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(73) Patenthaver: **Strix Beheer B.V., Bothoekweg 9, 7115 AK Winterswijk Brinkheurne, Holland**

(72) Opfinder: **VAN DER HEIDEN, Pieter, Mgr. Van De Weteringstraat 89, NL-3581 EE Utrecht, Holland**
BUEVINK, Marc, Dilleveld 67, NL-7006 TN Doetinchem, Holland
OOSTERHUIS, Nicolaas Marius Gerard, Landschrijverlaan 35, NL-9451 KT Rolde, Holland

(74) Fuldmægtig i Danmark: **NORDIC PATENT SERVICE A/S, Bredgade 30, 1260 København K, Danmark**

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DESCRIPTION

[0001] The invention relates to the field of biotechnology and industrial scale cell culturing. In particular, it relates to a method and an apparatus for cultivating cells utilizing wave motion.

[0002] Such a method and such an apparatus are known from US 6,190,913 B1, which discloses a method according to the preamble of claim 1 and an apparatus according to the preamble of claim 6. This known technique employs a pre-sterilized flexible plastic bag in which cells are cultivated. The bag is partially filled with growth media and the remainder of the bag is continuously purged with air or other oxygen-rich gas. The bag is secured to a rocking platform that is rocked to and fro about one horizontal axis by the alternate actuation of pneumatic pistons. The rocking motion promotes wave formation in the bag which provides liquid mixing and enhances oxygen transfer from the headspace gas to the liquid phase where it is essential for cell growth and metabolism.

[0003] FR 2 353 638 A1 is another document, which discloses a method according to the preamble of claim 1 and an apparatus according to the preamble of claim 6. Similar to the abovementioned document US 6,190,913 B1, also FR 2 353 638 A1 discloses a rocking apparatus. In FR 2 353 638 A1 a container is rocked to and fro about one horizontal axis.

[0004] Adequate gas-liquid mass transfer (oxygen supply and carbon dioxide removal) is one of the important parameters for bioreactor design and functioning, and also plays a decisive role in strategies for upscaling culturing systems (see Dhanasekharan, K., 2006, BioProcess Int. March 2006, pages 2-6).

[0005] It is an object of the invention to further improve cell growth and productivity in the cultivating of cells utilizing wave motion. Inter alia, it is an object to provide a cell culturing system which is suitable for industrial scale-up.

[0006] According to the present invention, this object is achieved by providing a method according to claim 1. The invention may also be embodied in an apparatus according to claim 6 and in an assembly according to claim 14.

[0007] The combination of the swivelling movement of the container with respect to the pivot axis and the cyclical movement of the pivot axis along the closed-loop path induces a wave motion of the liquid medium in the container, which wave motion results into enhanced oxygen transfer and liquid mixing. The wave motion can be optimized for a given cell culture to provide sufficient oxygen transfer for high-density cell culture without excessive foaming or shear damage. No mechanical mixing is required. Thus, an improved cell growth and productivity, e.g. of a recombinantly produced protein, can be achieved.

[0008] Further, the invention also discloses an improved flexible bag (container) for use in the present invention, the improvement consisting of a bag of which the volume can be changed *in*

situ e,g, by opening or closing one or more compartments and/or a bag which has essentially straight corners.

[0009] Particular embodiments of the invention are set forth in the dependent claims.

[0010] Further features, effects and details of embodiments of the invention are described below with reference to the accompanying schematic drawings.

Fig. 1 shows in side view an example of an embodiment of an apparatus according to the invention.

Figs. 2A-2D show the example of Fig. 1 at different stages during the movement of a container.

Fig. 3 shows in side view an example of a second embodiment of an apparatus according to the invention.

Fig. 4 shows in side view an example of a third embodiment of an apparatus according to the invention.

Figs. 5A-5H show the example of Fig. 4 at different stages during the movement of a container.

Fig. 6 shows the example of Figs. 4 and 5 in a perspective view.

Fig. 7 shows the effects of agitation speed of a bioreactor according to the invention on kia (volumetric oxygen transfer component) for two different bags A and B (see Fig. 10)

Fig. 8 shows effects of the tilt angle of a bioreactor according to the invention on kia for two different bags A and B (see Fig. 10)

Fig. 9 shows the effect of the liquid volume of a bioreactor according to the invention on kia.for two different bags A and B (see Fig. 10)

Fig. 10. Schematic representation of the top view shape of bags A and B.

Fig. 11. Viability of PER.C6® cells during pre-culture in a bioreactor according to the invention.

Fig. 12. Viability of PER.C6® cells during batch culture in a bioreactor according to the invention.

Fig. 13. Viable cell density (VCD) in a fed-batch culture in a bioreactor according to the invention compared to VCD in a traditional stirred bioreactor and a Wave bioreactor.

Fig. 14. Production of an IgG type antibody in a fed-batch culture in a bioreactor according to the invention compared to production in a traditional stirred bioreactor and a Wave bioreactor. **(A)** Antibody concentration versus time of culture. **(B)** Antibody concentration versus total amount of viable cells produced over time (IVC).

Fig. 15. Growth curve of a bacterial *E. coli* culture in a 3.5 L stirred batch reactor. Cell density measured at OD_{600}. **(A)** Linear Y-axis. **(B)** Logarithmic Y-axis.

Fig. 16. Comparison of growth curve of a bacterial *E. coli* culture in a 10 L bioreactor according to the invention compared to a 3.5 L stirred bioreactor. **(A)** and **(B)** as in Fig. 15.

Fig. 17A shows a container with two clamps, whereby reduction of the bioreactor volume can be obtained. Fig. 17B shows a detail of a possible embodiment for fixating the clamps in the platform support of the disposable container.

Fig. 18 shows a second embodiment for changing the bioreactor volume of a disposable container. Through a gas inlet the separate compartments on either side of the bag are inflated and thus reduce the volume of the inner (bioreactor) compartment.

Fig. 19. Example of a container for use in the invention.

[0011] Reference is first made to Figs. 1 and 2. These figures show an apparatus 1 (bioreactor) for cultivating cells in a container 2 utilizing wave motion of a liquid medium 3 comprising a cell culture in the container. In the shown example, the container 2 is a plastic bag having a single hollow interior chamber. The plastic bag is secured to a platform 7 of the apparatus 1. However, other types of containers and other types of retaining means for retaining the container may also be applied, such as a tray.

[0012] The apparatus comprises a driving mechanism for swivelling the platform 7, and hence the bag 2, with respect to a substantially horizontal pivot axis 5. In the example, the pivot axis 5 is the central axis of a hinge joint between the platform 7 and a rotating beam 8 of the apparatus 1. Many types of hinge connections between the platform and the rotating beam 8 can be applied. The rotating beam 8 is driven by a motor 4 to rotate around a rotation axis 9 which is distant and parallel to the pivot axis 5. The driving mechanism is arranged to swivel the bag 2 such that during said swivelling, the pivot axis 5 follows a cyclical closed-loop path 6. The closed-loop path 6 has a circular shape, which is simple to realize. However, by choosing different configurations of the driving mechanism, other shapes of closed-loop paths can be applied as well.

[0013] In the example, the platform 7 is furthermore swivelling with respect to a second pivot axis 15 which is distant and parallel to the pivot axis 5. The second pivot axis 15 is the central axis of a second hinge joint between the platform 7 and a second beam 18 of the apparatus 1. The second beam 18 is rotatable with respect to a second rotation axis 19 which is distant and parallel to the second pivot axis 15, such that the second pivot axis 15 of the second beam 18 is free to move back and forth along an arched path 16. The orientation of the first rotation axis 9 relative to the orientation of the second rotation axis 9 remains the same during the movement of the platform.

[0014] Figs. 2A-2D show four different stages during the thus obtained movement of the bag 2.

[0015] In the example, the closed-loop path 6 lies in a plane transverse to the pivot axis 5. This is favourable because then the movement vector of the pivot axis along such closed-loop path is in the same plane as the movement vector V of the waves, which turns out to be very efficient. High efficiency is also achieved when at least the projection of the closed-loop path onto a plane transverse to the pivot axis has a closed-loop shape.

[0016] However, closed-loop paths can be applied with any type of shape in space, for example a closed-loop path in a horizontal plane through the pivot axis or a closed-loop path in a vertical plane through the pivot axis.

[0017] Reference is now made to Fig. 3, which shows an example of a second embodiment of an apparatus according to the invention. The shown apparatus is an apparatus 101 for cultivating cells in a multiplicity of containers utilizing wave motion of a liquid media. The apparatus 101 comprises an apparatus 1 of the type shown in Figs. 1 and 2. This apparatus 1 is shown in the upper left part of Fig. 3, wherein the same reference numerals are used as in Figs. 1 and 2. The apparatus 101 is furthermore arranged for cultivating cells in a co-swivelling container 2A, shown in the upper right part of Fig. 3. For that purpose, the driving mechanism is arranged to swivel both the container 2 and the co-swivelling container 2A simultaneously. In the shown example, this is realized in the following manner.

[0018] The co-swivelling bag 2 is retained by a platform 7A, similar to the platform 7 of apparatus 1. This platform 7A has a hinge joint with the rotating beam 8 of apparatus 1, this hinge joint having the pivot axis 5 as central axis. The platform 7A is furthermore swivelling with respect to a pivot axis 15A which is distant and parallel to the pivot axis 5. The pivot axis 15A is the central axis of a hinge joint between the platform 7A and a beam 18A. The beam 18A is rotatable with respect to a rotation axis 19A which is distant and parallel to the pivot axis 15A, such that the pivot axis 15A of the beam 18A is free to move back and forth along an arched path 16A. The respective orientation of the rotation axes 9 and 19A remains the same during the movement of the platform 7A.

[0019] Cultivating cells in a multiplicity of co-swivelling containers simultaneously driven by a joint driving mechanism of an apparatus, offers a number of advantages. For instance, when compared to the use of a larger size single container, once culturing conditions are established for one container, there is no need for additional experimenting to scale up the cell culture volume. All that is required is placing several containers on the apparatus. Furthermore, cell culturing in parallel yet separate containers reduces the risk of culture failures, e.g. by infection or contamination. This contributes to the reliability of the culturing system.

[0020] In Fig. 3 the bag 2 and the co-swivelling bag 2A are positioned side by side as seen in a plane transverse to the pivot axis 5. Other types of side by side positions are also possible. For example, a side by side positioning as seen in a vertical plane through the pivot axis 5 is possible, wherein co-swivelling platforms are swivelling relative to the pivot axis 5 at different axial positions along the pivot axis 5.

[0021] The apparatus 101 is furthermore arranged for cultivating cells in two co-swivelling containers 2B, shown in the lower left and lower right parts of Fig. 3, respectively. In fact the configuration of the apparatus 101 shown in the upper left and upper right parts of Fig. 3 is repeated in the lower left and lower right parts of Fig. 3. Corresponding parts of the corresponding configurations are denoted by corresponding reference numerals, wherein the affix B is added to the reference numerals, respectively is replacing the affix A of the reference numeral in the upper parts of Fig. 3. In the shown example, this is realized in that the driving mechanism is arranged to swivel both the containers 2 and 2A and the two containers 2B simultaneously, for example by means of a driving belt 20 as shown. Thus, the apparatus 101 is arranged to perform the simultaneous swivelling in a condition in which different swivelling containers are positioned above one another. An advantage of placing a multiplicity of containers above one another is that it reduces costly plant space; the entire assembly of apparatus and containers occupies a much smaller surface area as compared to containers placed adjacent to each other in the horizontal plane. This allows for easy containment of the assembly in a cultivating room.

[0022] In the example of Fig. 3, the swivelling direction of the bag 2 relative to the joint pivot axis 5 is opposite to the swivelling direction of the co-swivelling container 2A relative to the joint pivot axis 5. This results into the movement vector V of the waves in the bag 2 and the movement vector VA of the waves in the bag 2A having opposite horizontal components. This turns out to be favourable with respect to energy economy and vibrations of the apparatus in operation.

[0023] This also holds for the lower configuration shown in Fig. 3. That is, the swivelling directions of the two shown bags 2B relative to their joint pivot axis 5B are mutually opposite, resulting into movement vectors VB of the waves in the bags 2B having mutually opposite horizontal components. The thus obtained advantage with respect to energy economy and vibrations is enhanced by arranging the apparatus such that the movement vector VB in the lower left part of Fig. 3 has a horizontal component which is opposite to the horizontal component V.

[0024] The combined effect of both side by side positioning and positioning above one another of containers, allows two dimensional and three dimensional arrangement of containers in one apparatus. This is favourable with respect to space requirements for carrying out methods for cell cultivation. It also is favourable with respect to costs of apparatuses for cell cultivation, because a single driving structure can be arranged to drive the swivelling motion of many containers.

[0025] Reference is now made to Figs. 4-6. These figures show an example of a third embodiment of an apparatus according to the invention. The figures show an apparatus 51 for cultivating cells in a container utilizing wave motion of a liquid medium in the container. For reasons of clarity, the container is not shown. Similar to the examples shown in Figs. 1-3, the container can for example be a plastic bag having a single hollow interior chamber. Such a bag can be secured to the shown platform 57 of the apparatus 51.

[0026] Also similar to the examples shown in Figs. 1-3, the apparatus 51 comprises a driving mechanism for swivelling the container with respect to a substantially horizontal pivot axis 55. In the example, the pivot axis 55 is the central axis of a hinge connection between the platform 57 and a rotating beam 58 of the apparatus 51. The rotating beam 58 is driven by a motor 54 to rotate around a rotation axis 59 which is distant and parallel to the pivot axis 55. The driving mechanism is arranged to swivel the container such that during said swivelling, the pivot axis 55 follows a cyclical closed-loop path 56.

[0027] The driving mechanism of the apparatus 51 is furthermore arranged to swivel the container such that during said swivelling the container carries out a second swivelling with respect to a second pivot axis 65 which is distant and parallel to the substantially horizontal pivot axis 55, during which second swivelling the second pivot axis 65 follows a second cyclical closed-loop path 66. The second pivot axis 65 is the central axis of a second hinge connection between the platform 57 and a scissor-beam assembly. In the example, the scissor-beam assembly comprises a second beam 68 and a third beam 78 which beams are interconnected via a hinge axis 77. The second beam 68 is rotatable with respect to a second rotation axis 69 which is distant and parallel to the second pivot axis 65. The rotation of the second beam 68 is driven by a motor 64. During the rotation of the second beam 68, the hinge axis 77 of the scissor-beam assembly follows a cyclical closed-loop path 76 which has circular shape. As an example, Figs. 4 and 5 show the second cyclical closed-loop path 66 which will be followed by the second pivot axis 65 when the first and second beams 58 and 68 are driven with the same rotation speed, however in opposite rotation directions. The orientation of the first rotation axis 59 relative to the orientation of the second rotation axis 69 remains the same during the movement of the platform 57. It is remarked that, instead of using two motors 54 and 64, also one motor could be applied driving the rotation of both the first beam 58 and the second beam 68, by utilizing suitable transmission means.

[0028] An advantage of such an apparatus based upon such second swivelling with respect to a second pivot axis 65 which is distant and parallel to the substantially horizontal pivot axis 55, is that in operation of the driving mechanism a wide range of varying slopes of the container can be obtained with only little vertical space requirement by the driving mechanism. In the example of Figs. 4-6, only short lengths of the first and second beams 58 and 68 suffice to create such wide slope range. When driving mechanisms such as those described with reference to Figs. 4-6 are incorporated in apparatuses wherein containers are positioned above one another, such as in the way as described with reference to Fig. 3, said little vertical space requirement will be benefited to an even greater extent.

[0029] As already discussed in the introduction, gas-liquid mass transfer is one of the important aspects in the design and operation of bioreactors. The oxygen transfer rate (OTR) depends on the difference between the actual concentration of oxygen in the medium and the maximal achievable concentration of oxygen (saturation concentration) in said medium and a factor k_1a which represents the mass transfer of the gas from the gas-liquid interfacial area in the medium as in the following formula: OTR = $k_la(c^*-c)$, where k_la is the volumetric mass

transfer coefficient, *c** is the saturated oxygen concentration, and c is the measured oxygen concentration at a certain time point. The same formula can be drawn up for carbon dioxide transfer. In the experimental section it is shown that the method and the apparatus according to the present invention dramatically increase the $k_l a$, thereby providing for more optimal growth conditions. The experiments also show that application of the method and apparatus of the invention indeed results in increased yields from the cultures, either measured in cell mass or in products (e.g. proteins, such as antibodies) excreted by the cells.

