

Fixed-Bed Bioreactor With Constant-Flow Pump / Tubing System

Related Applications

This application asserts priority to United States provisional patent filing Serial No. 62/322651, filed 14 April 2016, the contents of which are here incorporated by reference.

Government Interest

None.

Background

We have improved commercial-scale adherent cell culture by developing an improved bioreactor which provides a ~50% increase in productivity viz prior art bioreactors, while also eliminating a source of contamination.

Commercially-available adherent cell culture bioreactors include, for example, the laboratory-scale iCELLis™ Nano (commercially available from Pall Corporation, Cambridge Massachusetts) and the commercial scale iCELLis™ 500 bioreactor, which provides a volume of up to 65 liters of cell culture medium and a cell culture substrate of medical grade polyester microfibers which provide up to 500m² growth area available to the cells. We improved the function of such commercial-scale bioreactors by first, defining the process steps in a small scale and then scaling these up into a large scale. Pall Life Science, the manufacturer of the iCELLis™ brand of bioreactors, was recommending re-circulation or perfusion as a feeding strategy. The feeding strategy in small (Nano™) scale was tested by re-circulation, and later optimized by perfusion.

Perfusion is a process step where cells in a bioreactor are continuously feed with a fresh medium at the same time removing equal amount of spend medium, which enables the cell growth in high cell density (Vellinga et al. 2014). The perfusion rate can vary depending on the type of cell line used, the polypeptide product produced by those cells, the specific cell culture medium employed and the cell growth system used. An important aspect in the removal of the spent medium is also to remove the (possible toxic) metabolic side products from the cell culture. These side products may have a negative effect on cell viability, and further may impair the productivity of producer or host cells.

Options for perfusion include batch perfusion and fed-batch perfusion (where feeding of fresh medium is performed but no removal of spend medium is performed). The fed-batch type approach is also a re-circulation strategy, where the cell culture medium volume is only

enlarged by external medium container and medium is re-circulated from a media reservoir or container to the bioreactor and back again, wherein no actual removal of spend or used medium is done.

The manufacturer of the iCELLis™ line of bioreactors (Pall Life Technologies) markets the iCELLis™ 500 as suitable for perfusion feeding. We made a very surprising finding during our first experiment with iCELLis™ 500: as provided by the manufacturer, the equipment is not in fact capable of perfusion, if using the standard iCELLis™ 500 tubing and pump system set at the slowest pump output rate available.

We deconstructed the commercially-available apparatus and found out the reasons for failure. The commercially-available apparatus is manufactured with the feed-out tube of larger interior diameter than the feed-in tube. This configuration is intended to prevent unwanted overflow of the liquid media by ensuring that in-flow cannot be greater than out-flow. We surprisingly found that this configuration adversely impacts pump efficiency and the productivity of the cultured cells. Rather, we found the feed-out tube is best of equal or smaller interior diameter than the feed-in tube. We found that the commercially-provided (“stock”) medium feed-out tube was of too large interior diameter, providing a too-large interior volume space for the media flow rate. This large interior tube volume permitted formation of undesirable air pockets and bubbles in the tubing.

In addition, the stock feed-out tube was integrated into a too-weak pump system which could not provide an adequate media flow out from the bioreactor. This may be because formation of air bubbles in turn appears to affect the correct out feed-out pump capacity. The feed-out pump works by creating negative pressure in the feed-out tube, pulling media into the feed-out tube. Unlike liquid media, however, air bubbles expand in this negative-pressure environment, absorbing much of the negative pressure energy, thus frustrating pump work.

Brief Description

We resolved the failures inherent in the commercially-available apparatus by retrofitting the bioreactor with a bigger peristaltic pump able to provide the proper (lower) rate of fluid output, and replacing the feed-out tubing with replacement tubing having a smaller inside diameter, thus preventing formation of undesirable air bubbles in the feed-out tubing.

Another suggestion we have made for the manufacturer is to make bioreactors with smaller tubing diameter to improve the medium flow inside tubing.

We also have designed and tested a new sampling manifold configuration which eliminates the risk that a compromised aseptic filter will contaminate an entire manufacturing batch.

Brief Description Of the Drawings

Figure 1 schematically illustrates two different perfusion methods.

