube-shaped membrane.

In this connection, it may be provided for the outlet of the gas supplying means to be located in a space outside the space(s) formed by the tube-shaped membrane.

In a preferred embodiment the gas supplying means is a tube.

In a very particularly preferred embodiment the tube is arranged in the space in a concentric manner.

The membrane module of the invention may provide for the tube to have an internal diameter of from 0.2 mm to 3 mm.

In a particularly preferred embodiment the outlet of the gas supplying means is shaped like a nozzle.

According to the invention, the object is also achieved by using the membrane module of the invention [lacuna] a method according to the invention.

In addition, the object is achieved by using a membrane module which has a gas permeability coefficient which is high enough in order to ensure sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module, and/or has a suitably high area/volume ratio in order to ensure sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module.

More detailed information on the two factors mentioned and on how to determine said factors is found in the description of the figures and also in the description of the methods disclosed herein.

In addition, the object is achieved according to the invention by a reaction system for culturing cells comprising a container for dialysis fluid, a space for culturing cells and at least one membrane module inserted in between, said membrane module ensuring sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module.

In a preferred embodiment the membrane module is a membrane module of the invention.

In a further embodiment the container for dialysis fluid contains at least one gas-introducing device. In this connection, it is a technical precondition that the container contains at least one line for supplying gas(es) and at least a (second) line for removing gas(es).

In yet another embodiment the membrane of the membrane module has a gas permeability coefficient which is high enough in order to ensure sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module, and/or has a suitably high area/volume ratio in order to ensure sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module.

The invention is based on the surprising finding that introducing gas to the cells to be cultured during passage through the membrane module avoids the time-limited undersupply of said cells with gas, in particular with oxygen in the case of aerobic or facultatively aerobic cells and thus leads to higher cell densities.

The basic structure of the reaction system used within the scope of the method of the invention is depicted in Fig. 2. Although Fig. 2 depicts only one membrane module, the reaction system may of course comprise a plurality of parallel inserted membrane module units.

Within the scope of the method of the invention it may be provided for the gas required to be exchanged or supplied, for example in the form of supplying oxygen in the case of aerobic or facultatively aerobic organisms, directly to the culture fluid contained in the membrane module. This is herein referred to as directly introducing gas. In this connection, gas may be introduced directly such that gas is directly supplied to the culture fluid which normally contains cells via a gas supplying means (15, 16) integrated into the membrane module. For this purpose, it is possible within the scope of the invention to use the membrane module of the invention or for said membrane module to be included in the reaction system as it is used within the scope of the method of the invention.

Alternatively, the gas exchange required in the culture fluid contained in the membrane module or the gas supply required therefor, as described herein, may be carried out in the method of the invention by introducing gas indirectly. The indirect introduction or supply of gas is carried out by introducing gas to the dialysis fluid 18 in the container for dialysis fluid 1. A gas supply line 21 and a gas removal line 22 and normally a stirrer 23 are used here. The gas-enriched dialysis fluid is pumped through the membrane module 8 via a pump 9 and can give off the gas to the culture fluid via the membrane 3. Important parameters for the indirect introduction of gas are, in addition to the capacity of introducing gas into the container with dialysis fluid, the permeability coefficient of the membrane of the membrane module for the gas in question, i.e. the introduced gas, and the area/volume ratio of the membrane module.

In this connection, it is within the scope of the invention that both forms of introducing gas are applied simultaneously in the method of the invention, but there are quite possibly phases during culturing of the cells, in which only one of the two forms of introducing gas is applied.

The exclusively direct introduction of gas as well as the exclusively indirect introduction of gas are thus extreme cases.

Depending on the ratio of the membrane area available for introducing gas and also the permeability thereof for the gas to be transported and the culture volume into which gas is to be introduced and which is present in

the membrane module 8, the proportion of direct (via gas supplying means 15, 16) and indirect (via membrane 3) introduction of gas will be different. In this connection, it is in principle also necessary to take into account the amount of the gas in question which can be introduced into the container with dialysis fluid, more precisely into the dialysis fluid contained therein.

If, for example, indirect introduction of gas through the membrane module 8, due to its high area/volume ratio and/or the high permeability coefficient of the employed membrane for the gas in question, in particular oxygen in the case of aerobic and facultatively aerobic cells, makes a sufficient gas supply via the membrane possible, the direct introduction of gas via a special gas supplying means or using the membrane module disclosed herein can be dispensed with.