[0030] As said, the invention can be practised using many types of containers. In one embodiment, the container is a disposable container. On completion of the cell cultivation, the cell culture can be simply harvested from the container and the used container is discarded. A new container, provided with a new cell culture, can be immediately placed on the platform of the apparatus. In a preferred embodiment, the container is a pre-sterilized disposable container since this eliminates labour intensive cleaning, sterilization and associated validation. Such containers are known in the art. For example, US6,190,913 B1 discloses a pre-sterilized flexible plastic bag in which cells are cultivated. These single-use bioreactors are commercially available from Wave Biotech LLC, New Jersey ,USA. The container may be provided with additional means, such as means which facilitate the addition or removal of a substance, e.g. liquid medium or a sample, from the container (see for example US2003/0036192)

[0031] The invention can also be practised using a flexible or adjustable cell culturing container. At present, it is common practice to start a cell culture by introducing an inoculation cell culture in a relatively small container comprising a relatively small volume of growth medium, like 200 milliliter, such that the cell density is within an optimal range for cell replication. Subsequently, the starting culture is expanded by a step-wise transfer to containers of increasing size, for example up to a cell culture volume of 20 liters or even more, ensuring that the cell density is maintained within the required range. Presently known containers do not allow to perform the cell culture expansion from a small inoculation culture to a large 'working' culture in one single container. The dimensions of a container that can accommodate the volume of a working culture, e.g. 20 or 30 liter culture, are obviously unsuitable to accommodate the initial starting culture. Each step of the step-wise transfer of a cell culture to a larger container is a critical procedure as it carries the risk of introducing an infection or a contamination into the culture. Furthermore, it is very labour-intensive. To overcome these problems, the invention discloses a "flexible" container, that can be compartmentalized and that thus allows for modulating or adjusting the effective volume of the container during cell culturing. The term "effective volume" is meant to indicate the volume of the container which can be occupied by the cell culture. Modulating or restraining the effective volume involves in particular the restraining of one or more flexible walls of the container at the onset of the culturing process, while gradually or stepwise releasing the restrain along with expansion of the cell culture volume. Of course, adjusting the effective volume can also involve reducing the effective volume of the container. As a general rule of thumb, the initial effective volume is about 10% of the maximal volume of the container. Typically, during cell culturing 10 to 80% of the effective volume of the container which, as said, can be adjusted according to the volume of the culture, is filled with liquid medium and cells. There are many ways by which modulation

of the effective volume of a container can be realized. The container preferably has one or more flexible walls. It is for example a plastic bag whose side walls can be pushed downwards or towards each other by external means, like clamps, such that the volume of the bag is reduced. A more elegant system is to integrate (one of) the clamps with the disposable container. In this way, the position, which determines the volume, is fixed. With a fixed position, a part of the clamping system could even be used to mount the disposable container on the platform. The clamping system can be integrated with the bag in such a way that the bottom part of the clamps are forming one part with the bottom of the bag. The bottom clamp may, for instance, be glued to the bottom part of the bag. The clamp can be fixed in the supportive tray of the bioreactor system, e.g. by positioning the clamp in a groove in the tray bottom (see Fig. 17B). This figure shows a part of the cross section of a clamped bag, where the bottom side of the clamping system fits within the platform of the bioreactor system.

[0032] It is possible to clamp the bag between the clamps in such a way that the bag is closed water tightly at different places before use. For clamping the clamps together different mechanisms may be used: magnetic clamping, mechanical clamping by means of screws or bolts, etc. A person skilled in the art is capable of finding alternative ways to clamp the bag water tightly. The upper side of the clamp can be removed easily to increase the volume. After removal of the upper clamp, the bottom clamp can stay in place, providing for holding the bag in a fixed position in the container.

[0033] An alternative for using solid clamps is use of a peal-seal type of closing, in which the seal is integrated in the bag, e.g. as a groove in which a ridge from the opposite side of the bag is clamped. Alternatively, the seal on both sides of the bag consists of weakly adhesive strips. The seal will open by applying pull force from the outside or by applying push force from the inside.. In both above mentioned embodiments, mounting a plastic strip on the outside of the bag causes the seal to be closed and to be prevented from opening from inside or outside forces. Removing the strip causes the seal to open by the air and liquid pressure from the internal compartment.

[0034] Another way to implement a flexible volume system is to use the flexibility of the disposable bag. When on the outside of the bag inflatable compartments are sealed, filling these compartments with gas will decrease the culture volume in the bag. Dependant on the desired volume, these compartments can be (partially) filled and emptied. Due to the pressure provided by these inflatable compartments, controlling of the volume of the container is effected. It is possible to use the gas flow, which normally is led to the container for providing gas to inflate the separate compartments (see Fig. 18). To use the gas supply to control the working volume, the inlet of the gas has to be divided between the compartments to be inflated and the bioreactor. One port goes directly to the inlet filter of the bioreactor, the other port is equipped with a control valve, which controls the opening and closing of the gas flow to the inflatable compartments. In closed position, no gas flows to the inflatable containers, but gas will be present in the bioreactor, thereby forcing the bioreactor to expand to its maximal volume. Opening the valve will inflate the compartments, which then will force the wall of the bioreactor inwards, thereby decreasing the bioreactor volume. Due to the gas outlet of the

bioreactor, the pressure inside the inflated chambers will, when the valve is in the open position, always be larger than the pressure within the bioreactor. Releasing gas from the inflated chambers can be performed by using an exhaust switch at the valve.

[0035] In all embodiments, it is advantageous to also have a compartmentalization in the heating mats, which are located between the platform support of the apparatus and the container, to allow for differentiated temperature control of the different sections of the container.

[0036] Modulating the effective volume of the container during the cell cultivation process by changing the position of at least a flexible part of the container wall can be applied during cell cultivating methods wherein the container is moved in ways as explained above. However, said modulating can also be favourably applied for other cell culturing methods, wherein the container is moved in other ways, or wherein the container is not moved at all.

[0037] A third way to increase the working volume of the bioreactor and thereby reaching scale-up of the bacterial or cell culture makes use of an apparatus of the invention which accommodates more than one bioreactor, i.e. more than one container. In this case scale up can be reached by connecting the containers with each other through (disposable) tubing. If a connection between two or more containers is present, a culture can be grown to maximal volume in one container, where after a valve closing off the tubing can be opened, allowing the culture also to occupy a second container. A flow of the content of the container(s) can be exerted by inserting a pump in the tubing. In this way, the more than one container can be characterized as one batch in which the culture has undergone identical process conditions. Preferably, in the case of compartmentalized bioreactors, first the culture is grown in a minimalized bioreactor, then a connection between this reactor and one or more other (minimalized) bioreactors, which have been filled with medium, is established, and then the working volume of all bioreactors is gradually increased (e.g. by removing clamps or by deflating the inflatable compartments). In this way a full scale-up of the culture can be established, from a less than 100 mL culture to cultures of more than 200 L, without the need to repeatedly change containers.

[0038] Another important phenomenon is the scale-up strategy based on mass transfer principles. As long as the mass-transfer coefficient kia is kept the same at different scales the capability of oxygen transfer and CO_2-removal will stay the same at the different scales. In such a way the environmental conditions of the cells are the same. In stirred tank reactors and/or bubble columns this is not possible due to the impact of stirrer geometry on for example shear, superficial gas velocity (gasflow/reactor surface) on bubble coalescence and therefore shear damage to the cells and the lower specific liquid surface for surface aeration.
The wave bioreactor (US 6,190,913) is scaled-up into three dimensions (width, length and liquid height of the bag), thereby changing the mass transfer characteristics.

[0039] By keeping the wavelength (L) in the bag constant, as well as keeping the rocking speed and the rocking angle the same when scaling-up, the mass transfer characteristics will

not change. Important is that the shape of the bag is such that the edges are approx. 90° (+/- 15°) (see figure 10).

[0040] Another approach to keep the mass transfer properties the same in scaling up is to reduce the wavelength but to compensate this by changing the angle as well (at longer wavelengths the angle will decrease). The liquid height should be kept the same, as this mainly determines the specific gas-liquid contact area.
If the angle is changed, the rocking speed should be adjusted as well. This to create the same frequency of the wave. However, the range in between which the rocking speed might be varied, can be large.
Controlling the volume of the container by clamping or otherwise is preferably performed in such a way, that the initial small volume is localized central in the bag. In this way all necessary inlets and outlets can be centrally positioned and accommodate both the bags in minimal volume and in maximal volume. Further, advantageously, the volume in the bag is increased without changing the k_la value. This can be accomplished by causing the volume decreases and increases along with the direction of the wave motion that is made by the bioreactor according to the invention, i.e. parallel to the plane of the container in Fig. 1, see fig. 17A. Thereby, it is achieved that during increase of the volume, the parameter L, which stands for the wavelength within the container and which influences the kia, remains unchanged.

[0041] One example of a bag as schematically depicted in Fig 17A is depicted in Fig. 19. The position of the clamps is clearly visible, whereby the bag with clamps closed has a working volume of about 1/2 of the final volume. It will be clear that by adding clamps or taking other positions for the clamps more to the centre of the bag, the initial volume can be decreased. Also the availability of multiple inlets and/or outlets from the bag in the central position should be noted. These inlets and/or outlets can serve as inlet for medium and or inoculum, but also for inlet/outlet of gas (i.e. air or oxygen). Further, as described above, disposable tubing, as is visible in Fig. 19, can be connected to these inlets/outlets to provide for a connection with another bag. Further, the inlets/outlets can be used to take samples from the culture and/or to provide for temperature or other measurements of the culture liquid. Thus, the inlets and/or outlets also provide for coupling with other devices. These devices can be positioned outside the bag or can be integrated with the bag. Such a device can for instance be a pump for providing liquid culture flow between one or more bags, an aeration device, such as a hollow fibre, flat membrane or static mixer, a filter or any other device allowing for isolation of products and/or debris. In this way, e.g. continuous inflow of fresh medium and other essentials for the culture, and continuous outflow of cells and/or products could provide for a continuous culture.

[0042] It has also shown that the shape of the container can enhance the effects of the wave motion of the present invention. From Example 1 it can be learnt that the bags ideally would have essentially straight corners, i.e. corners of 90° ± 20° (see Figure 10), more preferably corners of 90° ± 15°, most preferably corners of 90° ± 10°. Because of the essentially square corners, the wave that is created by the apparatus of the invention 'rocks' against the end of the bag and rolls over its top. Thereby an intense mixing of air and medium is established. When the corners of the bag are rounded off, the wave dies out quickly due to giving the wave

a velocity direction towards the middle line of the bag and a less intensive mixing occurs. In all the experiments performed in Example 1, i.e. with different swiveling speeds, different tilt angles and different volumes, the bag with the square corners performed best

EXAMPLES

Example 1 k_la measurements

[0043] Several experiments were done to investigate the actual behaviour of the method and the apparatus according to the invention concerning the (oxygen) transfer rate. First these experiments were done with plain tap water as medium, later on a mixture, with physiological properties comparable to real medium, was used.

Experiment: determination kia-value

Materials:

[0044] 'Shocker' apparatus according to claim 8
WAVE Cellbag / Applikon media bag
Presens dissolved oxygen (DO) sensor, transmitter
N_2, compressed air

Purpose:

[0045] Determination of the performance of the Shocker system, concerning the k_la-values, by variation of several parameters, like agitation speed and tilt angle.

Operating procedure:

[0046] To calculate the k_la-value, the classic dynamic method is used. This means filling the headspace with an inert gas, N_2, to drive away the dissolved oxygen in the water.
When the DO-value reads 10 % or less, the headspace is filled with air, the machine is started and the DO-value is recorded. After changing the desired parameters the next measurement can start.
After the measurements the DO-values are exported to Microsoft Excel, where the data can be

plotted.
With two points of the graph (C_{L1}, t_1) and (C_{L2}, t_2) the kia-value can be calculated with the formula

$$k_L \cdot a = \ln \frac{\left(C_L^* - C_{L1}\right)}{\left(C_L^* - C_{L2}\right)} \cdot \left(t_2 - t_1\right)^{-1} ,$$

where (CL_1, t_1) is the DO-value at time ti, (C_{L2}, t_2) the DO-value at time t_2 and C^*_L the saturation concentration (the eventually achieved DO-value).
Plotting the graph on a semi-logarithmic scale will produce a straight line, on which the two points C_{L1} and C_{L2} are chosen.

Hypothesis:

[0047] The kia-value is dependent of the exchange area on the interface of liquid and gas. So it will increase for larger fluid displacements, i.e.
when agitation speed increases or when the tilt angle increases. It will also increase when the amount of liquid in the bag is reduced due to a lower liquid height (because in the above formula 'a' is the specific mass transfer area = surface / volume).

[0048] When the liquid level is increasing however, the wave created is more turbulent and flows back over the topside of the bag. This, at one hand creates a higher "a"- value but on the other hand also a higher k_l - value. Such way the mass transfer can increase at bigger volumes. This effect is most strongly at larger rocking speeds.

Measurement results

[0049] Measurements have shown the performance of the system as function of several parameters. These parameters, the agitation speed, the tilt angle and the liquid volume in the disposable were changed while the oxygen concentration in the liquid (water) was measured. With graphing the oxygen concentration against the time it is possible to determine the k_La-value with the 'dynamic method'. The results of these experiments are shown in the figures 7-9. During the experiments a distinction is made between the used bags, named 'bag A' and 'bag B' in the figures. The results show a significant effect on the oxygen transfer characteristics of the different bags. As the size was comparable, just as the amount of liquid during the experiments, the only variable was the shape of the disposables. The top views in figure 10 show these differences.

Example 2 Results on mammalian cell cultures

[0050] Three different experiments have been performed to investigate the growth and

performance of an antibody producing clone of PER.C6®-cells in the apparatus of the present invention (hereinafter also indicated by the name CELL-tainer™).
All the experiments have been performed in commercially available (standard) cultivation bags and standard media and protocols as have been used for PER.C6® cultivation.
The same feed regime (fed-batch) and pre-cultures were used in the comparison experiments.

Experiment 1

Pre-culture (see Figure 11)

[0051] After thawing the cells have been kept in pre-culture and the culture has been split two times. The culture has been kept viable during 12 days at viabilities > 98%. Conclusion, the CELL-tainer™ can be used for pre-culture.

Experiment 2

Batch culture (see Figure 12)

[0052] An ordinary batch culture has been performed. Comparison was made with a reference batch culture in a shaker (viable cell density maximum 5 x 10^6 cells/ml, blue line), a stirred 5L bioreactor (viable cell density maximum 7,5 x 10^6 cells/ml, green line) and the CELL-tainer™, 10L working volume (maximum viable cell density 8,0 x 10^6 cells/ml).
The viability was kept > 98% during the whole experiment which is slightly better than in a stirred bioreactor.
During the experiment, the shaking speed has been increased up to 80% (35 rpm) at the lowest shaking angle showing that the rocking speed does not impact the cells.

Experiment 3

Fed-batch culture (see Figure 13)

[0053] A fed-batch cultivation was performed in the CELL-tainer™ (working volume 10L) in comparison with a standard 10L stirred bioreactor, and a 20L Wave bioreactor (10L operational volume). During operation of the CELL-tainer™ the overhead space was gassed with a mixed gas, containing 5% CO_2. No pH-control has been used. The pH was kept constant

due to the buffering capacity of the medium as well as due to the exchange with the overlay gas. Here the performance was different from the STR (stirred bioreactor), where pH-control and stripping of CO_2 is needed to keep the pH constant.

The CELL-tainer™ results in a viable cell density (viability > 98%) of 13,0 x 10^6 cells/ml after eight days (CT1). During day 7 - 8 of the second run (CT2), there were operational problems with the gas-supply, resulting in a decreased growth during these days.

The STR equipment results in a cell density of approx. 11,0 x 10^6 cells/ml after eight days. The Wave bioreactor delivers a maximum of 7,0 x 10^6 cells/ml, and growth stops after 7 days.

[0054] During the whole experiment, the CELL-tainer™ system was running at 50 - 70% rocking speed at the minimum angle.

Productivity data

[0055] The production of an IgG type of antibody in the CELL-tainer™ has been compared with that of the production in a traditional stirred bioreactor and a Wave bioreactor.
As mentioned above, the cultivation of CT1 has been stopped after eight days. The second experiment in the CELL-tainer™, CT2, resulted in an IVC of 160 x 10^6 (cells x day/ml) and a final antibody concentration of 1,5 g/L. (see Figure 14A and B)

Example 3 Results on bacterial cell cultures

Materials and methods

Fermentation

[0056] Laboratory experiments were performed in a standard 3.5 L batch stirred bioreactor (Applikon 5L type Z61100005), with external temperature control (cryostat). Temperature was controlled at 37 °C. For growth studies *E. coli strain EC1000* was used. Medium used was standard TY medium (Trypton (Becton, Dickinson and co.) 1g/100 ml (1% end conc.); yeast extract (LS ferm powder DSM) 0.5g/100 ml (0.5% end conc.); Sodium chloride (Merck) 0.5g/100 ml (0.5% end conc.); pH 7.2

[0057] Growth was monitored for 24 h by measuring the optical density at 600 nm (OD_{600}) on samples of 2-3 ml.

