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Operation of a Benchtop Bioreactor

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Title: Operation of a Benchtop Bioreactor

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Abstract: Fermentors are used to increase culture yield and productivity of bioengineered cells. After screening multiple microbial or animal cell culture candidates in shake flasks, the next logical step is to increase the selected culture's biomass with the fermentor. This video demonstrates the setup and operation of a typical benchtop bioreactor system.

Long Abstract: Fermentation systems are used to provide an optimal growth environment for many different types of cell culture. The ability afforded by fermentors to carefully control temperature, pH and dissolved oxygen concentrations in particular makes them essential to efficient large scale growth and expression of fermentation products. This video will briefly describe the advantages of the fermentor over the shake flask. It will also identify key components of a typical benchtop fermentation system and give basic instruction on setup of the vessel and calibration of its probes. The viewer will be familiarized with the sterilization process and shown how to inoculate the growth medium in the vessel with culture. Basic concepts of operation, sampling and harvesting will also be demonstrated. Simple data analysis and system cleanup will also be discussed.

Introduction: Basic fermentation technology is an extension of the simple shake flask technique for growing cultures. It grew out of the desire to control growth environments for live cultures in a more complete and quantitative way. Batch culture shake flasks are usually limited by imprecise control of temperature. Temperature uniformity in an incubated shaker or warm room is highly variable, sometimes straying 5 degrees C or more from the intended setpoint. Since the shake flask is normally agitated at a fixed speed, oxygen uptake and gas exchange is limited. Once the available ambient oxygen is depleted, most cultures fail to thrive. There is no pH control in shake flasks. In many cases, if the culture is not limited by feed stock, it becomes acidic to the point of detriment to the culture, and respiration slows dramatically. Most shake flask cultures are also run as a 'batch', which means that they are fed only once at or near the beginning of the culture's inoculation. After this initial carbon source is consumed, the culture stops growing. In some cases its metabolism may shift and begin to consume other metabolites in the culture broth, sometimes changing the characteristics of the resultant biomass or protein. Shake flasks are also usually subject to media evaporative loss in warmer culture environments, typically 10% of volume per 24 hours at 37 degrees C. This loss changes the density of the culture and prohibits longer term operation of the system. Finally, the user may encounter foaming from the media after agitation. The occurrence of foam in the headspace above the culture will limit gas exchange and further stifle growth.

The basic fermentation system is designed to address all of these limitations. Careful temperature control is achieved in fermentation vessels by the use of impeller agitation and a heating jacket. A sensor inserted into the vessel and feedback control of heating and cooling of this jacket usually results in temperature control ± 0.1°C around the setpoint. Benchtop fermentors generally provide control of pH via liquid reagent addition through a pump. The pH value is continuously monitored in an effort to keep the environment optimal for cell growth. Proper aeration is maintained by the aforementioned mixing impeller or by the infusion of air or oxygen supplemented gas directly into the culture. With shear-sensitive cultures, oxygen supplemented gas is the primary mechanism for maintenance of oxygen level in the culture. Measurement of oxygen in solution is usually achieved by a polarographic probe which is not normally available for use in shake flasks. It is also possible to continuously or periodically add feed to the vessel to maintain growth in a linear or exponential fashion. The exit gas condenser provides a cold surface for vapor in the exhaust gas flow to condense, thus preserving culture volume and density. Periodic addition of antifoam surfactant is actuated by a conductivity probe in the culture, reducing foam on the surface and allowing gas exchange.

The vessel, with all probes, fittings, impellers, harvest pipes and tubes, is assembled and sterilized in a standard autoclave. After final probe calibrations and stabilization to operating environment, the culture is added to the vessel. The system can then be used to characterize the culture in a way that is more quantitative and precise than with a shake flask method. Tight control of temperature, pH, oxygen content, feed consumption, liquid evaporation and foam levels all contribute to a much higher biomass and better protein yield.

Protocol:

1. Begin operation with vessel setup. A conventional fermentor has the following components that need to be installed (Figure 1):

1.1. pH probe - for measurement of pH in the live culture. Calibrate probe before installation and sterilize with the vessel. Install probe in the headplate.

1.2. pO2 probe - for measurement of dissolved oxygen content inside the vessel during fermentation. Install in the headplate.

1.3. Harvest pipe for sampling the culture. Install this adjustable height component in the headplate.

1.4. Gas sparger - resides at the bottom of the vessel and provides gas infusion to the culture. Hook sparger up to the gas source on the fermentor and mount in the headplate.

1.5. Impeller shaft and impeller - the impeller stirs the culture and is critical for maintaining culture uniformity in the vessel.