The membrane used in the membrane modules described may generally comprise, independently of the type of gas introduction, Cuprophan (regenerated cellulose), polyamide, polypropylene, polysulfone or other natural or synthetic substances. This may be a dialysis membrane (cutoff: 10 000 dalton), a microporous membrane (cutoff: 0.2 µm) or membranes whose permeability (cutoff) is in between.

Within the scope of the present invention, the membrane is functionally a dialysis membrane in the membrane module of the invention too and/or in the reaction system of the invention.

In connection with the method of the invention as well as with the membrane module and reaction system of the invention, all membranes are like dialysis membranes.

In the prior art, membranes made of regenerated cellulose (for example Cuprophan) are commonly denoted dialysis membranes. Said membranes are unsuitable for use as filters, since there is no hydraulic flow through said (dialysis) membranes. The hydraulic flows occurring in a dialysis membrane such as a Cuprophan membrane at transmembrane pressure differences are very low. The actual function of said (dialysis) membrane is based on the fact that concentration differences can balance out via the membrane by diffusion.

If, for example, a microporous membrane with pores of less than 0.2 µm is taken and formation of a transmembrane pressure difference is avoided, then, in this case too, transport through the membrane is carried out via diffusion mechanisms. Hydraulic flowing through is substantially avoided under said conditions.

Within the scope of the method of the invention, which is normally carried out without a transmembrane pressure difference, porous and in particular microporous membranes are thus not different from hydraulically tight membranes (the actual dialysis membranes).

The term chosen herein, namely that the membrane is functionally a dialysis membrane, is to be understood in view of said contexts. In other words, within the scope of the method of the invention or the membrane module of the invention or the reaction system of the invention there may be provision for a dialysis membrane in the proper sense to be used. However, there may also be provision for a porous and in particular microporous membrane to be used, as long as said membrane functionally acts like a dialysis membrane, i.e. shows no hydraulic flowing through, or, if hydraulic flowing through occurs, the extent thereof is relatively small overall, compared with the substance transport via the membrane by diffusion. The said change in character of the porous or microporous membrane may be due to the manner in which the membrane is utilized or operated, more precisely due to not applying across the membrane any pressure, as is applied, for example, in perfusion systems.

The examples illustrate the considerations which have to be made in this connection by the skilled worker.

The invention is now further illustrated below on the basis of the attached drawings which result in further advantages and embodiments of the invention and in which

Fig. 1 shows a reaction system according to the prior art for culturing cells comprising a culture space 2, a container for dialysis fluid 1 and a separate membrane module 8 containing a dialysis membrane 3;

Fig. 2 shows the reaction system of the invention which is used within the scope of the method of the invention,

Fig. 3 shows the membrane module of the invention; and

Fig. 4 shows a plate module from Gambro Medizintechnik GmbH.

Fig. 1 which depicts the basic structure of the generic reaction system, with that version indicated, in which the membrane module 8 containing a dialysis membrane 3 is located outside the culture space 2, has already been discussed briefly in the introduction of the description.

Culture space 2 contains culture fluid 17, and in the inoculated state the cells to be cultured which are supplied with oxygen via supply line 5. Oxygen may be supplied such that air, air/oxygen mixtures or pure oxygen are supplied. The gas bubbles 7 formed at the outlet of the supply line 5 move upward in the culture fluid and are further dispersed by the stirrer 4 in culture space 2. The remaining gases, where appropriate together with the gaseous reaction products are then removed from the culture space via discharge line 6. Culture space 2 is connected to the membrane module 8 containing the dialysis membrane 3 via lines 13 and 14. By means of pump 10, culture fluid is pumped from culture space 2 into the membrane module and from there fed again to culture space 2 via line 14. The container for dialysis fluid 1 is filled with dialysis fluid and connected to the membrane module 8 containing the dialysis membrane 3 via lines 11 and 12. Dialysis fluid is pumped from the container for dialysis fluid 1 through the membrane module 8 by means of the pump 9 and reintroduced into the container for dialysis fluid 1 via line 12.

Further devices for the reaction system, as they are familiar to the skilled workers, such as, for example, measuring devices, sampling devices, devices for supplying and discharging media and also for supplying and discharging substrates, devices for exchanging the dialysis fluid and the like, are not depicted in said diagrammatic representation.