[0058] The bioreactor was inoculated with 50 ml preculture shake-flask culture using same

media.

Results and conclusion

Fermentation on TY in a 3.5 L stirred bioreactor

[0059] The growth characteristics of the experiments are summarized in Table 1. In Fig 15 the growth curve is presented, Fig. 15A represents a linear scale of the measured OD_{600} values, Fig. 15 B gives the same data on a logarithmic scale.

Table 1. Characteristics of fermentation of *E. coli* EC1000 on TY in a stirred bioreactor

	Growth yield (OD_{600})	Max. Growth-rate (h^{-1})
	6.5	1.4

Fermentation on TY in CELL-tainer™ compared to a 3,5 STR

[0060] The growth curves and growth characteristics as shown in Fig 16A and B and in table 2 were at least similar, some higher maximum growth rate was observed in the CELL-tainer™.

Table 2. Characteristics of fermentation of *E. coli* EC1000 on TY in a batch reactor and Cell-tainer™.

	Growth yield (OD_{600})	Max. Growth-rate (h^{-1})
Stirred bioreactor	6.5	1.4
Cell-tainer™	6.9	1.75

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US6190913B1 [0002] [0003] [0030]
- FR2353638A1 [0003] [0003] [0003]
- US20030036192A [0030]
- US6190913B [0038]

Non-patent literature cited in the description

- **DHANASEKHARAN, K.**BioProcess Int., 2006, 2-6 [0004]

Patentkrav

1. Fremgangsmåde til celledyrkning ved hjælp af bølgebevægelse, der omfatter følgende trin:

- tilvejebringelse af en beholder (2; 2A; 2B), holdemidler (7; 7A; 7B; 57) til at holde beholderen og en drivmekanisme (4, 8; 4, 8, 8B, 20; 54, 58, 64, 68, 78) til drejning af beholderen i forhold til en i alt væsentlig horisontal drejeakse (5; 5B; 55);

- indføring af et gasholdigt oxygen, et flydende medium (3) og en cellekultur i beholderen;

- bevægelse af beholderen, således at beholderen drejer i forhold til den i alt væsentligt horisontale drejeakse (5; 5B; 55) for derved at inducere en bølgebevægelse til det flydende medium (3) i beholderen, hvilken bølgebevægelse bidrager til den nødvendige oxygenoverføring og blanding, der er krævet for cellevækst,

kendetegnet ved, at drejeaksen (5; 5B; 55) er defineret som en central akse af et hængselsled placeret ved en fastgørelse af holdemidlerne (7; 7A; 7B; 57) til en roterende stang (8; 8B; 58) af drivmekanismen (4, 8; 4, 8, 8B, 20; 54, 58, 64, 68, 78), og **ved, at** under drejningen af beholderen drejeaksen (5; 5B; 55) følger en cyklisk banen med lukket sløjfe (6; 6B; 56).

2. Fremgangsmåde ifølge krav 1, hvor projektionen af banen med lukket sløjfe på et plan på tværs af drejeaksen (5; 5B; 55) har form af en lukket sløjfe.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor den cykliske bane med lukket sløjfe (6; 6B; 56) har en cirkelform.

4. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor

- beholderen (2; 2A; 2B) omfatter en præsteriliseret plastpose med et enkelt hult indvendigt kammer;

- indføringen af gassen, det flydende medium og cellekulturen i beholderen sker ved:

- delvis fyldning af posen med gassen for derved delvist at oppuste posen;

- indføring af det flydende medium og cellekulturen i posen, hvor det flydende medium og cellekulturen omfatter mellem 10 og 80 % af posens volumen;

- fyldning af det resterende af posen med gassen, således at posen bliver stiv;

- posen holdes af holdemidler (7; 7A; 7B; 57);

- bevægelsen af beholderen (2; 2A; 2B) sker ved bevægelse af holdemidlerne (7; 7A; 7B;

57).

5. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor beholderens (2; 2A; 2B) effektive volumen moduleres under celledyrkningsprocessen.

6. Indretning til celledyrkning in en beholder (2; 2A; 2B) ved hjælp af bølgebevægelse af et flydende medium i beholderen, der omfatter holdemidler (7; 7A; 7B; 57) til at holde beholderen og en drivmekanisme (4, 8; 4, 8, 8B, 20; 54, 58, 64, 68, 78) til drejning af beholderen i forhold til en i alt væsentlig horisontal drejeakse (5; 5B; 55), **kendetegnet ved, at** drejeaksen (5; 5B; 55) er defineret som en central akse af et hængselsled placeret ved en fastgørelse af holdemidlerne (7; 7A; 7B; 57) til en drejende stang (8; 8B; 58) af drivmekanismen (4, 8; 4, 8, 8B, 20; 54, 58, 64, 68, 78), og **ved, at** drivmekanismen er indrettet til at dreje beholderen, således at under drejningen drejeaksen (5; 5B; 55) følger en cyklisk bane med lukket sløjfe (6; 6B; 56).

7. Indretning ifølge krav 6, indrettet til celledyrkning i en samtidigt drejende beholder (2A; 2B), hvor drivmekanismen er indrettet til at dreje både beholderen og den samtidigt drejende beholder samtidigt.

8. Indretning ifølge krav 7, indrettet til at udføre den samtidige drejning i en tilstand, hvori beholderen (2) og den samtidigt drejende beholder (2A) er placeret side om side.

9. Indretning ifølge krav 8, indrettet til at udføre den samtidige drejning i en tilstand, hvori beholderen (2) og den samtidigt drejende beholder (2B) er placeret over hinanden.

10. Indretning ifølge et hvilket som helst af kravene 6-9, indrettet til at udføre den samtidige drejning, således at den i alt væsentligt horisontale drejeakse (5) fungerer som fælles drejeakse for både drejningen af beholderen og drejningen af den samtidigt drejende beholder.

11. Indretning ifølge krav 10, indrettet til at udføre den samtidige drejning, således at drejningsretningen of beholderen i forhold til den fælles drejeakse (5) er modsat drejningsretningen for den samtidigt drejende beholder i forhold til den fælles drejeakse.

12. Indretning ifølge krav 6, indrettet til celledyrkning i mindst to samtidigt drejende beholdere (2A, 2B), hvor drivmekanismen er indrettet til at dreje både beholderen (2) og det

mindst to samtidigt drejende beholders (2A, 2B) samtidigt i en tilstand, hvori beholderen (2) og mindst én (2A) af de mindst to samtidigt drejende beholdere er placeret side om side, og hvori beholderen (2) og mindst en anden én (2B) af de mindst to samtidigt drejende beholdere er placeret oven på hinanden.

13. Indretning ifølge et hvilket som helst af kravene 6-12, hvor drivmekanismen er indrettet til at dreje beholderen, således at under drejning beholderen foretager en anden drejning i forhold til en anden drejeakse (65), der er distant og parallel med den i alt væsentlig horisontale drejeakse (55), hvorunder den anden drejning af den anden drejeakse følger en anden cyklisk bane med lukket sløjfe (66).

14. Anordning til celledyrkning, der omfatter en indretning (1; 51; 101) ifølge et hvilket som helst af kravene 6-13, og en beholder (2) til celledyrkning, når den er holdt af indretningens holdemidler (7; 7A; 7B; 57).

15. Anordning ifølge krav 14, hvor beholderen er en beholder til engangsbrug, fortrinsvis en præsteriliseret beholder.

16. Anordning ifølge krav 14 eller 15, hvor beholderen har en mindst delvist fleksibel væg, og hvor anordningen endvidere omfatter midler til modulering af beholderens effektive volumen under celledyrkningsprocessen ved ændring af positionen af mindst en fleksibel del af væggen.

17. Anordning ifølge et hvilket som helst af kravene 14-16, hvor beholderen er en steril plastpose til engangsbrug med mindst ét indløb og ét udløb, **kendetegnet ved, at** den har i alt væsentligt rette hjørner på 90° ± 20°, fortrinsvis hjørner på 90° ± 15°, mere fortrinsvis hjørner på 90° ± 10°.

18. Anordning ifølge et hvilket som helst af kravene 14-17, hvor beholderen er en steril plastpose til engangsbrug med mindst ét indløb og ét udløb, **kendetegnet ved, at** posens effektive volumen kan ændres.

19. Anordning ifølge et hvilket som helst af kravene 16 til 18, hvor ændringen kan foretages ved oppustning af lukkede ikke-vævskulturholdige rum.

20. Anordning ifølge et hvilket som helst af kravene 16 til 18, hvor ændringen kan foretages ved lukning og/eller åbning af ét eller flere rum af beholderen, hvor lukningen er foretaget med én eller flere klemmer eller forseglinger.