Figure 2 shows a way to connect a bioreactor vessel to a sampling manifold.

Figure 3 shows a version of our improved sampling manifold.

Detailed Description

We have implemented a way to improve medium feed in and feed out strategy in large capacity adherent cell culture bioreactors such as, for example, the iCELLis™ 500 bioreactor. Commercially-available large scale bioreactors are designed to *recirculate* media at *high velocity*; we found that *perfusing* media at a *low velocity* unexpectedly increases producer cell output. Our approach is the only suitable way to achieve acceptably slow and constant cell culture medium flow into and out of the bioreactor vessel. Constant medium flow supports high viral productivity for infected, transfected or transduced producer cells, while avoiding excessive medium usage.

Adherent cell culture bioreactor vessels provide a substrate on which cultured cells can adhere and grow. That substrate is housed in a container which contains liquid cell culture media.

Commercially-available large capacity adherent cell culture bioreactors are provided with “Feed In” and “Feed Out” pumps. These pumps respectively pump cell culture media into and out of the container which contains the substrate, thus providing the cultured cells with fresh culture media, and removing spent media and its appurtenant culture waste products.

Example 1 - Intermittent Pumping

Pumps can be programmed to run constantly. Run constantly at minimum speed, the prior art iCELLis™ 500 Feed In and Feed Out pumps produce at least about 42.3 L of medium flow per day (running at the slowest rate possible, 24 rpm). The capacity of the bioreactor vessel being about 25 liters, the medium in the reactor is completely exchanged almost twice a day.

We used a commercially-available large capacity adherent cell culture bioreactor vessel to culture transfected “producer” cells which express viral polypeptides and produce viral particles. We found that these producer cells are most productive when the cell culture

medium is perfused at a rate slower than the slowest pump output volume available in commercially-available apparatus. To be able to get a properly low medium perfusion rate for our purposes, we programmed the pumps to run for certain time interval, followed by an interval where the pumps did not pump at all.

Because of this need to vary pump output, we have seen in practice that the stock iCELLis™ 500 bioreactor Feed Out pump provided by the manufacturer is not capable of removing medium out from the bioreactor vessel in a well controlled manner. We thus began to test the viability of lower-output pumps able to provide an adequately low output flow.

Example 2 - Constant Low-Velocity Pumping

We then investigated whether the variable media flow used in the prior art might impact cell culture in some way. To do this, we replaced the prior art Feed In pump provided with the iCELLis™ 500 bioreactor with a replacement pump which was able to provide a lower output volume, about 16.7 L/day. For a 25 liter capacity bioreactor vessel, this means the media in the vessel would be exchanged once every 3 ½ to 4 ½ days, rather than the nearly twice a day typical in the prior art. This reduces the flow across *e.g.*, a 100m² substrate surface area from at least 42.3 L per 100 m² per day to 16.7 L per 100 m² per day. This lower Feed In pump rate enabled us to for the first time to run the Feed In pump constantly, without periodic stoppages.

During these runs it was observed that the Feed Out pump provided by the manufacturer was not able to perform the removal of the media from the bioreactor vessel throughout the process as planned. We therefore similarly replaced the prior art Feed Out pump provided by the manufacturer with a lower-output pump.

We performed a commercial-scale manufacturing run, using recombinant adherent producer cells to produce a recombinant adenovirus bearing a transgene (useful for *e.g.*, gene therapy), using in the adherent cell culture process a combination of the stock iCELLis™ 500 Feed In pump and our own lower-output Feed Out pump. We have used several transgenes (*e.g.*, coding for interferon, or for herpes simplex thymidine kinase). Our method is generally advantageous in producing vector with any kind of transgene (including therapeutic transgenes and marker transgenes such as green fluorescent protein), or any other genetic element or nucleotide sequence (*e.g.*, viral vector containing RNA transgene, shRNA, lncRNA, eRNA etc.).

We also performed three commercial-scale manufacturing runs, wherein we used our own lower-output Feed Out pump and also replaced the stock iCELLis™ 500 high-output

131 Feed In pump also with a lower-output pump. We received significantly higher adenoviral
132 production in the adherent producer cells in each of these three runs. In these three runs, the
133 productivity of viral particles per cell had increased 49.4 % as compared to productivity using
134 the prior art higher-output pumps.