In this connection, the method of the invention may use the inventive reaction system depicted in Fig. 2, which has been developed further from the reaction system depicted in Fig. 1. It comprises a container for dialysis

fluid, a space for culturing the cells and a membrane module inserted in between, which acts as dialysis module. The membrane module may be either the membrane module of the invention, as depicted in an embodiment in Fig. 3, or a membrane module having a gas permeability coefficient high enough to ensure sufficient indirect gas supply, while the culture fluid is passing through the membrane module, and/or having a suitably high area/volume ratio, in order to ensure sufficient gas supply, while the culture fluid is passing through the membrane module which is used according to the invention in the reaction system of the invention. However, it is also possible that both ways of introducing gas are used simultaneously and the reaction system is then organized as depicted in Fig. 2.

The various realizable ways of introducing gas are discussed below. If in this connection air or oxygen is the gas stated, the aspect described in each case is not restricted to said particular gas but serves merely for illustrating by way of example. In principle, it is possible to use any gas within the scope of the present invention.

In this connection, the way in which gas is directly introduced is that tubes 16 as gas supplying means are arranged concentrically in the bottom part of the tube-shaped membranes in the membrane module, which tubes in this case represent the gas supplying means. In the present embodiment, oxygen-containing gas is introduced under increased pressure through said tubes into the inner space of the membrane, i.e. the space formed by the tube-shaped membrane, which contains culture fluid together with the cells to be cultured. In the present case, the tubes are interconnected by a supply line 15. The tubes whose internal diameter is 0.2-3 mm may contain in the region of the gas outlet a narrowing which may form a nozzle in order to limit the amount of escaping gas. The supply line 15 supplies the culture of organisms with air which may also be admixed with oxygen. It is also possible to use pure oxygen as the gas introduced. It is furthermore possible to subject the membrane module entirely to increased pressure in order to increase the partial pressure of oxygen in the gas mixture described and thus to increase likewise the oxygen supply into the suspension containing the organisms.

In the method of the invention, gas is introduced indirectly by introducing gas into the dialysis vessel 1 via a gas supply 21 and thus increasing the oxygen concentration in the dialysis fluid. The gas having a reduced oxygen concentration leaves the dialysis container via the line 22. The oxygen-containing dialysis fluid is transported via the pump 9 into the membrane module 8 where the oxygen is given off via the dialysis membrane to the suspension containing the cells to be cultured, for example microorganisms. The indirect gas supply, i.e. oxygen supply, hangs substantially on the ability of the membrane to transport, in the present case, oxygen and/or on the available membrane area in relation to the volume to be supplied.

The membrane module from Gambro, as it is depicted diagrammatically in Fig. 4, has said properties. Here, flat membranes 3 are arranged at a certain distance, with in each case two membranes forming a space through which the culture fluid 17 flows. The dialysate 18 flows through the external part. The membranes are supported by a supporting structure 19.

A detailed description of the membrane module is given in connection with Fig. 4.

As already described, it is possible within the scope of the method of the invention, apart from introducing gas directly, to introduce gas indirectly by using a membrane module as shown in Fig. 4. In this connection, it may also be provided for the indirect introduction of gas via the dialysis fluid possibly to be dispensed with when introducing gas directly is sufficient. Conversely, the direct introduction of gas via the membrane module provided with gas supplying means, as it is described in Fig. 3, may also be dispensed with when the membrane module ensures sufficient gas supply, due to its high area/volume ratio and advantageous transport behavior.

The operation of the reaction system demonstrated, its technical equipment and the method for culturing cells, which can be realized by using said reaction system, otherwise correspond by analogy to the reaction system, equipment and method, respectively, as illustrated in connection with the inventive apparatus in Fig. 1. In this connection, nearly identical hydrostatic pressure is applied preferably to both spaces formed by the membrane. It

is furthermore preferred that the pressure level in both containers whose volumes are connected to the membrane is nearly identical.

Experiments involving the *E.coli* culture and the arrangement indicated in Fig. 2 and using the membrane module depicted in Fig. 4 and the oxygen supply discussed there have indeed produced excellent results in our own experiments. The cell densities achieved of 160 g of dry weight/liter correspond to those achieved using a reaction system in which the membrane was directly integrated into the reaction space. The experiments show that growth is not impaired by time-restricted lack of oxygen.

If, in one case, it is desired to further increase oxygen supply via the membrane, further measures are available therefor. It is possible, for example, to introduce additionally oxygen gas into the membrane module on the dialysis fluid side via a line 20 (Fig. 2). Said oxygen is dissolved in the dialysis fluid in the course of the membrane passage. Thus the negative concentration gradient $(c_1\text{-}c_2)_{mean}$ which is critical for oxygen supply is increased.