DRAWINGS

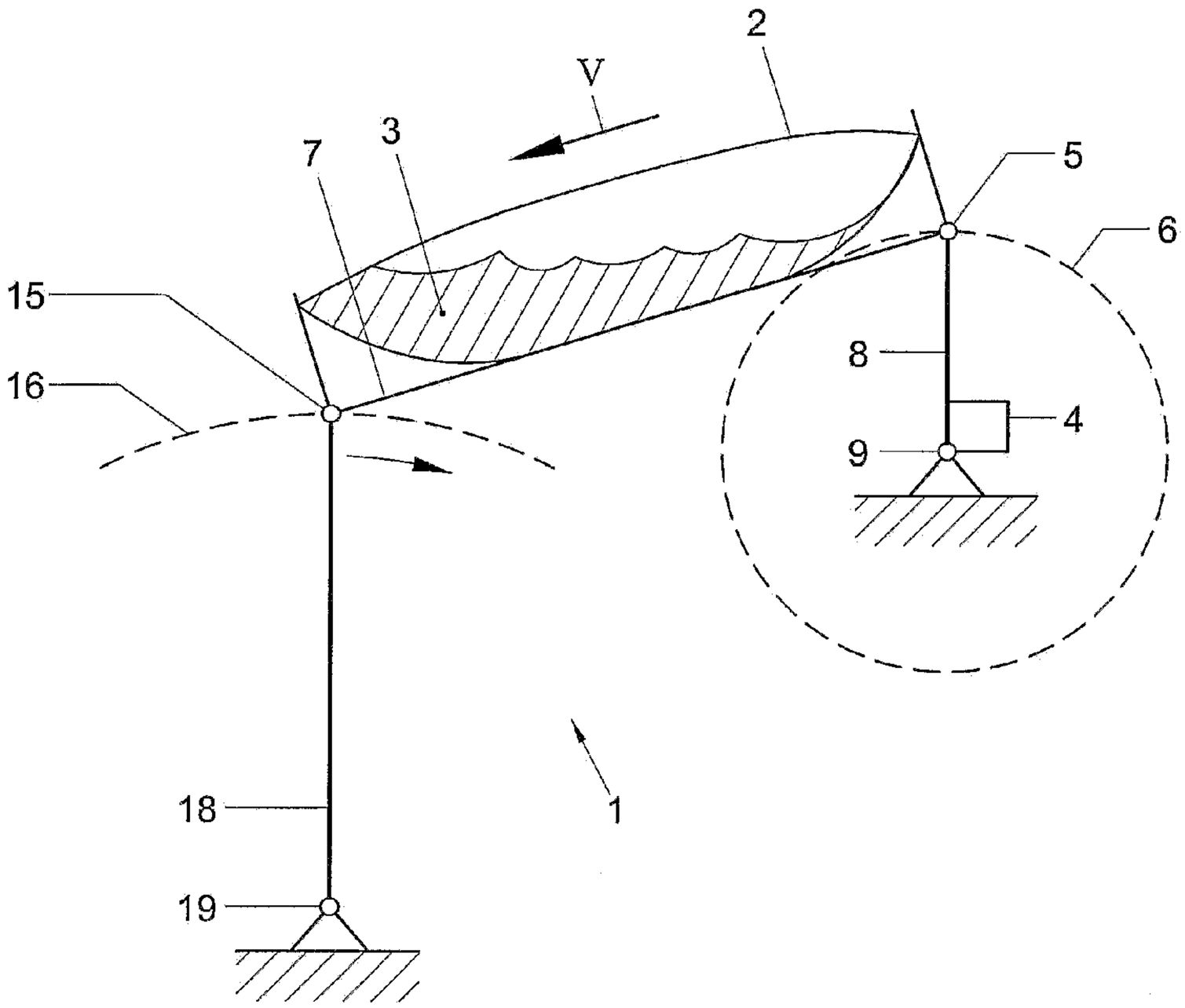


Fig. 1

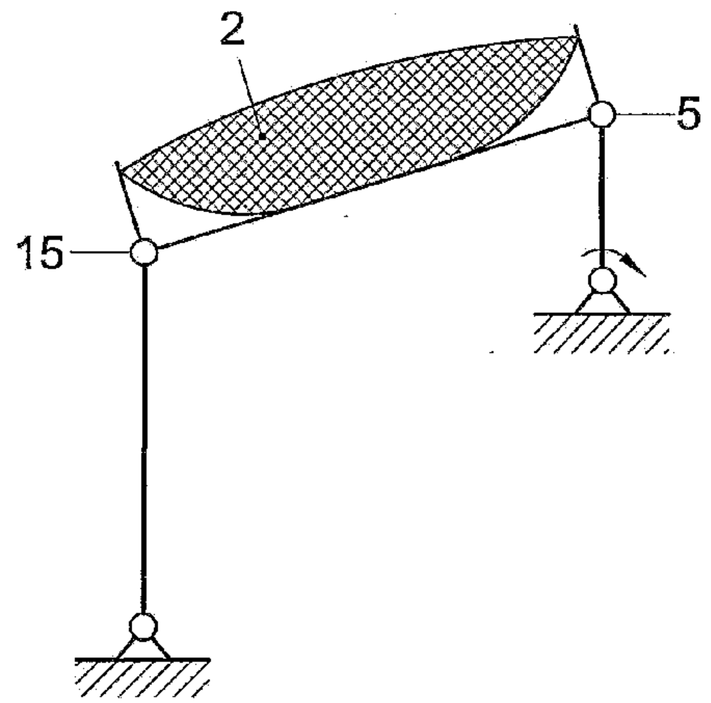


Fig. 2A

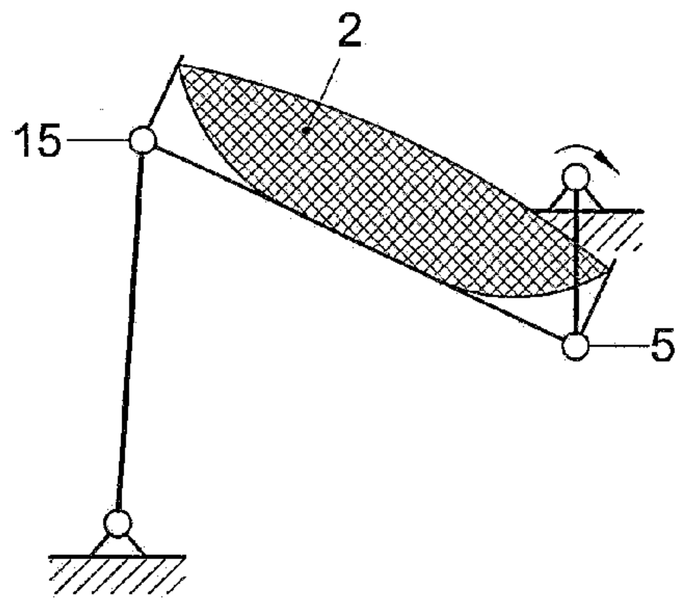


Fig. 2C

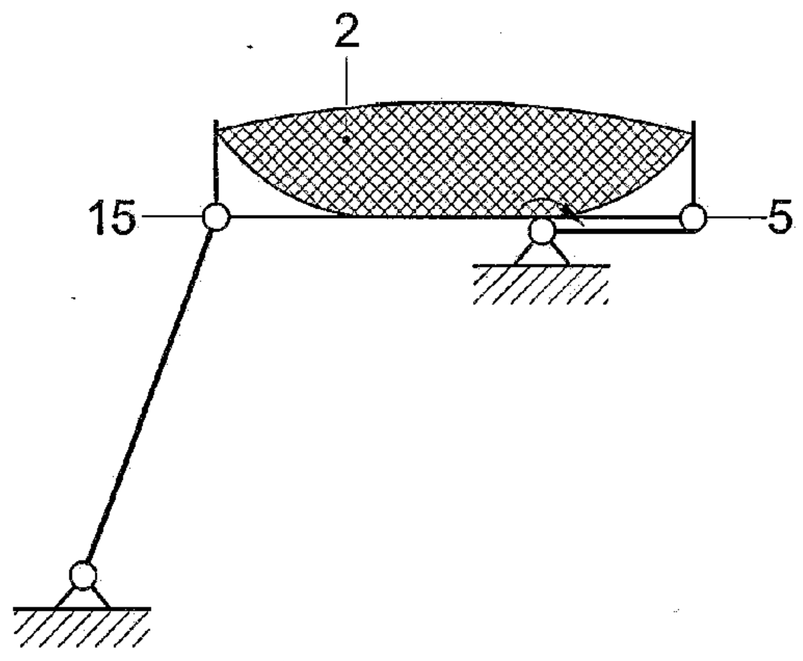


Fig. 2B

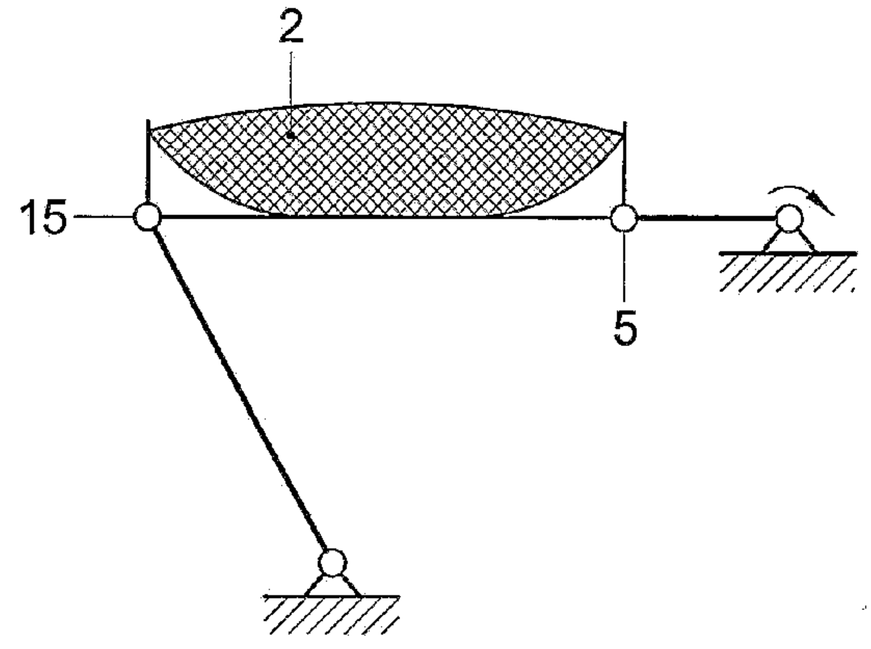


Fig. 2D

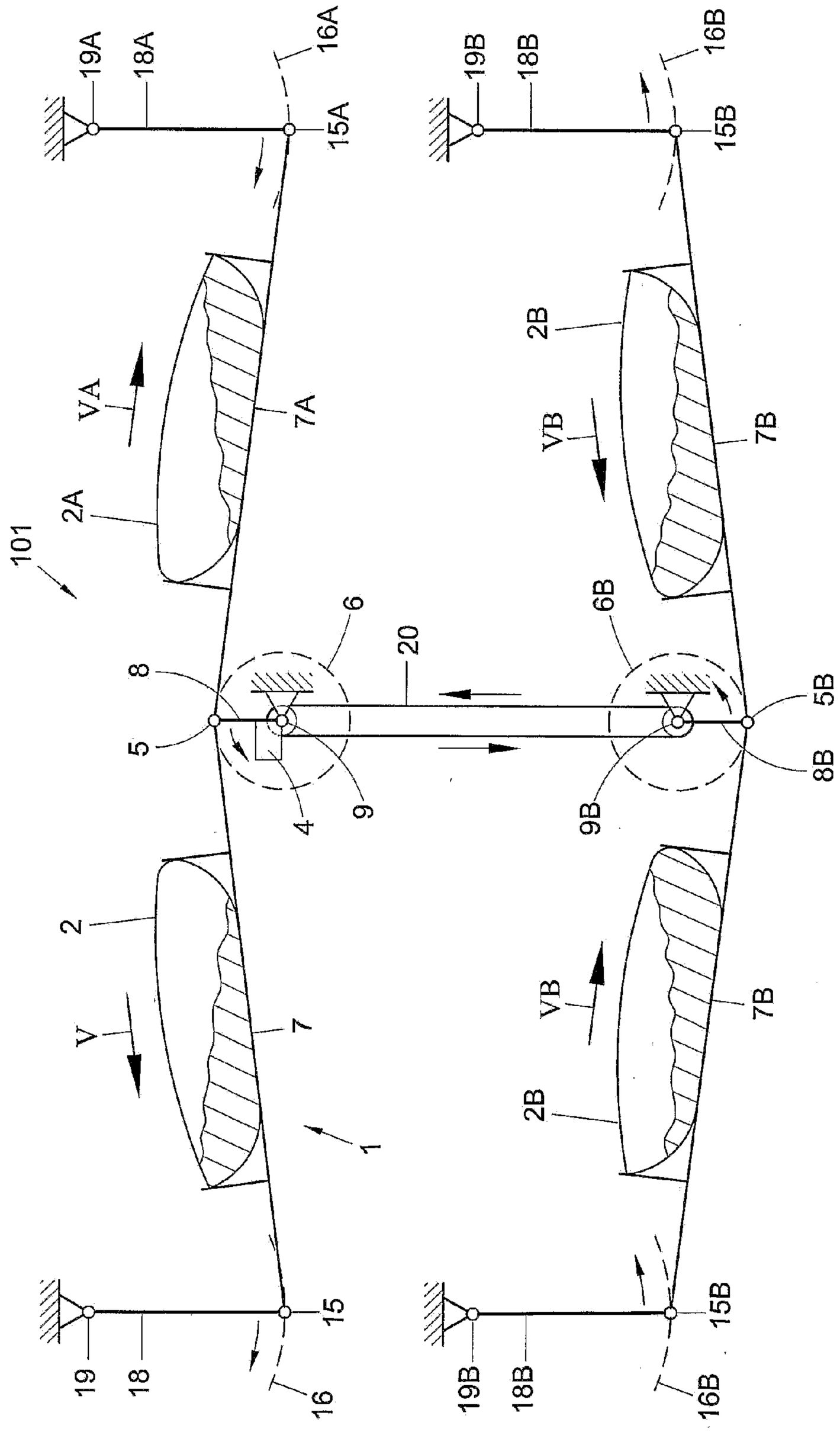

101
1
2
2A
2B
4
5
5B
6
6B
7
7A
7B
8
8B
9
9B
15
15B
16
16B
18
18B
19
19B
20
V
VA
VB

Fig. 3

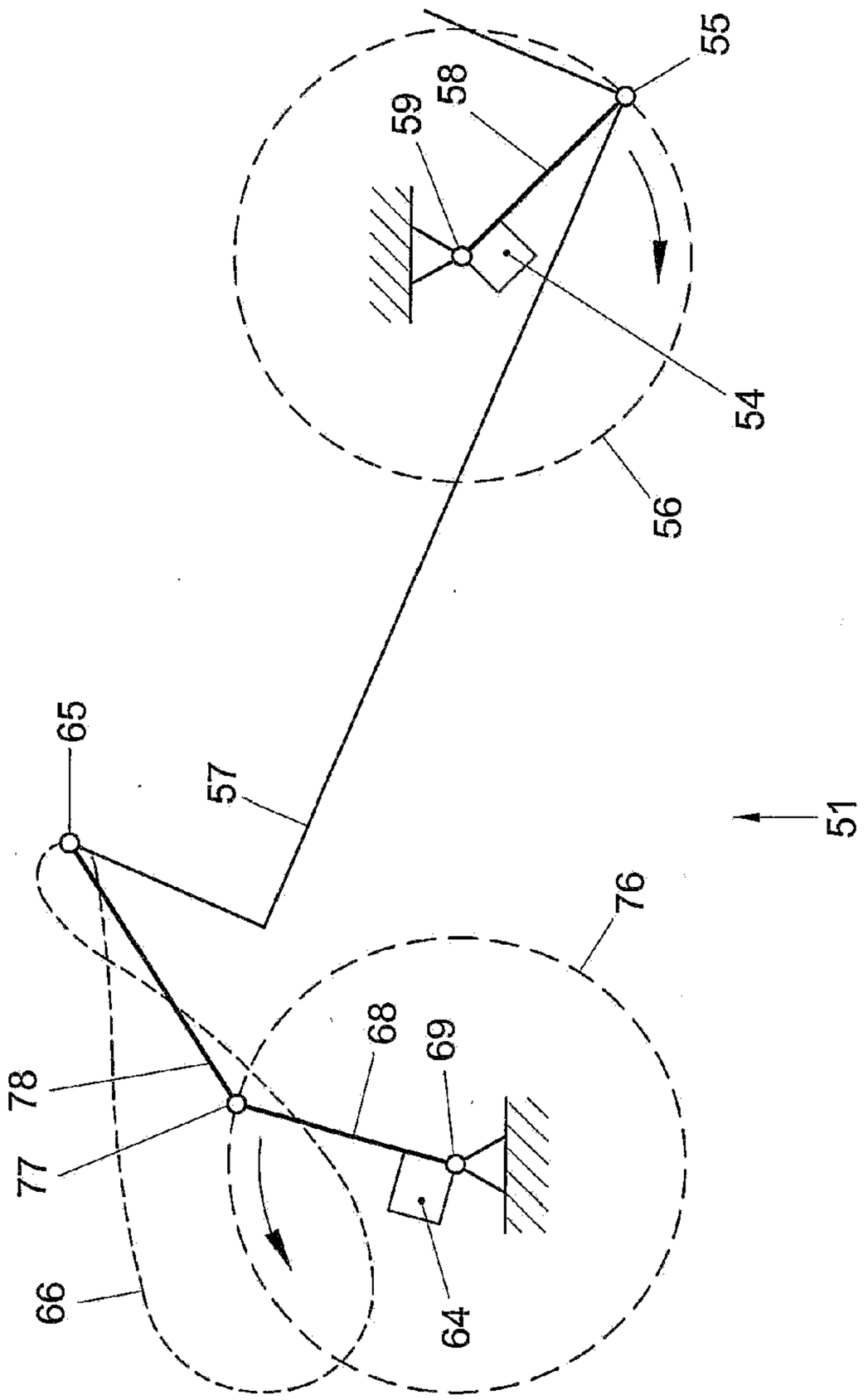


Fig. 4

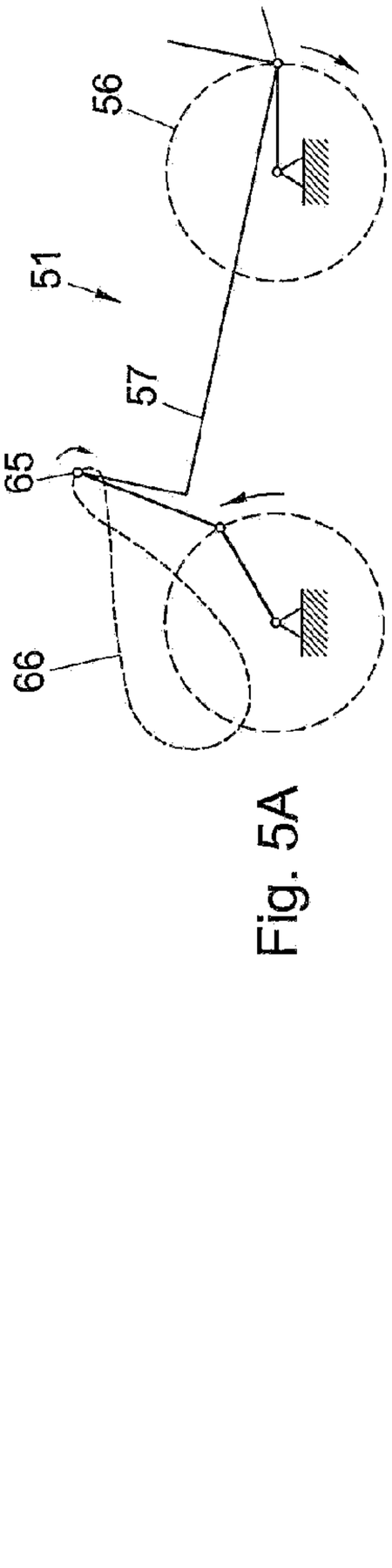


Fig. 5A