135 When we changed the process to work with lower-output pumps, our viral
136 productivity per cell surprisingly increased 49.4%. Without intending for the legal coverage
137 of our patent to be bound by any scientific causal theory, this improvement may be due to
138 feeding the bioreactor vessel constantly with fresh medium, thereby keeping stable the level
139 of nutrients in the media in contact with the cultured cells. For example, we have found that
140 adherent cells are most productive when the media flow is substantially constant and slow
141 enough to maintain in the cell culture medium a level of lactate of not more than about 1.6
142 grams lactate / liter of culture medium. Similarly, we have found that certain adherent
143 producer cells are most productive when the concentration of glucose in the culture media is
144 maintained at between about 0.5 and about 1.0 grams of glucose per liter of media. Other
145 adherent producer cells are most productive with a glucose concentration which is higher
146 (*e.g.*, at least about 2.9 grams per liter) but nonetheless maintained at a relatively constant
147 concentration due to low-velocity but substantially constant media flow. Alternatively, this
148 may be due to the slow but constant flushing away of unwanted cellular waste products from
149 the cell surface. Alternatively, this could be due to avoiding the physical shear stress placed
150 on the cultured cells when using the higher-velocity media flow required by prior art
151 hardware. Alternatively, this increase could be due to slower media flow enabling each
152 producer cell a longer time to produce virus-like particles. Whatever the cause (or causes),
153 we found that a slower, constant medium flow surprisingly and significantly increased
154 adherent producer cell productivity.

155 This increase is particularly surprising in light of the fact that suspension cell culture
156 (*e.g.*, the CultiBag RM™ suspension cell culture bag, commercially available from Sartorius
157 Corp., Cambridge Massachusetts) provides more-or-less constant media flow across the cell
158 surface, yet suspension cell culture is known in the art as less productive than is adherent cell
159 culture.

160 Lower-output pumps may also be successfully used for inoculation of the host cells
161 into the adherent bioreactor vessel, for drawing samples of the cell culture media during cell
162 growth, and for harvesting *e.g.*, culture media at the end of the cell growth. We have
163 surprisingly found that using a lower-output pump to perform these functions counter-
164 intuitively makes each of those processes faster than with the prior art high-output pumps.

One may replicate our system by simply obtaining a commercially-available large-scale adherent cell culture bioreactor vessel, disconnecting the Feed In and Feed Out tubing from the pumps provided with that bioreactor, and connecting that tubing to external pumps. With external pumps, however, the operator may lose the ability to control the external pumps through the computer which controls the bioreactor (and its integral pumps). For example, commercially available large scale adherent bioreactors typically include a sensor which measures the level of liquid cell culture medium in the bioreactor vessel, to monitor whether the cell culture substrate is adequately submerged in medium. External pumps may not be automatically controlled by the feedback control loops that are automatically activated by the liquid cell culture medium level sensed in the bioreactor vessel. Similarly, commercially available large scale adherent bioreactors typically include a recording device which records the running speeds of the pumps. Using external pumps, the output may not be recorded automatically to the computer. One may overcome these disadvantages by calibrating the external pumps before use and manually monitoring the culture medium flow in and out from the bioreactor vessel regularly, and by recording all steps manually to the batch manufacturing records. Alternatively, one can connect the new pumps to the bioreactor control computer, so that the low output pumps can be controlled and monitored similarly and simultaneously, as for the bioreactor itself.

The pump(s) can be controlled by software or through external calibration, or by controlling by nominal motor speed (RPMs), or using a balance or weight-controlled system. Alternatively, the pump(s) may be controlled manually, or have an automatic speed control or any other suitable way to control the pump. The precise control method is not critical, as long as the pump is able to provide the consistent low-flow output we have surprisingly found is favorable to increased adherent cell production.

Our lower flow rate is effective for larger-area adherent cell cassettes (e.g., a 500 m² cassette). Lower flow rate may also be effectively used with smaller-area substrate cassettes, such as 66 m² fiber cassettes.

Our system can be used to culture adherent producer cells which express viral polypeptide and thus produce recombinant virus or virus-like particles (we use the term “virus” in our appended legal claims to encompass both viruses and virus-like particles).