The oxygen concentration in the dialysate may also be increased by increasing the entire pressure level of the arrangement according to Fig. 2. In the case of doubling the pressure from the ambient pressure of 10^5 Pa to $2 \cdot 10^5$ Pa, to give an example, the corresponding oxygen contents in the dialysate can also be doubled. As a result, the oxygen supply of the culture medium in the membrane module or dialysis module is increased accordingly (the terms membrane module and dialysis module may be used synonymously herein.).

The inventive method for culturing cells comprises one or more of the following steps, with culturing in such a generic reaction system being known in principle to the skilled workers in the field: a) providing a reaction system comprising a culture vessel (culture space), an external membrane module which comprises the apparatus of the invention, i.e. the membrane module of the invention or a commercially available membrane module having the necessary properties (area/volume ratio, oxygen permeability coefficient), and a container for dialysis fluid, and the reaction system, as well as the liquids contained therein, were preferably sterilized, b) inoculating the culture vessel with the cells to be cultured, c) providing a

suitable temperature, substrate supply and oxygen supply, d) leading the culture fluid containing the cell suspension to be cultured from the culture vessel through the dialysis apparatus and leading the culture fluid back to the culture vessel with simultaneous circulation of the dialysis fluid from the container for dialysis fluid through the dialysis apparatus back into the container for dialysis fluid, with the two liquid streams preferably being led countercurrently, and e) harvesting the cultured cells.

Although the above embodiments have been carried out mostly in connection with the culturing of microorganisms, in particular E.coli, the use of the apparatus, the reaction systems or the method of the invention is in no way limited thereto.

The cells used in connection with the apparatus, the use, the reaction systems and the methods of the present invention may also be photosynthetic cells, irrespective of whether they are prokaryotic or eukaryotic cells and therefore photosynthetically active microbial or plant cells.

One aspect of the present invention thus relates to supplying cells with oxygen.

Another aspect of the present invention relates to the removal of gases or gaseous metabolic products, as they appear in cultures of cells. In this connection, by the idea that the process of introducing gases is in principle, at least with respect to the physicochemical processes involved, similar to that of removing gases plays a part. In this connection, it is possible to remove, under the influence of introducing, in particular blowing in, a gas, another or the same gas from the liquid. In the present case, said liquid may be the culture space fluid (medium) and/or the dialysis fluid. In this respect it is possible to use the apparatus of the invention for removing gases from the liquid in the reaction system(s) or a part thereof. This is also true for the case of the inventive of the use of a membrane module.

Some of those culturing systems in which stripping of gaseous metabolic products has proved advantageous are to be given by way of example.

In the case of photosynthetically active cells, for example plant cells or algae, carbon dioxide is typically added to the culture medium. This may be carried out using an appropriately designed membrane module or the apparatus of the invention, with calculations for the design of the gas-introducing apparatus or the membrane module used to be made, which are similar to those above for the oxygen supply of Escherichia coli cultures and which allow the skilled worker to supply the culture with the required amount of carbon dioxide. At the same time it is in the case of high-density cultures of such cells necessary to remove from the medium oxygen generated due to the photosynthetic activity of the cells. This may also be achieved by using the inventive apparatus or through the use of a membrane module. Typically, the gas will be introduced using a mixture of air and carbon dioxide, with the setting of suitable partial pressures.

Another example of the aspect of the invention relating to the removal of gases from liquids according to the present invention is the culturing of Pyrococcus. Here, introducing nitrogen or a mixture of nitrogen and carbon dioxide succeeds in bringing about high cell densities. The corresponding disclosure on this by Krahe, M. FEMS Microbiology reviews 18 (1995) 271-285 is incorporated herein by way of reference.

Whenever the supply or removal of gas appears to be helpful or necessary for culturing cells or, in other words, the gas metabolism of a culture is important for culturing the cells, the skilled worker will thus (be able to) use the apparatus, use, reaction systems and method according to the present invention.