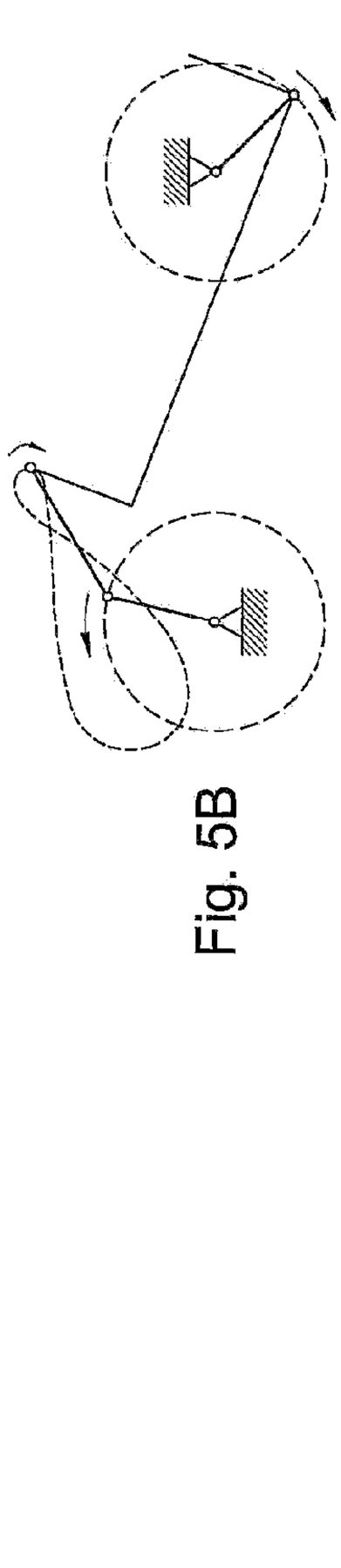


Fig. 5B

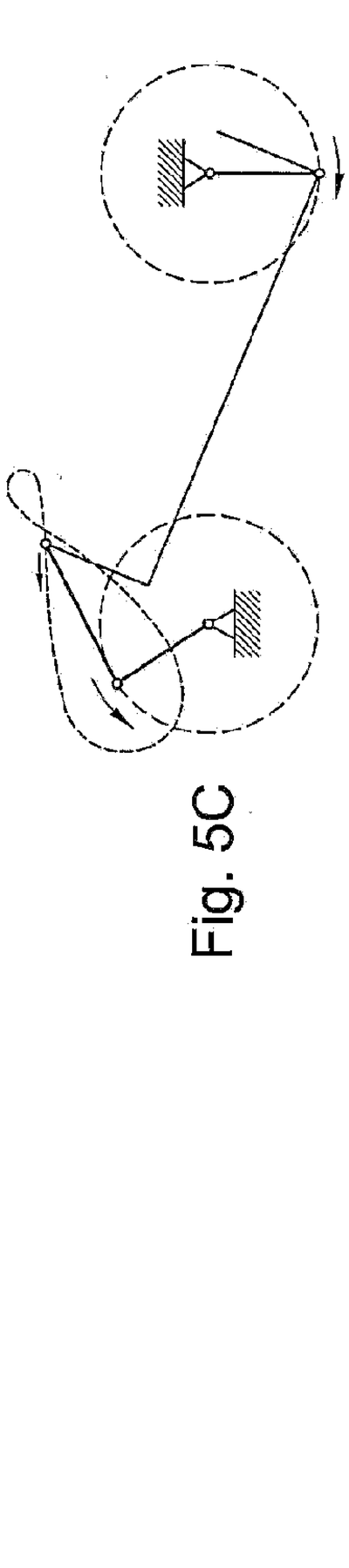


Fig. 5C

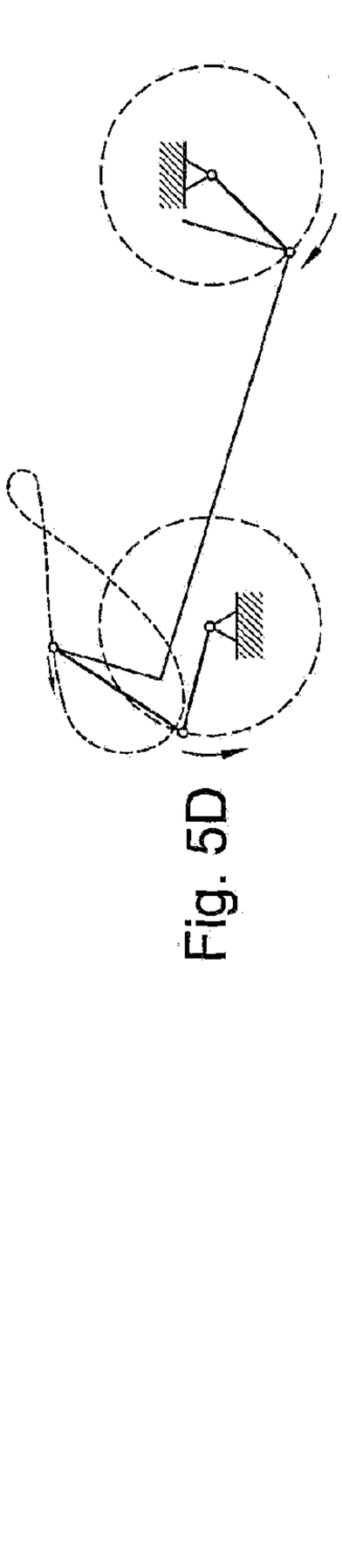


Fig. 5D

Fig. 5E

Fig. 5F

Fig. 5G

Fig. 5H

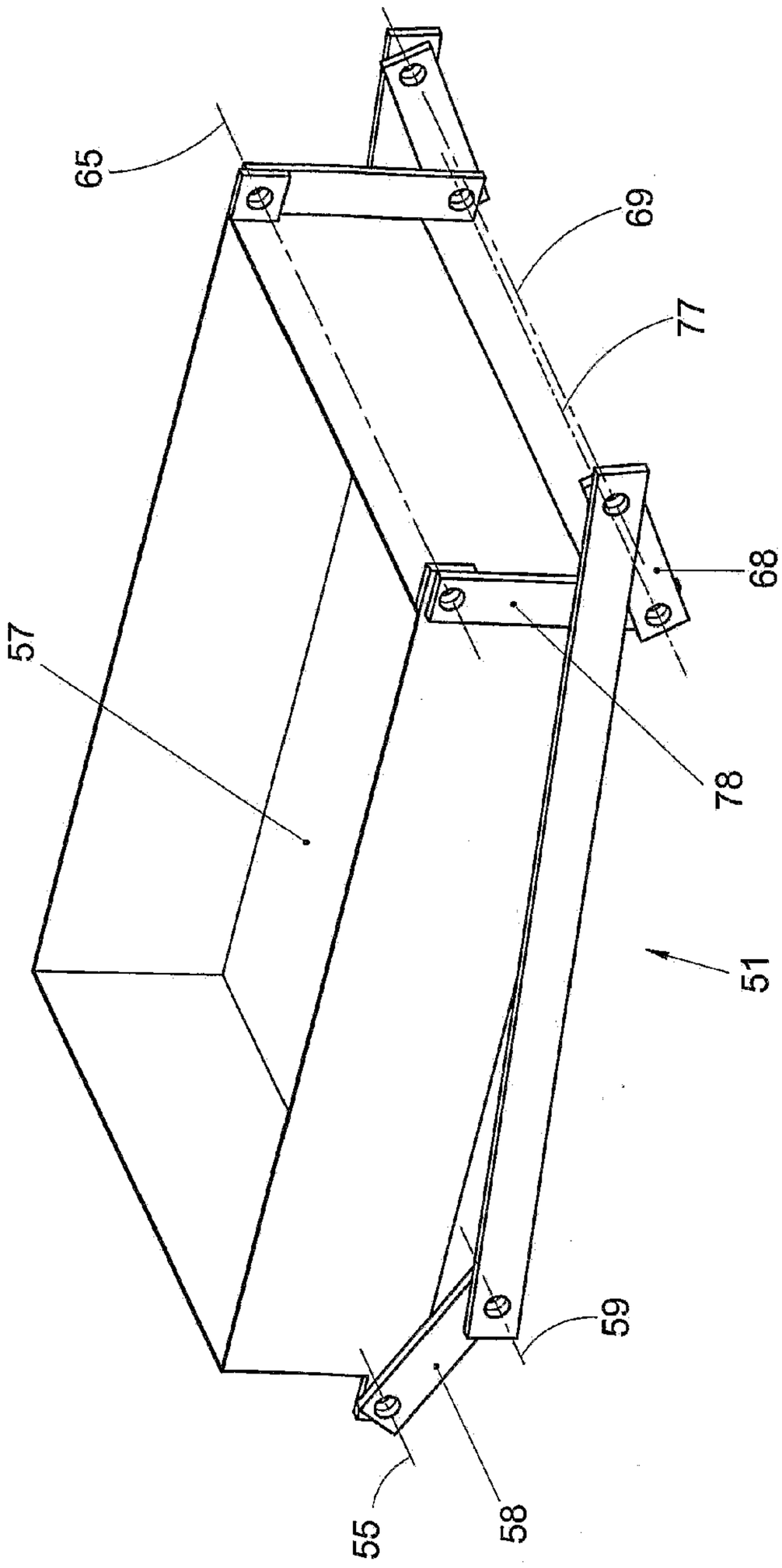


Fig. 6

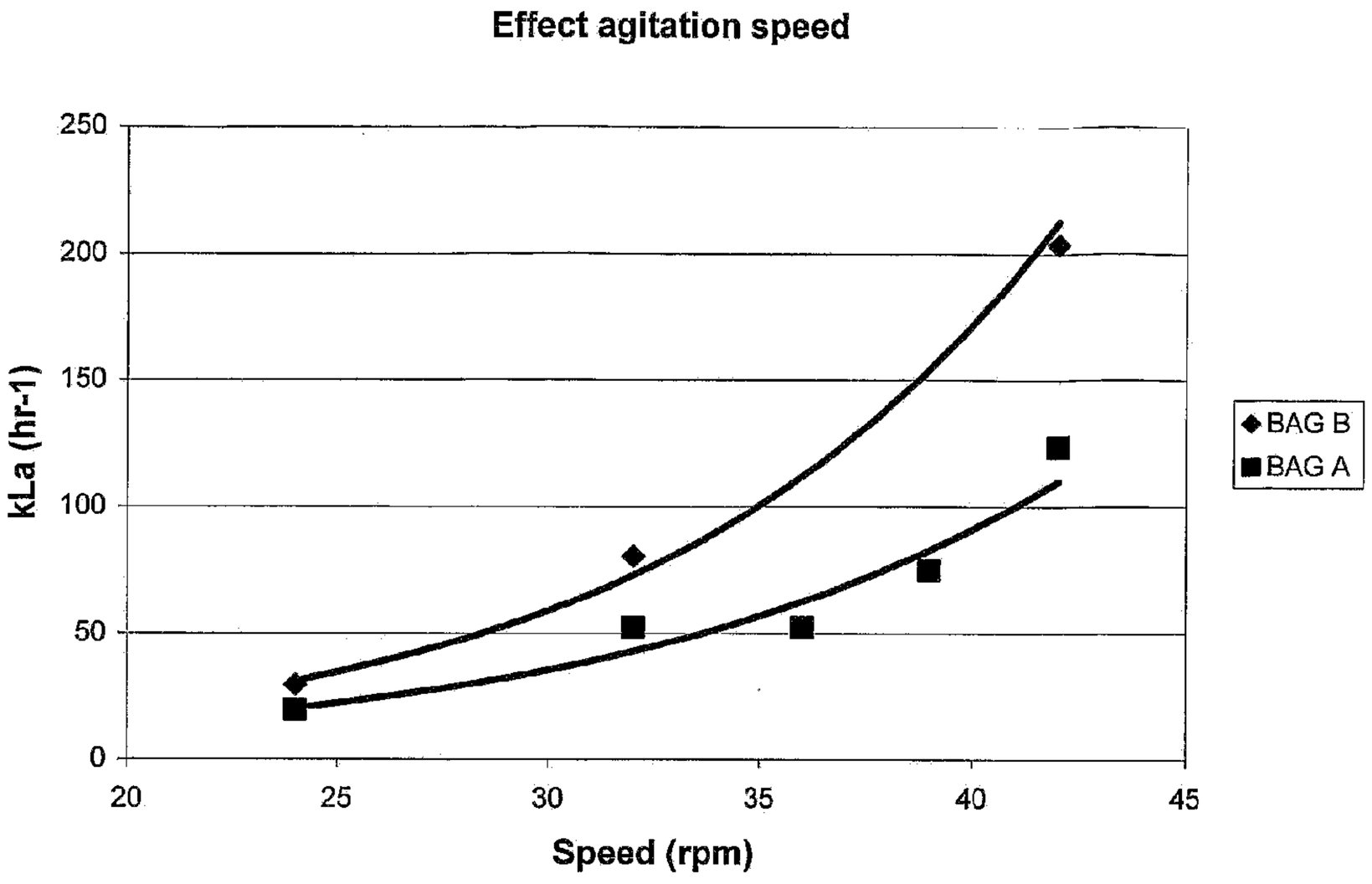

Effect agitation speed
kLa (hr-1)
250
200
150
100
50
0
20
25
30
35
40
45
Speed (rpm)
BAG B
BAG A

Fig. 7

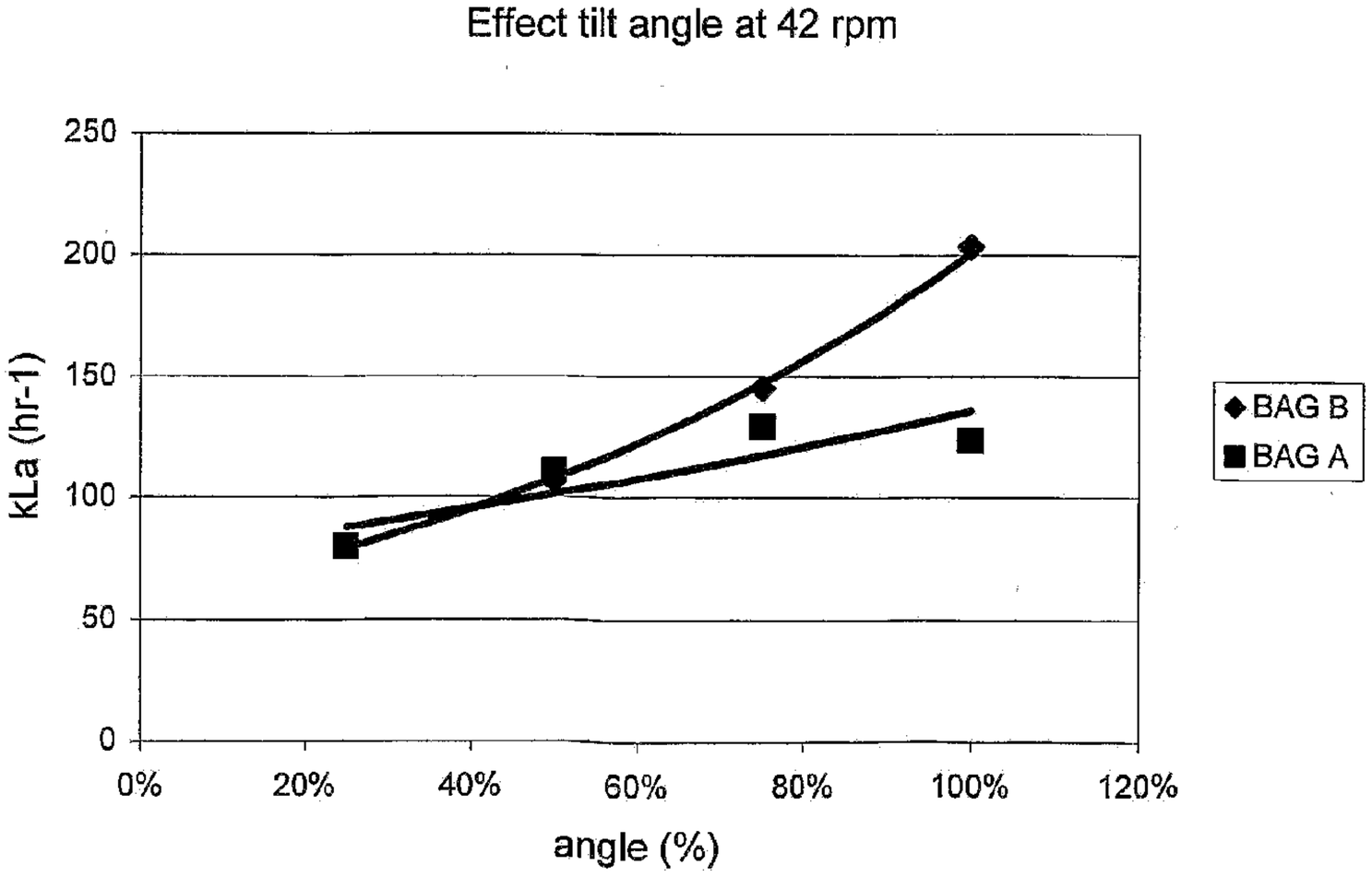

Effect tilt angle at 42 rpm
kLa (hr-1)
250
200
150
100
50
0
0%
20%
40%
60%
80%
100%
120%
angle (%)
BAG B
BAG A