Example 3 - Unidirectional Sampling Manifold

196 A bioreactor sampling manifold is used for taking liquid samples of the cell culture
197 medium from the bioreactor vessel to e.g., check pH, or to measure glucose, metabolites and
198 cell waste products (e.g., lactate) or other substances in the medium.

199 We unexpectedly found that the stock sampling manifold offered as part of the
200 commercially-available iCELLis™ is vulnerable to contamination. We have successfully
201 designed a new sampling manifold for the bioreactor for removing the need of pumping
202 medium in and out from the bioreactor vessel into a sampling bottle. This way we minimize
203 the risk of contamination due to a sampling.

204 A schematic drawing of an adherent culture bioreactor is shown in Figure 2. The
205 bioreactor is comprised of a vessel [1] which contains a cell culture substrate [2] to which
206 cells adhere when cultured in adherent mode. The vessel [1] is filled with liquid cell culture
207 medium which irrigates the cell culture substrate [2]. The vessel [1] has a vent which
208 includes an air filter [3] having an exit vent [4]. The exit vent [4] is able to vent excess gas
209 out of the bioreactor vessel and the filter [3] filters the gas flowing through the filter to
210 prevent particulate contaminants (e.g., viral particles, fungal spores) from exiting or entering
211 the bioreactor vessel. The filter [3] is preferably detachably connected to the vessel [1] via a
212 vent tube [5]. Alternatively, the filter may be integrated into the body of the vessel [1], albeit
213 this risks enabling liquid cell culture media to splash onto the filter and occlude it.
214 Preferably, the vent tube has a clamp [6] which enables a technician to close the vent tube
215 [5] as and when desired.

216 The vessel [1] also has a sampling tube [7] with an input end [7a] which passes into
217 the liquid cell culture medium and thus is able to intake liquid medium. Preferably, the
218 sampling tube [7] also has a clamp [8] which enables a technician to close the sampling tube
219 [7] as as when desired. The sampling tube [7] has a connection [9] enabling it to be
220 connected to a sampling device. The sampling device may be a detachable sampling bag or
221 bottle. We prefer, however, the sampling device comprise a sampling manifold [Figure 3]
222 able to connect to a pair of detachable sampling bags or bottles.

223 The sampling manifold [Figure 3] is connected [9] to the sampling tube [7]. The
224 sampling manifold comprises a waste tube [10] having an input end [10a] and an output end
225 [10b], and the sampling tube [7] output end [7b] able to connect to a removable sampling
226 container [11]. The sampling container [11] may be, for example, a bag or a bottle.

227 The prior art commercially-available sampling manifold is based on the idea that first
228 approximately 200 ml of cell culture media is merely a rinse; it is pumped through the
229 manifold to rinse the interior of the manifold. That rinse media passes out of the manifold

230 through an exit opening into a temporary storage bottle. Additional cell culture media is then
231 pumped through the manifold, and a sample is collected. After the sample is taken, the
232 approximately 200 ml of cell culture rinse media which was initially collected in the
233 temporary storage bottle is pumped from the bottle back into the manifold and back into the
234 bioreactor vessel [1], so that as little as media as possible remains inside the sampling
235 manifold. The temporary storage bottle and the tubing can be emptied because the prior art
236 configuration provides an aseptic filter in the sampling bottle, which filter permits ambient air
237 to exit and enter the bottle (when liquid is pumped into and out of the bottle, respectively),
238 the filter filtering airborne contaminants in the ambient air and thus preventing contamination
239 of the cell culture medium.

240 If the integrity of the filter is compromised, however, this can pose a major
241 contamination risk which is difficult to detect until an entire manufacturing batch has been
242 contaminated. Using the commercially-available prior art configuration, we have in fact
243 incurred a contamination of a commercial-scale manufacturing batch. In evaluating the
244 failure, it was observed that the aseptic air filter in a sampling bottle was broken, but it
245 remained unclear how it had broken.

246 We thus designed a new sampling manifold using a different sampling technique. We
247 have tested it successfully in at least four commercial-scale manufacturing batches, and have
248 encountered no problems.