The above-described reaction system may be used for the method of the invention. In this connection, it may be provided for either the container 1 with the dialysis fluid or the actual culture vessel, i.e. the culture space, or both to be operated batchwise, semi-continuously or continuously. Apart from introducing gas into the apparatus of the invention, gas may be introduced into the culture space and/or into the container for dialysis fluid, more precisely into the dialysis fluid (or dialysate 18). In principle, it is possible to culture in the culture vessel any forms of cells, i.e. prokaryotic and eukaryotic cells, in particular microorganisms, fungal cells, animal cells and plant cells. The cells are typically present in the culture vessel in suspension. Regarding the operation, in particular of the culture vessel, any

conceivable feeding strategy is possible, such as, for example, and in particular also fedbatch or split feed strategy which has been described, for example, in Ogbonna, J. Ch. and Märkl, H.; Biotechnology and Bioengineering Vol. 41, pp. 1092 - 1100 (1993) whose disclosure content is in its entirety incorporated herein. Otherwise limiting substrates such as, for example, a carbon source, a nitrogen source and/or particular salts are selectively added to the culture, according to the nutrient conditions.

Fig. 3 shows the membrane module of the invention, which can be incorporated as membrane module 8, for example, into the reaction system depicted in Fig. 1 or Fig. 2, i.e. it can be inserted as separate membrane module 8 between culture space 2 and the container for dialysis fluid 1.

The membrane module 8 is constructed from in total two groups of spaces of which in each case two or more are present, on the one hand those spaces in which culture fluid 17 is located or through which said culture fluid flows, and on the other hand those spaces which contain the dialysis fluid. In the present case, the dialysis membrane in the membrane module 8 forms tubes, summarily denoted tube-shaped membrane herein, through which the culture fluid is led. The dialysis fluid flows through the spaces located in between and separated from the culture fluid by the membrane material. The module is connected with the culture vessel via the feed line 13 and the discharge line 14. In the case of a suspension culture in culture space 2, the cells here flow through the interior of the tube-shaped membrane(s) from bottom to top. The dialysis fluid which is exchanged with the container for dialysis fluid 1 via the lines 11 and 12 flows around the outer wall of the membrane. In this respect, feed line 13 and discharge line 14 and feed line 11 and discharge line 12 form in each case a set for supplying and discharging liquid medium to and from the space formed by the tube-shaped membrane(s) and, respectively, to and from the space located outside the space formed by the tube-shaped membrane(s). In this connection, the present embodiment provides for only in each case one feed line and discharge line, respectively, to be provided for the two sides of the dialysis module, i.e. dialysis fluid, and the liquid to be dialyzed, i.e. culture fluid, respectively. However, it is also possible to provide for in each case a plurality of said feed lines and discharge lines on each side.

If the membrane module of the invention is present in incorporated form in the reaction system of the invention, the pressure in the membrane module is expediently increased by simultaneously increasing the pressure in the container for dialysis fluid 1 and the culture space 2. This measure makes it possible, as our own experiments have demonstrated, to supply oxygen in the required range between 5 and 15 g/(1 h) [i.e. kg O_2 per m^3 of culture fluid and per hour] without problems.

The gas or gas mixture flows through the supply line 15 through the internal space of the membrane 3, leaves the membrane module through the discharge line 14, reaches from there the culture vessel 2 and leaves said culture vessel through the opening 6 in the lid of the culture space 2, together with the waste gas of the culture space 2 which is likewise supplied with oxygen.

The gas is introduced with a certain excess pressure via the tubes 16 into the internal space of the membrane 3. Depending on the amount of excess pressure, the gas obtains a certain velocity with which it enters the suspension containing the microorganisms. Since the gas is slowed down by the liquid, an impulse is conferred upon the liquid, which contributes to pumping the liquid in the interior of the tube-shaped membrane 3. Another pumping effect arises due to the high gas content which is established in the internal space of the membrane. Since said gas content is, with correspondingly high gas supply, higher than in the actual culture space 2, an additional pumping effect is established (mammoth pumping effect). For this reason, it is possible, with an appropriately large amount of gas being introduced into the membrane module, to dispense with the pump 10 in a reaction system containing the apparatus of the invention.

The membrane module 8 is normally placed as close as possible to the culture space 2 so that the feed line 13 between culture space and membrane module, into which no gas is introduced, is as short as possible and, consequently, the cells contained in the culture fluid are only very briefly subjected to the conditions of a non-optimal gas supply.

Fig. 4 depicts a cross section through a membrane module which can be used according to the invention for introducing gas into culture fluid for culturing cells. The cross section specifically depicted in Fig. 4 originates

from a commercially available plate module denoted Gambro LunDia: Alpha 600, Gambro Medizintechnik GmbH. The membrane material is Cuprophan®. The membrane wall thickness is 8 µm (in the dry state), the membrane area is 1.3 m^2 and the number of membrane layers per module is 70. The volume of the space 17 which contains culture fluid and cells during operation according to the invention is 100 ml.