Fig. 8

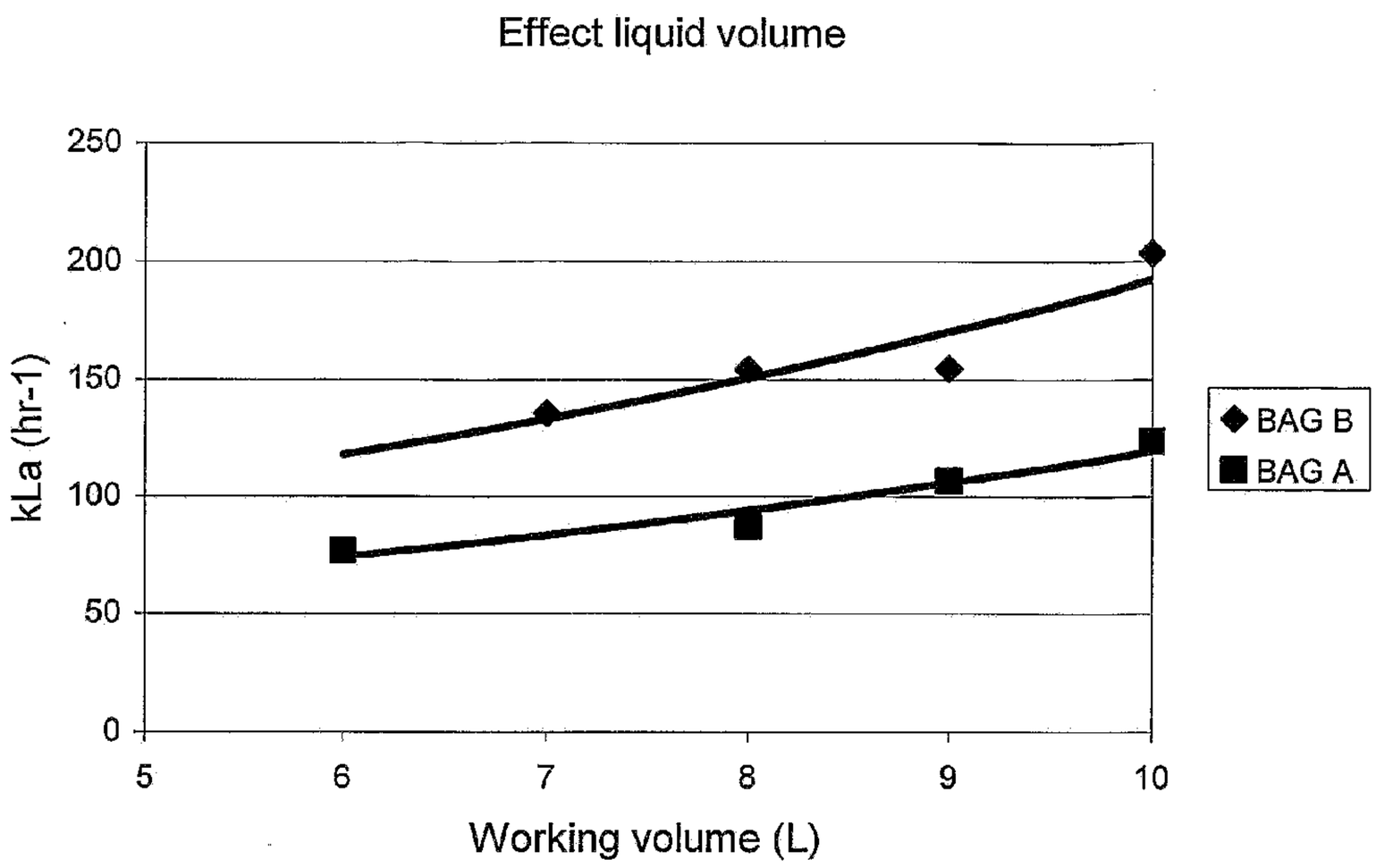
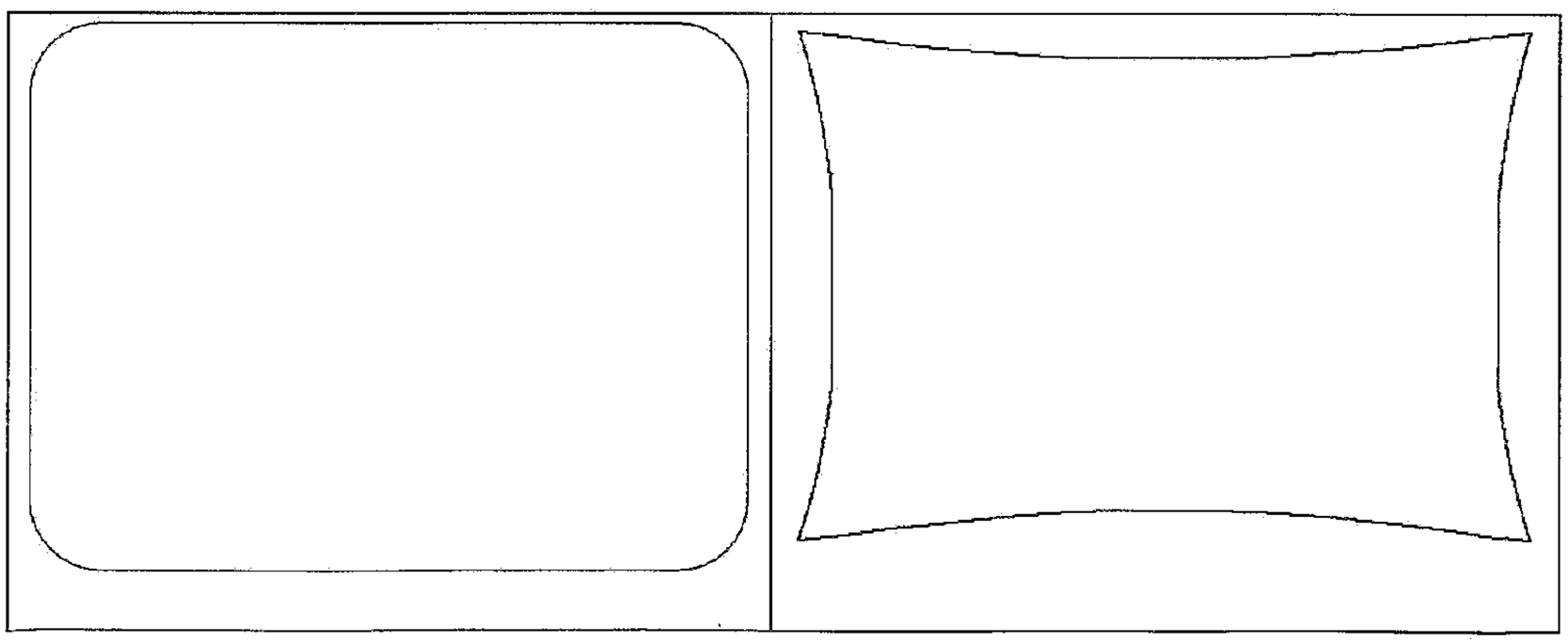


Fig. 9



Top view 'Bag A'

Top view 'Bag B'

Fig. 10

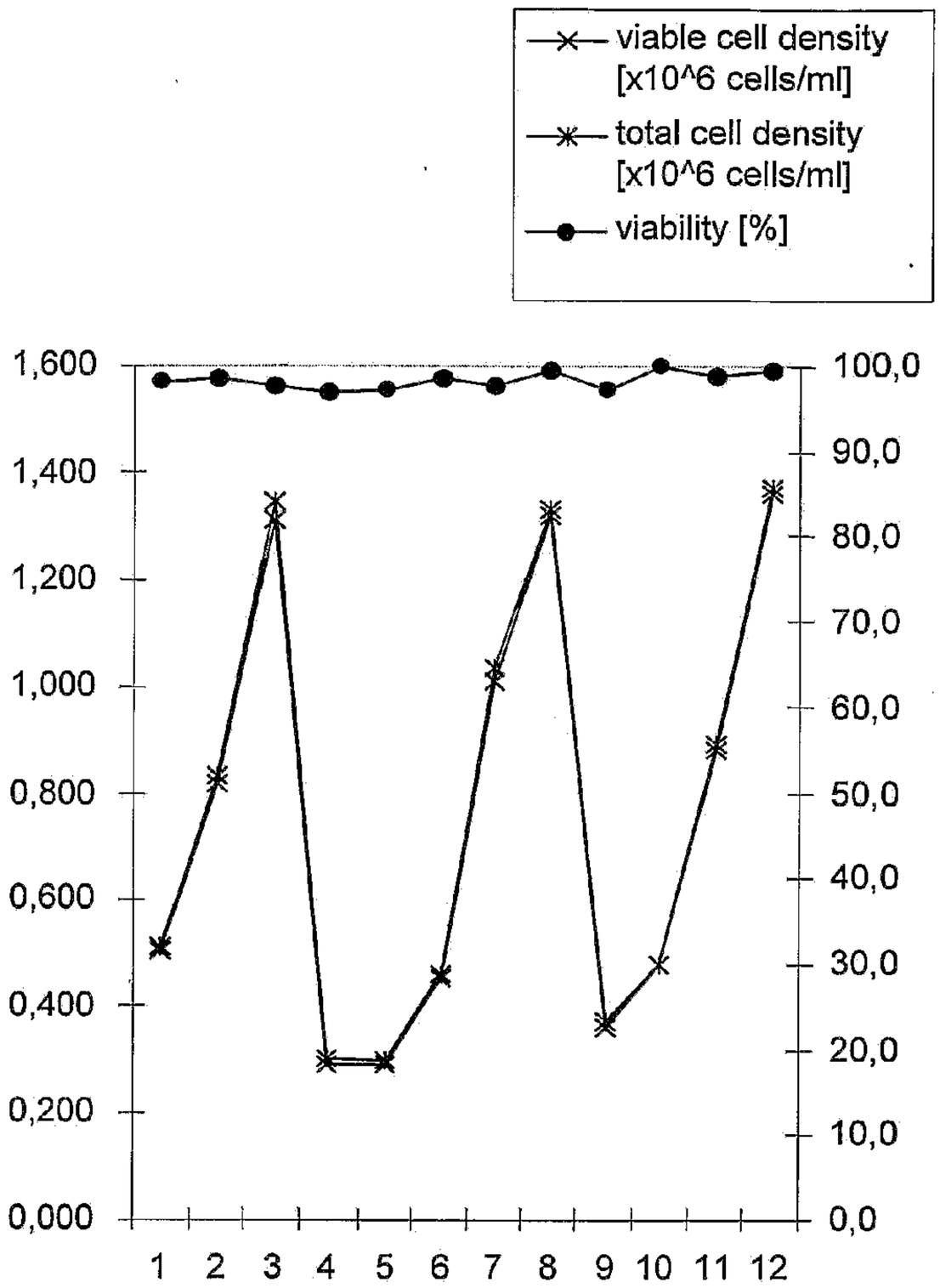

viable cell density [x10^6 cells/ml]
total cell density [x10^6 cells/ml]
viability [%]
1,600
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 11

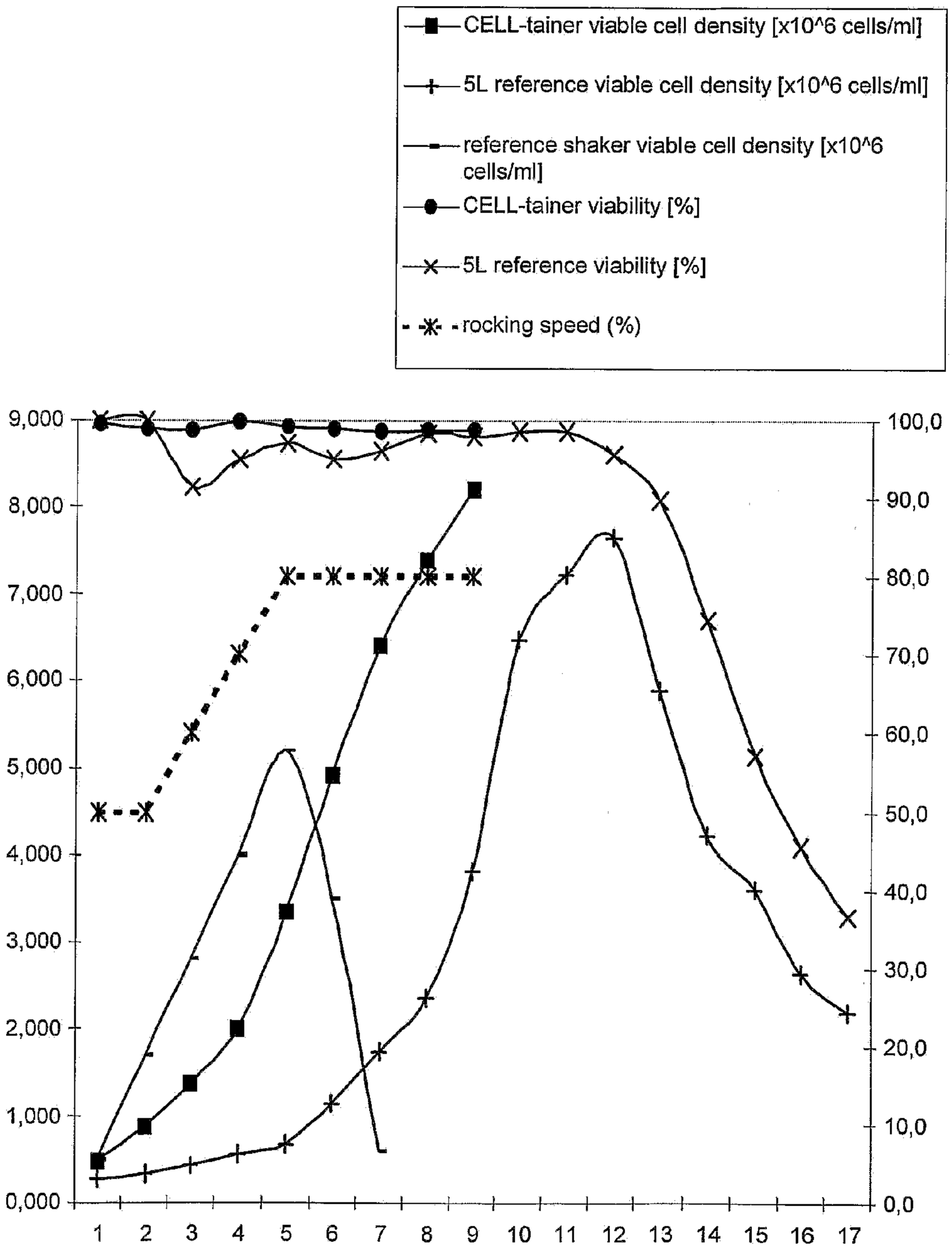

CELL-tainer viable cell density [x10^6 cells/ml]
5L reference viable cell density [x10^6 cells/ml]
reference shaker viable cell density [x10^6 cells/ml]
CELL-tainer viability [%]
5L reference viability [%]
rocking speed (%)
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0,000
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 12

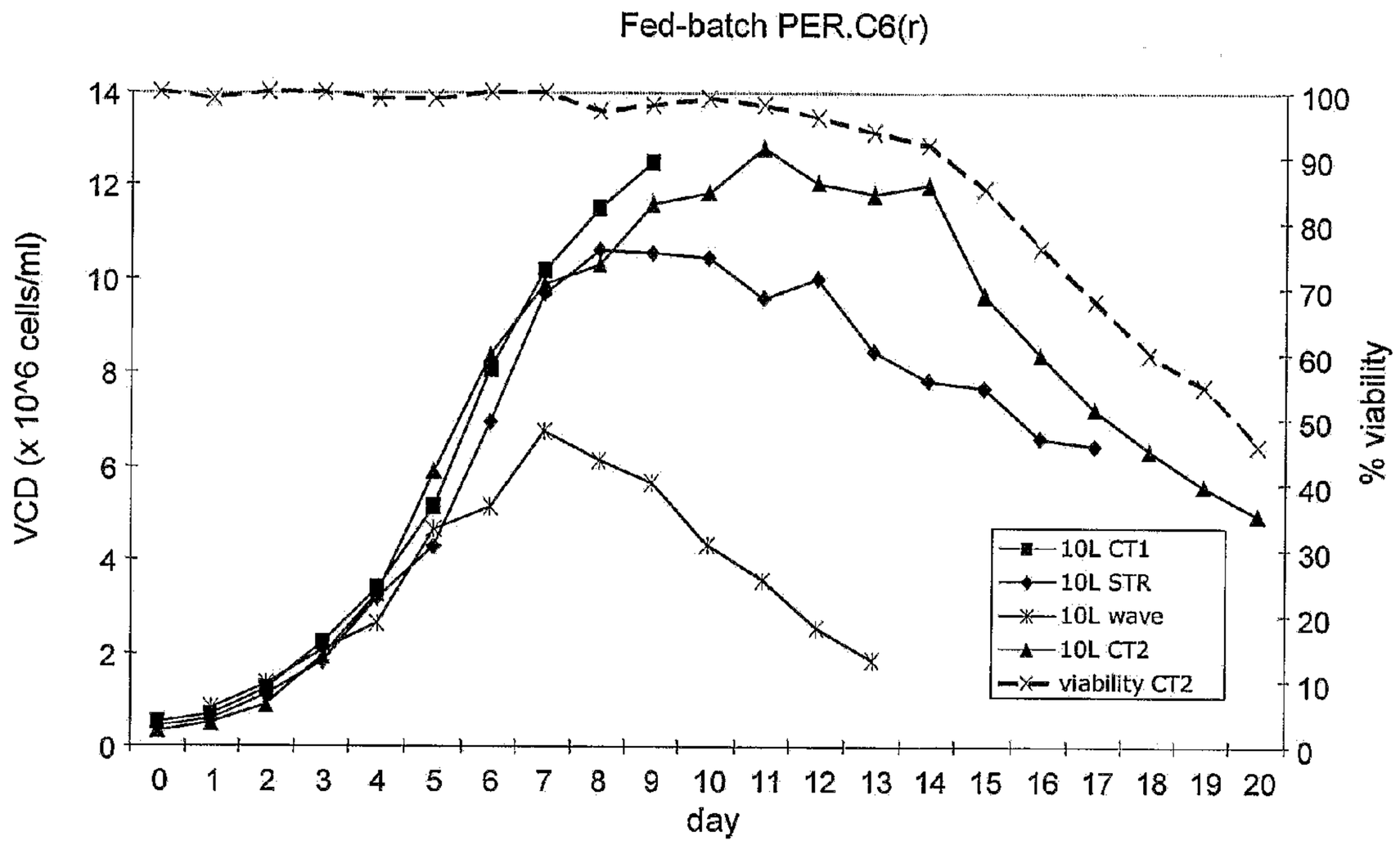

Fed-batch PER.C6(r)
VCD (x 10^6 cells/ml)
% viability
day
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
10L CT1
10L STR
10L wave
10L CT2
viability CT2