1.6. Exhaust gas condenser - for curbing evaporative loss in the vessel by cooling the exhaust gas path.

1.7. Reagent Pump lines for pH control or feed addition.

2. Clean the vessel and headplate with soap and water. A soft brush is recommended for scrubbing the vessel and headplate. Bleach or cleaners with chlorine cannot be used to clean the vessel.

3. If the media is not heat labile, add it to the clean vessel. Only add until maximum working volume is reached. In this instance, maximum working volume is 3.3 L for a 5 L total volume vessel.

4. Mount the vessel headplate, ensuring that the o-ring seal is properly seated.

5. Install the exhaust gas condenser.

6. Install the antifoam probe into its 10 mm port.

7. Install the harvest pipe into its 10 mm port. Put a piece of tubing on the top of it and clamp it off.

8. Install tubing and a 0.20 micron filter on the sparge inlet and then clamp this off.

9. Calibrate the pH probe:

9.1. Gather 2 reference buffers in small beakers, a wash bottle and a spare wash beaker.

9.2. Hook the pH probe up to the fermentor and turn the fermentor on.

9.3. Scroll to the pH parameter and using the menu button, scroll to the 'cal' option and press 'enter'.

9.4. Select '2' for 2 point calibration.

9.5. Wash off the probe tip and submerge in the low reference buffer. The value on the fermentor will stabilize. Using the + and – keys, adjust the displayed value to match the value of the pH reference buffer. Once it stops blinking, press 'Enter'.

9.6. Wash the probe off and insert it into the second reference buffer. Allow the value to stabilize and then use the keypad to make the displayed value match the high reference buffer value. Press 'Enter' to confirm.

9.7. Wash the probe tip off and disconnect it from the fermentor. Put the protective cap on the connection and install it in the headplate.

10. Open the pO2 probe tip and check to ensure that there is enough electrolyte to cover the tip. If not, add some to the reservoir and close it back up.

11. Install the pO2 probe in the headplate and make sure that the autoclave cap is put on to protect its electrical connections. It will be calibrated after sterilization.

12. Obtain a bottle for reagent feed with a dip tube and air filter. Add pump tubing to the dip tube port and attach the other end to the inlet port on the fermentor.

13. Place tubing on all unused ports then clamp tubing off.

14. Install a 0.45 micron filter on the exhaust gas condenser. This filter ensures that the vessel does not pressurize in the autoclave.

15. Check that all tubes that go below the level of the media are clamped off to prevent media from coming out.

16. Put the vessel in the autoclave for 25 to 30 minutes at 121 C, liquid cycle. Caution: When it comes out it will be very hot.

17. Install the vessel on the fermentor base.

18. Hook up the pH probe cable.

19. Attach the pO2 probe cable.

20. Hook the sparger up to the gas addition rotameter.

21. Sterilely add 1 M NaOH reagent to the bottle with the dip tube and install the pump head on the ‘Base’ pump head spindle.

22. Put the temperature sensor in the thermowell on the headplate. Ensure that it goes all of the way to the bottom of the thermowell.

23. Lower the agitation arm onto the vessel’s impeller coupling.

24. Make sure that the fermentor is on.

25. Check that water is available to the fermentor.

26. Turn on the air supply to the fermentor.

27. Scroll to the temperature parameter and set the temperature to 37 C. Press the ‘Enter’ button to start the temperature control. The vessel will heat up in 15 minutes or less.

28. Turn the gas flow to 1 vessel volume of media per minute or less. For this set up, gas flow is 3 L/min. Bubbles should appear at the bottom.

29. Scroll to the agitation parameter and set to 300 rpm. Press ‘Enter’ to turn on the agitation.

30. Once the temperature has reached 37 C, scroll to the pH parameter and set it to 6.8. Turn the pH control on by pressing the ‘Enter’ button.

31. Set agitation to the maximum speed of 1000 rpm for the run.

32. Ensure that the pO2 probe is polarized properly to display proper values. After 2 hours, scroll to the pO2 parameter and use the ‘Menu’ button to select the ‘calibrate’ function and select the 1 point calibration.

33. After 15 minutes, use the cursors to set the value to 100 and press ‘Enter’. This will calibrate pO2.

34. Scroll to agitation and set to 300 rpm.

35. Using the ‘menu’ button, turn the ‘Cascade’ to ‘on’ in the agitation menu. This will increase the speed of agitation in an effort to chop up air bubbles and force more oxygen into solution as growth demand increases.