Membrane modules of this kind are currently used only in hemodialysis and are prepared for this purpose, but are not used in culturing cells and in particular not in a reaction system of the generic type, as it is depicted in Fig. 1 and Fig. 2 diagrammatically for the case of the membrane module being arranged as an independent unit.

Within the scope of studies it was surprisingly found that under particular preconditions said commercially available membrane modules may also be used if, due to their area/volume ratio and the permeability coefficient for oxygen, indirect oxygen supply sufficient for the cells is ensured.

To this end, a few considerations, stated below, have to be made and, in particular, the permeability coefficients for oxygen have to be determined, but this can be carried out within the scope of the abilities of the average skilled worker. The determination of permeability coefficients is described, for example, for the compound glycerol in Märkl, H. et al. Appl. Microbiol. Biotechnol. (1993) 39: 48-52.

In the plate module Gambro LunDia Alpha 600, mentioned here by way of example, the predetermined construction makes possible an extremely high ratio of membrane area to volume to be provided for.

The active membrane area of the model LunDia Alpha 600 is 1.3 m^2. The volume of the space to be supplied with oxygen is 100 ml so that the area/volume ratio is

$$F/V = 13\ m^2/l\ (\text{LunDia}).$$

For comparison, the corresponding area/volume ratio for a membrane tube as it is employed in the arrangement drawn in Fig. 2 is to be stated. In this case, the internal space of the tube has to be supplied. The available

transport area is the circumferential surface area of the cylinder-shaped membrane area. A calculation for the example of a tube of 5 mm in diameter gives

$$F/V = 0.8\ m^2/l\ (5\ mm\ tube).$$

For a tube diameter of only 1 mm a value of

$$F/V = 4\ m^2/l\ (1\ mm\ tube)$$

is obtained.

An exemplary calculation shows that using the membrane module LunDia Alpha 600 it is indeed possible to achieve a sufficient indirect oxygen supply via the membrane:

Based on the volume to be supplied, the oxygen transport is:

$$S = F/V * P * (c_1-c_2)_{mean}.$$

Here $F/V = 13\ m^2/l$.

P is the permeability coefficient. Said coefficient was determined in our own experiments to P = 0.066 cm/min for a membrane, Cuprophan type, as is employed in the module mentioned. This value is valid for a membrane of 20 µm in thickness (thickness measured in the dry state). The membrane module LunDia Alpha 600 uses a Cuprophan membrane of 8 µm in thickness so that the actual permeability coefficient is rather greater and the calculation given above gives a rather "low" value.

The negative concentration gradient (c_1-c_2) across the membrane is assumed to be

$$(c_1-c_2)_{mean} = 11.45\ mg/l.$$

This calculation example assumes that a dialysate having an oxygen content of 30 mg/l flows from the container for dialysis fluid 1 to the membrane module. It is assumed here that pure oxygen is introduced into

the container (the saturation concentration of pure oxygen is approx. 32 mg/l). In the membrane module the dialysate is diluted to a concentration of 4 mg/l. The dialysate is fed back to the container at said concentration. Thus, on the dialysate side an oxygen content c_1 which is reduced from 30 mg/l (module entrance) to 4 mg/l (module exit) is to be expected. The oxygen content on the culture side should be a constant value of c_2 = 1 mg/l which is sufficient for growth of the organisms. Using these assumptions,

$$(c_1 - c_2)_{mean} = \frac{(c_{1entrance} - c_2) - (c_{1exit} - c_2)}{\ln((c_{1entrance} - c_2)/(c_{1exit} - c_2))} = 11.45\,mg/l$$

The assumed data give a value for the oxygen supply, based on the culture volume, of

$$S = 5.9\ g/(1*h).$$

Since the actual permeability coefficient which indicates the permeability of the membrane for oxygen is in reality markedly greater than the value of 0.066 cm/min assumed in the calculation, due to the low membrane thickness of only 8 µm, the oxygen supply of the suspension of organisms in the membrane will in reality be better than indicated here by calculation. Thus it can be assumed that the oxygen supply for microorganisms in an external membrane module via the membrane is in a range which can be regarded as "sufficient", if the area/volume ratio is sufficiently large and the membrane has good oxygen permeability.

The term "sufficient" is oriented toward the need of the particular cells or toward the supply as provided in the culture vessel 2. Usual values for an industrial culture of E.coli cells, to give an example, are in the range between 5 and 15 g/(1 h).