Fig. 13

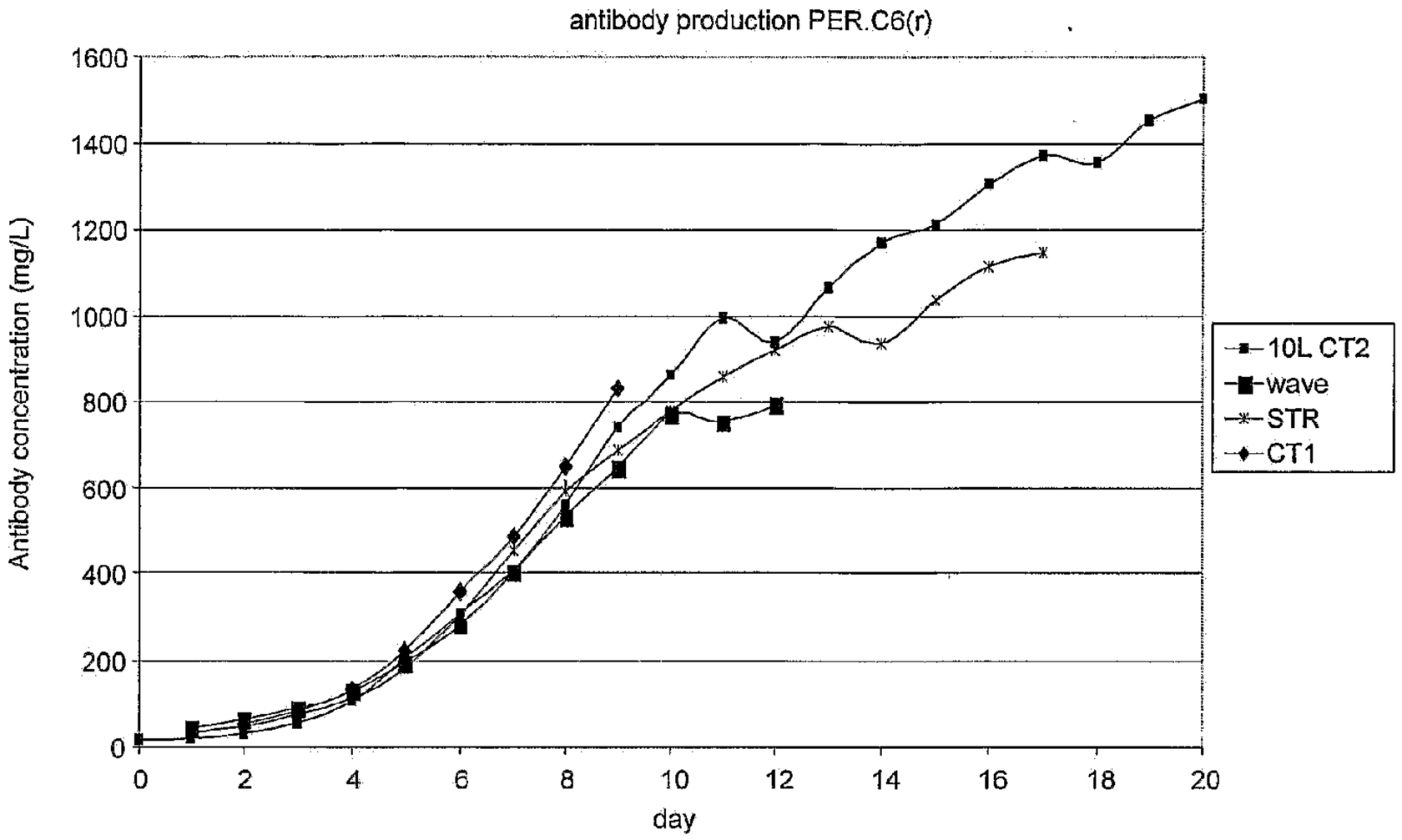


Fig. 14A

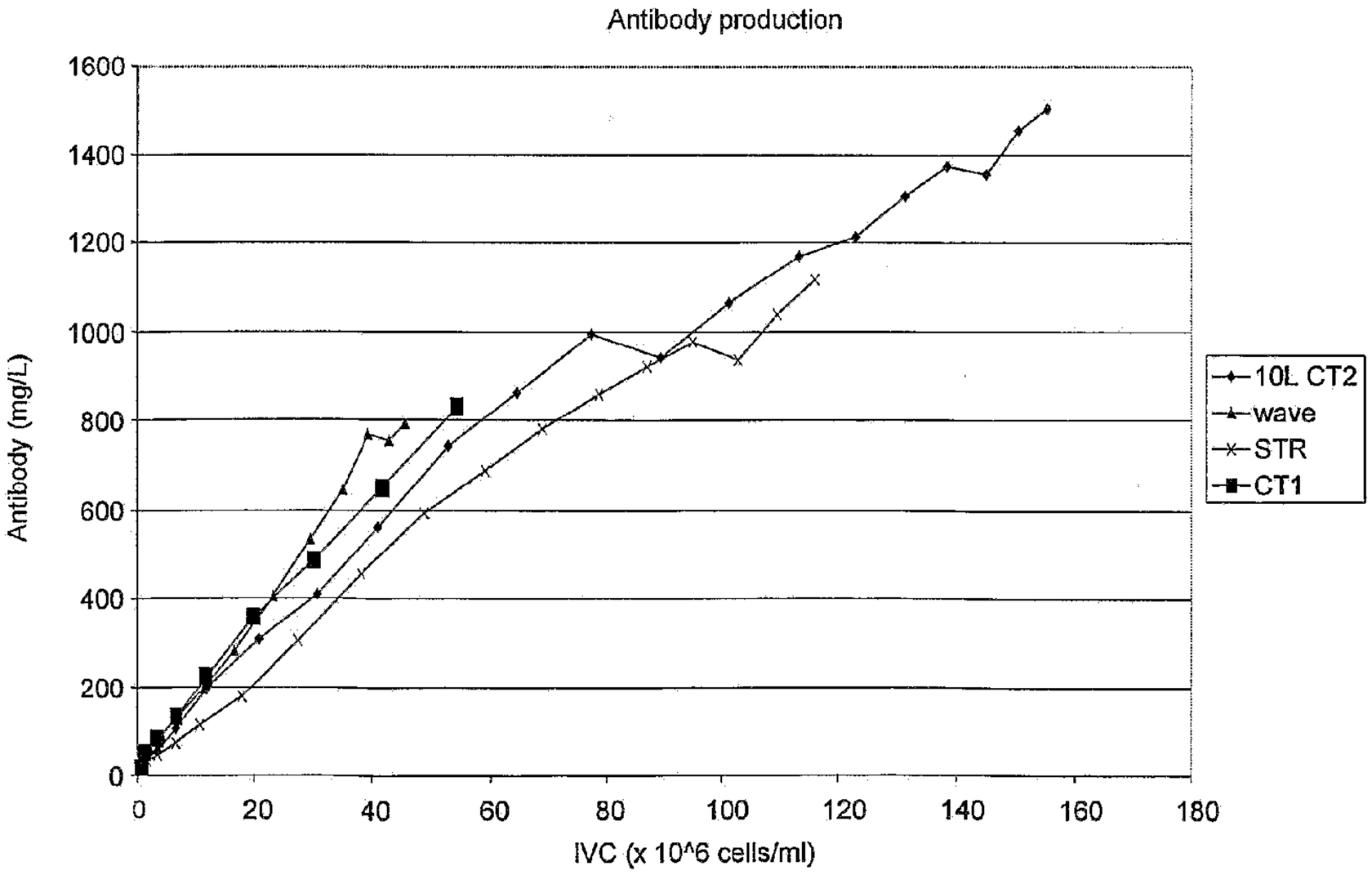


Fig. 14B

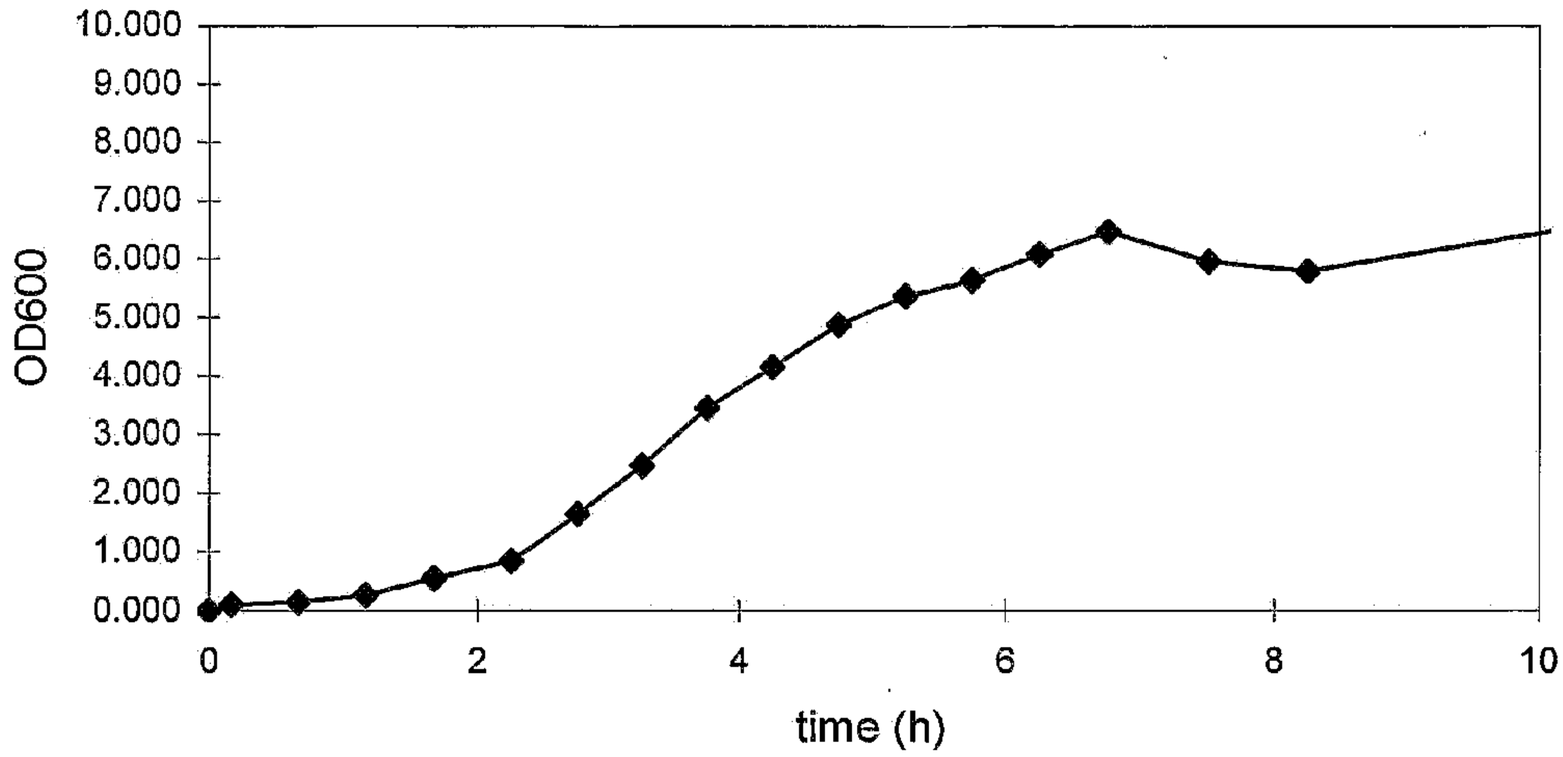


Fig. 15A

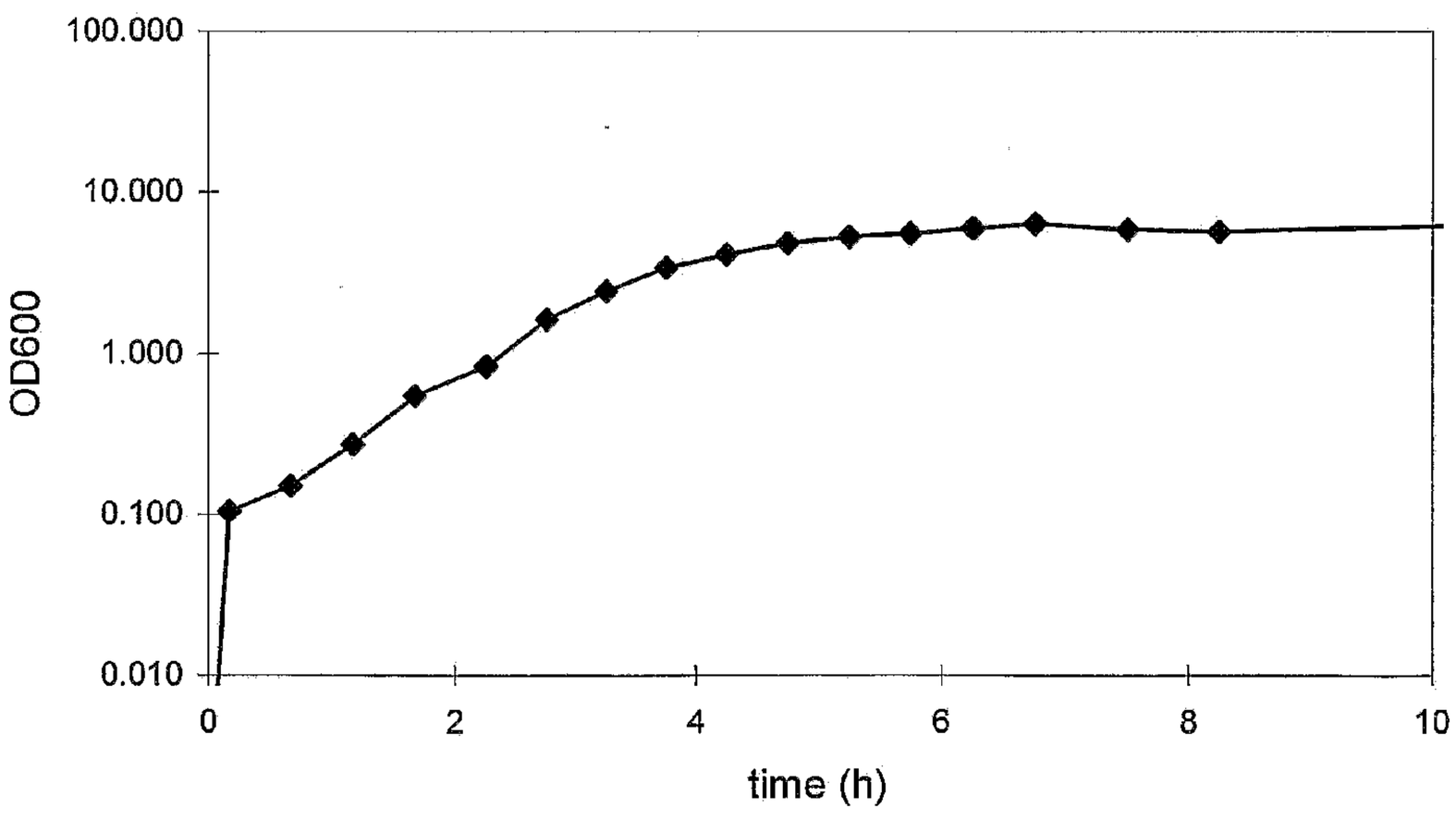


Fig. 15B

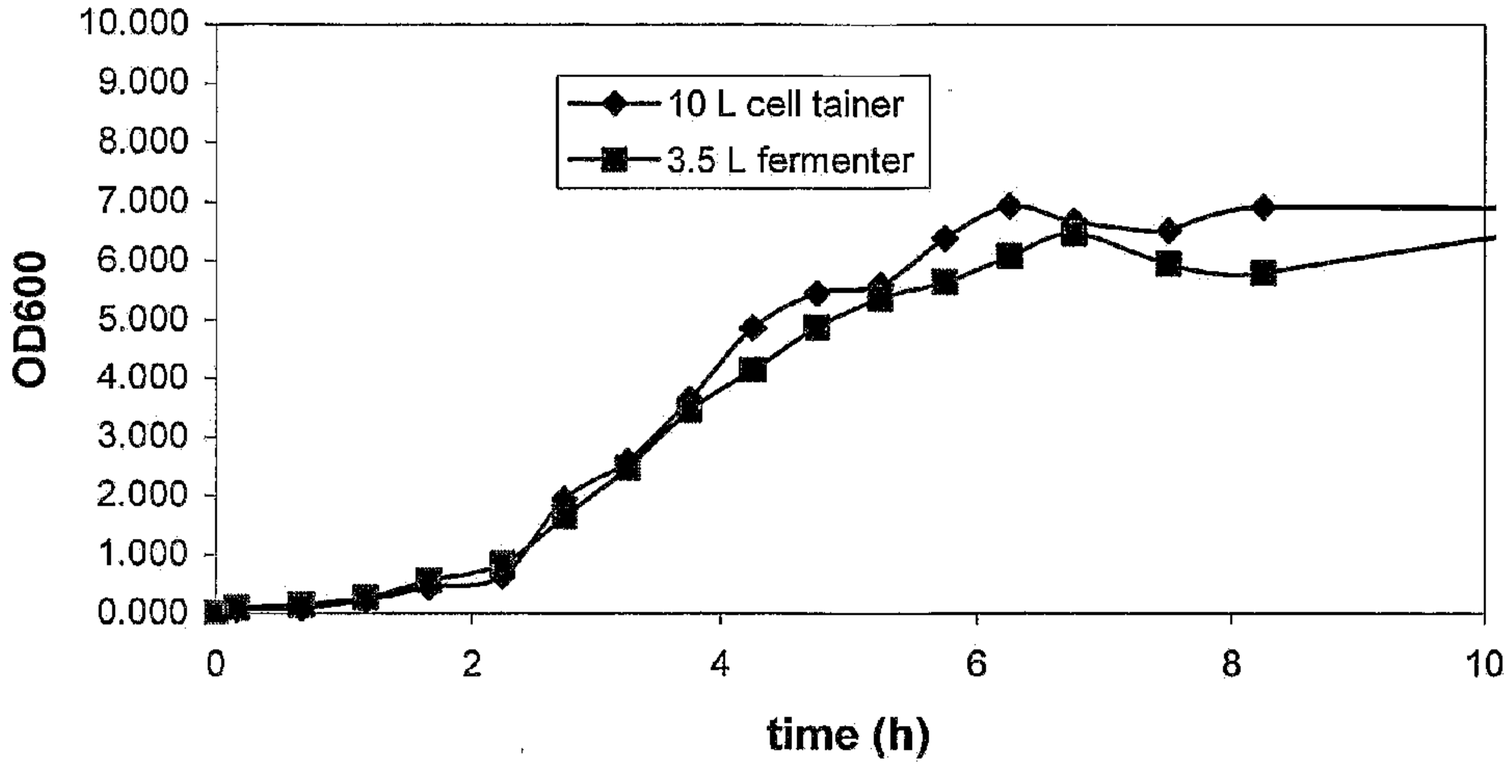


Fig. 16A