249 With our sampling manifold we eliminate the need to pump cell culture media liquid
250 out from the bioreactor vessel and then back again. Rather, with our sampling manifold,
251 when liquid is taken from the bioreactor vessel, it is never pumped back in again. This
252 reduces the risk of contamination significantly.

253 Our sampling manifold is described in Figure 3. We prefer to make our sampling
254 manifold from commercially-available C-flex tubing, Masterflex™ tubing, clamps, and
255 commercially-available polymer tube connectors. The tubing can be non-sterile or sterilized
256 by e.g., temperature- or moisture-based techniques such as autoclaving, or gamma-irradiated
257 or gas-based sterilization such as ethylene. The tubing can be ready-made, or custom-made
258 by the user. We merely prefer it to be compatible with commercially-available sterile
259 connections, thus able to be branched with any type connections, and suitable for sealing and
260 welding.

261 Our sampling manifold can be connected to a bioreactor such as the iCELLis™ 500
262 bioreactor through MPC-connector. When used with an iCELLis bioreactor, our sampling
263 manifold can entirely replace the sampling manifold offered by Pall Life Sciences as part of

their commercially available Starter Kit, which is marketed together with the iCELLis™ 500 bioreactor.

In our sampling manifold, we use a pair of tubes to take one sample. One tube is used to rinse the manifold interior, the other tube is used after rinsing to take the sample. For example, one can use tube [15] to rinse the manifold and then use tube [7] to take a sample.

To begin, a single-use sampling bag or bottle [11, 17, 19] is aseptically attached (we prefer through welding) to the sampling tube [7]. One may (and we prefer) to also attach a sampling bag [19] to the first (rinse) line of the pair [15]. Alternatively, the first line may drain into a waste bottle etc.

To use our sampling manifold, liquid cell culture media is pumped from the bioreactor vessel [1] or an accompanying media reservoir through the manifold to rinse the interior. This is for rinsing the manifold and getting fresh medium/liquid from the vessel [1] into the manifold. This rinse media is removed from the manifold via a rinse tube [15] into a rinse media container [19]. Eherinse tube [15] is then sealed or closed. This may be done with a tubing clamp [12] (as illustrated). Alternatively, the tubing may be sealed with an end cap [on 15b], or welded closed [at 15b], etc.: the manner of sealing is not particularly important here. The rinse tube [15] thereafter remains sealed off for the remainder of the manufacturing run. In the appended legal claims, we refer to sealing off for the remainder of the manufacturing run as “permanent” sealing because in the context of a given manufacturing run, it is effectively permanent (*i.e.*, lasting to the end).

The sampling tube exit end [7b], going to the sample container [11], is then opened [13]. Fresh medium/liquid is pumped from the bioreactor vessel [1] (*e.g.*, from the part of the bioreactor which houses the adherent cell culture substrate) into the sampling tube [7], and then out of it [7b] into the sampling container [11]. While we illustrate one sample container ([11]), one could alternatively use several sample containers and take several physically-separate samples. The sampling tube [7] is then closed [13]. The sampling container [11] may then be sealed and removed from the sampling tube output [7b]. After taking the sample, the pair of manifold branches [15, 7] remain sealed off for the remaining duration of the manufacturing run. We prefer both tubes be drained to remove residual media, to reduce risk of contamination.

Sampling bags [11] provide a sampling port used for aseptically drawing liquid out of the sampling bag to assay. Several samples can thus be taken from one bag aseptically.

To enable sequential sampling at different times, we prefer to provide several pair of sampling tubes. With this, a second (or subsequent) sampling may be performed in a similar

298 fashion as the first one, using a different pair of manifold tubes and a second sampling bag
299 attached to the second tube of that second pair of tubes. For example, to take a second
300 sample, one may use line [10] as the rinse tube and line [16] as the sampling tube, with the
301 sample being taken in container [17]). Media/liquid that has remained in the manifold since
302 the first sampling is discarded by pumping it out of the manifold [10b], preferably into a
303 rinse or waste receptacle or container until fresh media/liquid from the bioreactor substrate
304 has filled the manifold. This second rinse tube [10] is then sealed or closed. This may be
305 done with a tubing clamp [12] (as illustrated). Alternatively, the tubing may be sealed with
306 an end cap, or welded closed, etc.: the manner of sealing is not particularly important here.
307 The second rinse tube [10] thereafter remains sealed off for the remainder of the
308 manufacturing run.