Owing to these relationships, it is possible for the skilled worker with knowledge of or after determination of the oxygen permeability coefficient and also the area/volume ratio of the module used in each case to readily determine whether said module is suitable for the use of the invention. In the case that the membrane module cannot ensure the desired supply with

gas, a membrane module according to Fig. 3 may be used, followed by both indirect and direct oxygen supply.

The inventor completely surprisingly recognized the relationships described above and, in particular, the possibility of using the system otherwise used merely for hemodialysis as a separate membrane module in the generic reaction system in which, as is further illustrated in connection with Fig. 2, oxygen is introduced into the dialysis fluid of container 1 and the dialysis fluid enriched in this way transports enough oxygen in order to supply the culture space 2 and the cells contained therein and, in a further aspect, to supply in particular those cells suffering from oxygen limitation while passing the membrane module in a sufficient and necessary manner with oxygen so that the cell density attainable in the culture fluid is not limited by said oxygen limitation, while the cells pass through the membrane module. Although the use of dialysis membranes for removing toxic metabolic products was known, as also already illustrated at the beginning, it was not known that when using appropriate membranes it is possible at the same time to ensure the oxygen supply described above. Thus, there is a relationship between the apparatus of the invention and the use of the invention such that the problem to be solved was to avoid oxygen limitation of the cells, while said cells were passing through the membrane module. In the case of the apparatus of the invention this is achieved by blowing oxygen preferably into the internal space of the membrane-shaped membranes and, in the case of the inventive use of the commercially available plate modules, selecting those modules which have a sufficiently high area/volume ratio and also a sufficiently high oxygen permeability coefficient so that the dialysis fluid which is enriched with oxygen in the container for the dialysis fluid 1 can give off said oxygen via the dialysis membrane to the cells passing through the membrane module. In both cases, the problem of oxygen limitation of the cells passing through the membrane module is solved.

A previously described membrane module depicted diagrammatically in Fig. 4 may be used in the generic reaction system, as, for example, depicted in Fig. 2.

The features of the invention disclosed in the description above, the claims and the drawings may be essential, both individually and in any desired

combinations, for the realization of the invention in its various embodiments.

List of reference numbers

1	Container for dialysis fluid
2	Culture space
3	Dialysis membrane
4	Stirrer (in culture space)
5	Gas supply line
6	Exhaust air line
7	Gas bubbles
8	Membrane module
9	Pump (for dialysis fluid)
10	Pump (for culture fluid)
11	Line (for dialysis fluid)
12	Line (for dialysis fluid)
13	Line (for culture suspension)
14	Discharge line (for culture suspension)
15	Supply line (for tube 16)
16	Tube
17	Culture fluid
18	Dialysis fluid
19	Supporting structures
20	Line (to membrane module)
21	Gas supply line
22	Exhaust air line
23	Stirrer (in container for dialysis fluid)

Claims

1. A method for culturing cells using a reaction system, in which the reaction system comprises a container for dialysis fluid, a space for culturing cells and a membrane module which connects both spaces with one another and which has at least two spaces which are separated by a membrane and through one of which a dialysis fluid flows and through the second of which a culture fluid containing cells flows, and a first gas is introduced into the culture fluid in the space for culturing the cells, characterized in that a second gas is introduced into the culture fluid in the membrane module and the membrane is functionally a dialysis membrane.

2. The method as claimed in claim 1, characterized in that gas is passed directly into the culture fluid present in the membrane module by introducing the second gas directly into the culture fluid in the membrane module.

3. The method as claimed in claim 1, characterized in that gas is passed indirectly into the culture fluid present in the membrane module by introducing the second gas into the dialysis fluid in the container for dialysis fluid and the gas reaching from there the culture fluid present in the membrane module via the membrane of the membrane module.

4. The method as claimed in claim 2 or 3, characterized in that gas is introduced both directly and indirectly at the same time.

5. The method as claimed in any of claims 1 to 4, characterized in that the membrane module comprises at least one gas supplying means and the gas supplying means supplies at least one of the spaces separated by the membrane and carrying a liquid with the second gas.

6. The method as claimed in claim 5, characterized in that the gas supplying means has an outlet which is located in particular in the membrane module space carrying the culture fluid.

7. The method as claimed in claim 5 or 6, characterized in that the gas supplying means is a tube.

8. The method as claimed in any of claims 5 to 7, characterized in that the gas supplying means has the shape of a nozzle.