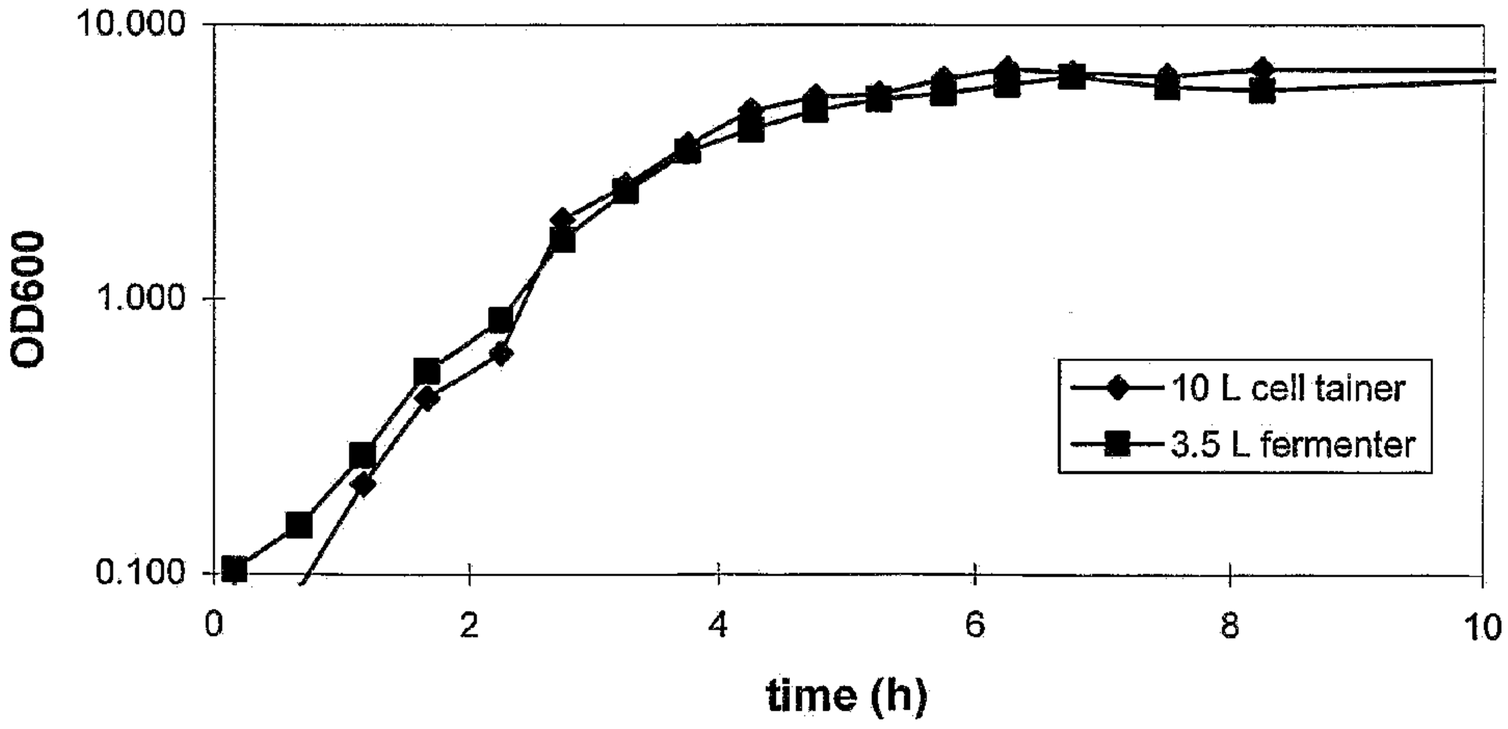


Fig. 16B

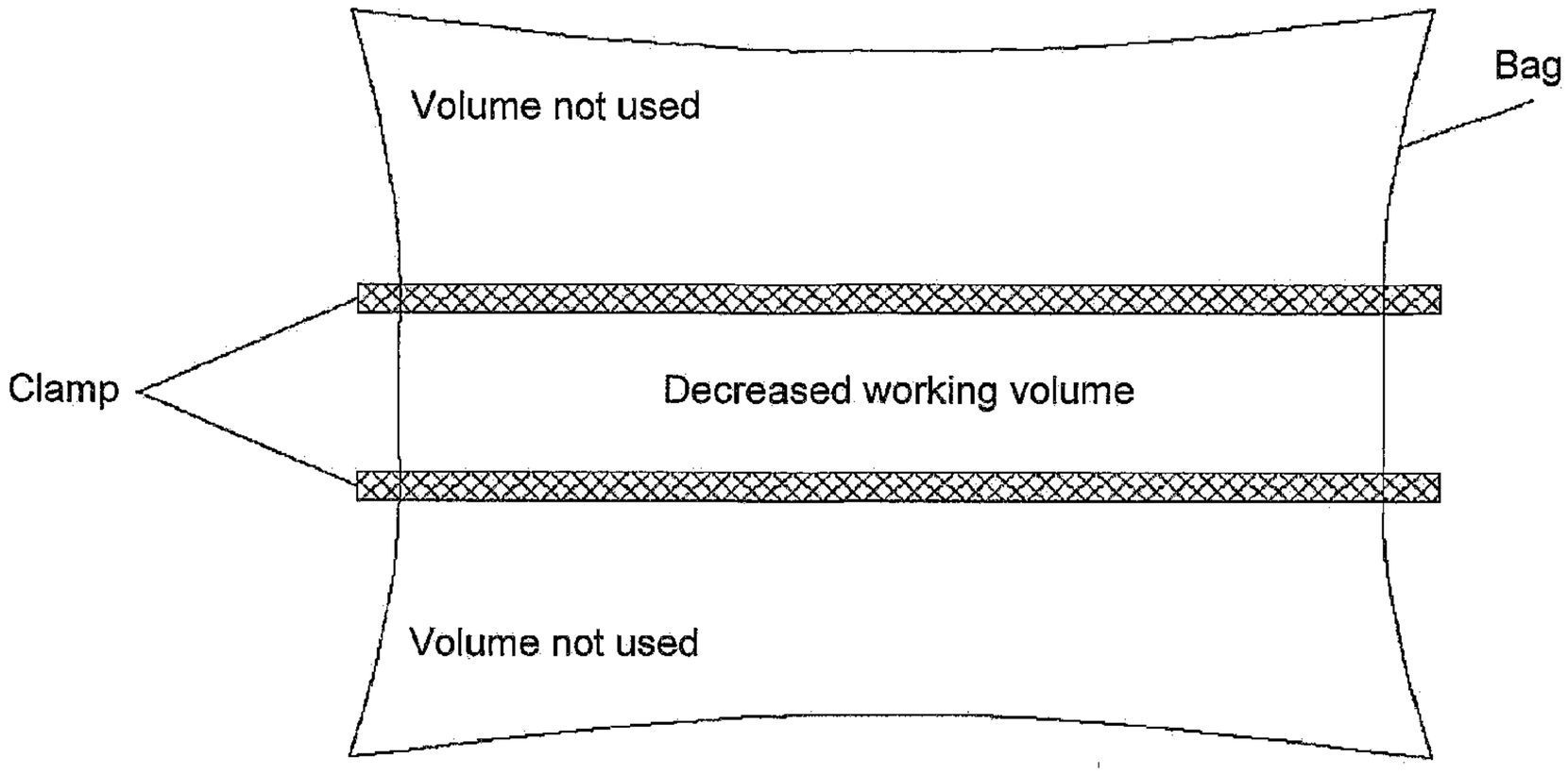


Fig. 17A

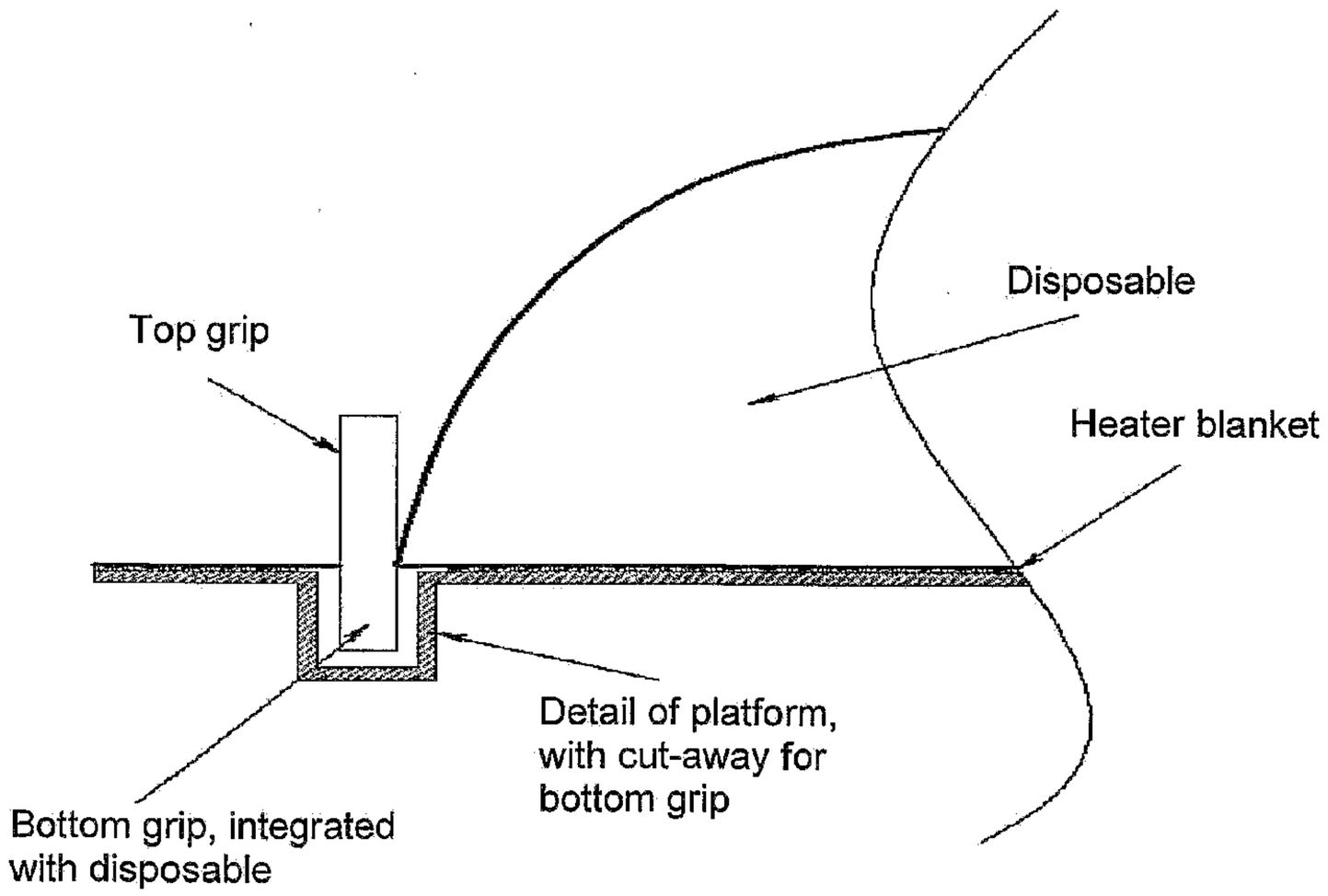


Fig. 17B

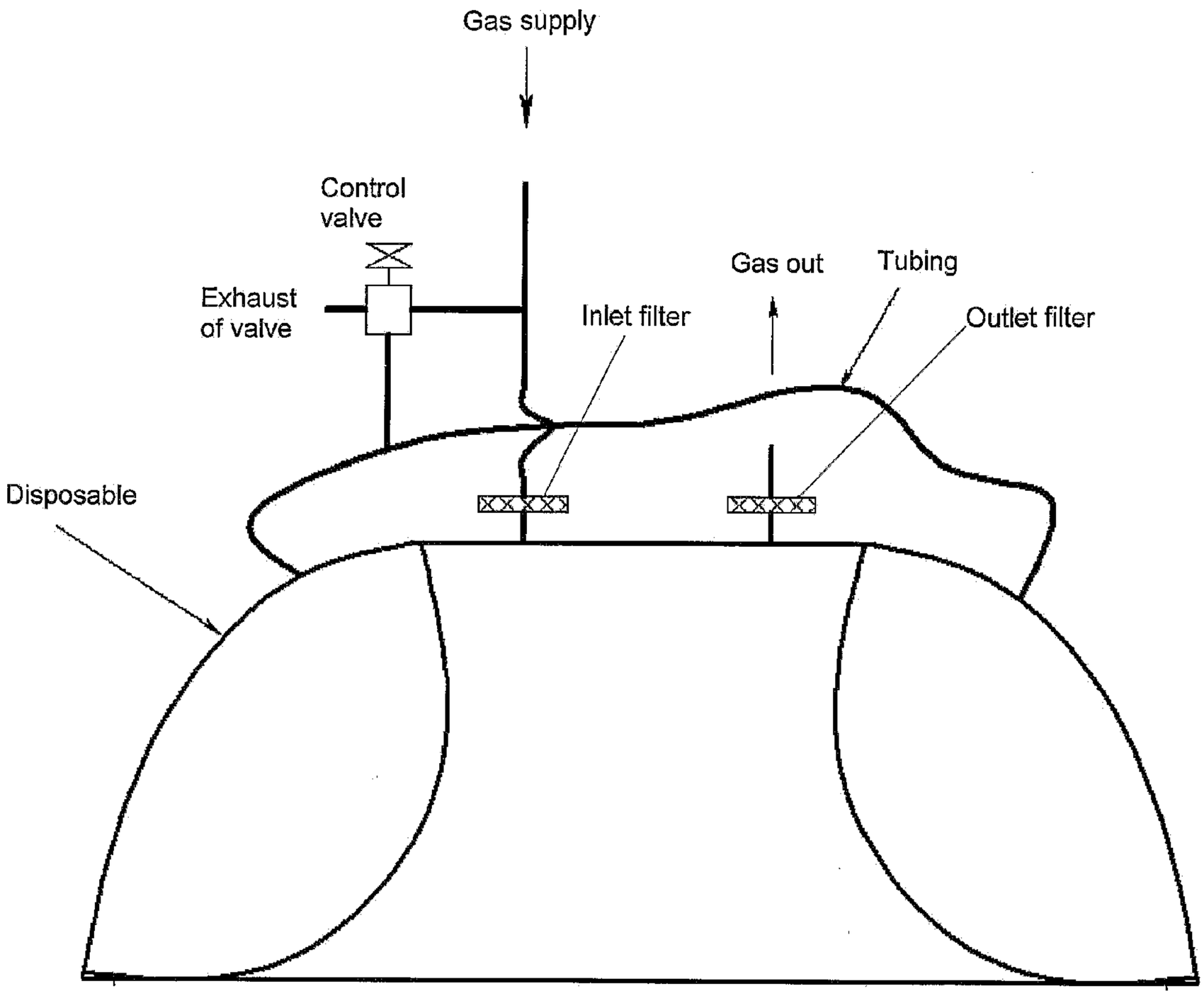

Gas supply
Control valve
Exhaust of valve
Inlet filter
Gas out
Tubing
Outlet filter
Disposable

Fig. 18



Fig. 19