309 The second sampling tube [16] is then opened [13]. Fresh medium/liquid is pumped
310 from the bioreactor vessel [1] (*e.g.*, from the part of the bioreactor which houses the adherent
311 cell culture substrate) into the second sampling tube [16] and then out of it [16b] into a
312 second sampling container(s) [17]. The the sampling tube of the second pair [16] is then
313 closed [13]. The sampling container(s) [17] may then be sealed and removed. After taking
314 the sample, the pair of manifold branches [10, 16] remain sealed off for the remaining
315 duration of the manufacturing run.

316 This configuration may be repeated as desired, providing as many pair of rinse and
317 sampel tubes as desired to enable as many different sampling times as desired.

318 With our sampling port, a disadvantage at the moment is the need to construct the
319 manifold (rather than be able to purchase it commercially). Another disadvantage could be
320 the use of several sampling bags for each manufacturing batch. These disadvantages,
321 however, are more than off-set by the reduced possibility of contaminating a manufacturing
322 batch, which in commercial-scale manufacturing poses a significant financial risk.

We claim:

1. An adherent cell culture bioreactor comprising a substrate for adherent cell growth, a feed-in pump and a feed-out pump, wherein the feed-in pump and the feed-out pump are configured to provide a constant-rate output of cell culture media from the bioreactor at an output volume of less than 50 mL media per m² substrate area per day, the bioreactor configured to contain at least 20 liters of cell culture media.
2. The adherent cell culture bioreactor of claim 1, the substrate comprising at least 60 m² of surface area.
3. The adherent cell culture bioreactor of claim 2, the substrate comprising at least 400 m² of surface area.
4. The adherent cell culture bioreactor of claim 1, wherein the feed-out pump is connected to the bioreactor via a tube having an interior diameter sized to accommodate the pump output volume of cell culture media without the formation of air bubbles inside the tube.
5. A method for culturing adherent cells on a substrate comprising:
 - a. obtaining the bioreactor of claim 1, and then
 - b. adding to the bioreactor cell culture media and cells, and then
 - c. culturing the cells in adherent mode on the substrate, while circulating the cell culture media about the cells at a rate of less than 50 mL of media per m² substrate area per day.
6. The method of claim 5, wherein the cells in adherent mode produce a recombinant polypeptide.
7. The method of claim 6, wherein the cells in adherent mode produce a virus.
8. The method of claim 7, wherein the virus comprises a viral capsid containing a non-viral transgene.
9. The method of claim 5, the substrate comprising at least 60 m² of surface area.
10. The method of claim 5, the substrate comprising at least 400 m² of surface area.
11. An adherent cell culture bioreactor comprising a substrate for adherent cell culture, a feed-in pump and a feed-out pump, wherein the feed-in pump and the feed-out pump are configured to provide a constant-rate output of cell culture media from the bioreactor sufficient to exchange the cell culture media in the bioreactor less than once every 3 ½ days, the bioreactor configured to contain at least 20 liters of cell culture media.

12. The adherent cell culture bioreactor of claim 11, wherein the feed-in pump and the feed-out pump are configured to provide a constant-rate output of cell culture media sufficient to exchange the cell culture media in the bioreactor less than once every 4 ½ days.
13. The adherent cell culture bioreactor of claim 11, the substrate comprising at least 60 m² of surface area.
14. The adherent cell culture bioreactor of claim 11, the substrate comprising at least 400 m² of surface area.
15. A method for culturing adherent cells on a substrate comprising:
 - a. obtaining the bioreactor of claim 11, and then
 - b. adding to the bioreactor cell culture media and cells, and then
 - c. culturing the cells in adherent mode on the substrate, while circulating the cell culture media about the cells at a rate sufficient to exchange the cell culture media in the bioreactor less than once every 3 ½ days.
16. The method of claim 15, wherein the cells in adherent mode produce a recombinant polypeptide.
17. The method of claim 15, wherein the cells in adherent mode produce a virus.
18. The method of claim 17, wherein the virus comprises a viral capsid containing a non-viral transgene.
19. The method of claim 15, wherein the substrate comprises at least 400 m² of surface area.