9. The method as claimed in any of claims 1 to 8, characterized in that the membrane of the membrane module is composed of a material which is selected the group comprising regenerated cellulose, polyamide, polypropylene and polysulfone.

10. The method as claimed in claims 1 to 4 and 9, characterized in that the membrane module is a plate module.

11. The method as claimed in claim 10, characterized in that the membrane of the membrane module is a dialysis membrane.

12. The method as claimed in claim 11, characterized in that the dialysis membrane material is Cuprophan.

13. The method as claimed in any of claims 1 to 12, characterized in that the membrane module provides for a gas exchange sufficient for the cells, due to its area/volume ratio and its gas permeability coefficient.

14. The method as claimed in claim 13, characterized in that the area/volume ratio is at least about 5 m^2 per liter, in particular at least 10 m^2 per liter.

15. The method as claimed in claim 14, characterized in that the area/volume ratio is about 13 m^2 per liter.

16. The method as claimed in any of claims 13 to 15, characterized in that the oxygen permeability coefficient is about 0.066 cm per minute or higher.

17. The method as claimed in any of claims 1 to 16, characterized in that the container for dialysis fluid has a means for supplying gas and/or a means for removing gas.

18. The method as claimed in any of claims 1 to 17, characterized in that the membrane module, the space for culturing the cells and/or the container for dialysis fluid have increased pressure.

19. The method as claimed in any of claims 1 to 18, characterized in that the first and the second gas are individually and independently of one another selected from the group comprising air, oxygen, nitrogen, carbon dioxide and mixtures thereof.

20. The method as claimed in claim 19, characterized in that the second gas is oxygen.

21. The method as claimed in claim 19, characterized in that the second gas is carbon dioxide.

22. The method as claimed in any of claims 1 to 21, characterized in that the cells are selected from the group comprising microbial cells, fungal cells, animal cells and plant cells.

23. The method as claimed in claim 22, characterized in that the cells are Escherichia coli cells.

24. A membrane module which comprises at least two spaces separated by a membrane and through each space a liquid flows, the liquid in one space being dialysis fluid and in the other space culture fluid, characterized in that the membrane module comprises at least one gas supplying means and the gas supplying means in at least one of the spaces separated by the membrane has an outlet.

25. The membrane module as claimed in claim 24, characterized in that the membrane has the shape of a tube and its volume forms one of the two spaces.

26. The membrane module as claimed in claim 25, characterized in that the diameter of the space formed by the tube-shaped membrane is about 3-10 mm, preferably 6-8 mm.

27. The membrane module as claimed in claim 25 or 26, characterized in that the membrane module comprises a plurality of spaces formed by a tube-shaped membrane.

28. The membrane module as claimed in claim 27, characterized in that a gas supplying means outlet is located in at least one of the spaces formed by the tube-shaped membrane.

29. The membrane module as claimed in any of claims 24 to 28, characterized in that the gas supplying means outlet is located in a space outside the space(s) formed by the tube-shaped membrane.

30. The membrane module as claimed in any of claims 24 to 29, characterized in that the gas supplying means is a tube.

31. The membrane module as claimed in claim 30, characterized in that the tube is arranged in the space in a concentric manner.

32. The membrane module as claimed in claim 30 or 31, characterized in that the internal diameter of the tube is from 0.2 mm to 3 mm.

33. The membrane module as claimed in any of claims 24 to 32, characterized in that the gas supplying means outlet is shaped like a nozzle.

34. The use of the membrane module as claimed in any of claims 24 to 33 as membrane module in a method as claimed in any of claims 1 to 23.

35. A reaction system for culturing cells comprising a container for dialysis fluid, a space for culturing cells and at least one membrane module inserted in between, said membrane module ensuring sufficient gas supply during passage of the culture fluid through the

membrane module or sufficient gas exchange in the culture fluid located in the membrane module.

36. The reaction system as claimed in claim 35, characterized in that the membrane module is that as claimed in any of claims 24 to 33.

37. The reaction system as claimed in claim 35 or 36, characterized in that the container for dialysis fluid contains at least one gas-introducing device.

38. The reaction system as claimed in claim 35 or 37, characterized in that the membrane of the membrane module has a gas permeability coefficient which is high enough in order to ensure sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module, and/or has a suitably high area/volume ratio in order to ensure sufficient gas supply during passage of the culture fluid through the membrane module or sufficient gas exchange in the culture fluid located in the membrane module.