36. Scroll to the pO2 parameter and set the value to 30. Press the ‘Enter’ button to start pO2.

37. Start the control software package on the PC.

38. Once the probes are calibrated, agitation is stable at 300 rpm, temperature is at 37 C and pH is close to 6.8, it is time to inoculate. Using alcohol, sterilize the port that will be used for inoculation.

39. Draw the inoculum into a syringe and add it into the sterilized port. Close the port.

40. Mark the time of inoculation in a log book.

41. It is also important to take a sample at sterilization time. Sterilize the end of the harvest pipe tube with alcohol.

42. Open the clamp covering the harvest port and use a syringe to draw a sample. This first sample is usually discarded because it has been sitting in the pipe.

43. Use another syringe to pull another sample.

44. With a third syringe, push air back through the pipe to remove as much dead volume from the line and clamp the line off.

45. Determine the cell density and pH and log the values. With microbial cultures, it is useful to log values every hour. Interval is culture dependent.

46. Harvest methodology depends upon the intent for the culture after growth is completed. In this case, sample the culture a final time to receive endpoint values for cell density and

possible pH or measurement of wet cell weight. Open the headplate disposed of the contents in a designated “kill tank” with bleach or other antimicrobial agents.

Representative Results:

The bioreactor can be run with or without the IRIS software, however to capture data, it is best to use the software. Before addition of bacteria, the pH and oxygen sensors must be calibrated, the impeller speed set and the temperature set. In Figure 2, the data output for a bioreactor run is presented. The temperature was set to 30 C, the impeller speed at 200 rpm. The parameters may be different for each experiment but before addition of bacteria, the system should be at steady state. In figure 3, the change in oxygen concentration with the addition of the bacterial culture is shown. The set-points for this experiment are 37 C, stirrer at 200 rpm, O_2 at 70%. At time 19:20, the feed pump delivers the bacterial seed culture at an OD of 0.1 causing an immediately drop in the O_2 level. The bioreactor responds to the changing O_2 level with an increase in the airflow and impeller speed. The set-points for the increases are set in the cascade (step 35). The reactor pH was monitored in real time, with the pH decreasing then increasing as demonstrated by the pH line fluctuation over time (figure 4). The entire run can be analyzed and parameters adjusted for subsequent experiments.

Discussion:

Stirred tank bioreactors are the standard in the Biotechnology industry and have been used for over 40 years (1). The small stirred tank has been important for scale-up, scale-down, strain optimization, characterization and process development. It may also have an important role in the development of individualized medicine (2). The small scale bioreactor is most similar to *in situ* conditions for cell growth because it can be monitored and optimized throughout a run. Most often, initial experiments are performed using shake flasks but the conditions in the small scale bioreactor differ significantly from the shake flask. In one experiment we found that the conditions for optimal growth of *E. coli* and production of Green Fluorescent Protein (GFP) in the shake flask did not translate to the stirred tank (unpublished data).

Other methods of growing cells in large scale include roller bottles, single use rocking platform bioreactors (3) and larger single use bioreactors with working volumes from 50 to 5000 L. Each method provides challenges for scale-up, but has found a place in production. The single use rocking platform bioreactor is similar to the stirred tank, and provides a regulated environment. It differs from the stirred tank in that mixing occurs due a rocking motion to generate waves to prevent cell settling and provide oxygenation. The hydrodynamics for this method are different from the stirred tank and maximum volume is limited to 1000 liters. The differences can affect cell growth and product production. Other single use systems combine the stirred tank with the disposable reactor to, provide a platform with a minimum of infrastructure and associated overhead, and the capability for high-throughput bioprocessing (4).

New users of benchtop Bioreactors may have trouble determining initial setpoints for pH, pO2 and temperature; however, published research can be referenced for this information (5, 6, 7, 8, 9). With bacterial cultures in particular, it is recommended to start agitation at the same speed as the shaker flask and the temperature at the same setpoint. Culture pH from previous shake flasks runs can also be used as a starting point. Setting of the pO2 value is more difficult and is typically determined empirically, however, beginning with 50% pO2 is a recommended starting point.

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Disclosures: Author A. Magno is an employee of ATR Biotech that produces reagents and instruments used in this article.

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Figure Legends:

Figure 1 – Benchtop bioreactor with all parts labeled and connected

This figure shows the stirred tank reactor at the beginning of a run. The parts of the reactor are labeled and include the sensors, impeller, temperature gauge, feed & sampling bottles, exhaust port, air pressure gauge, heating jacket, condenser tower, and control panel.

Figure 2 – Screen image of bioreactor software at the beginning of run

At the start of a stirred tank run, the temperature was set at 30^{o}C (yellow line), impeller speed at 200 rpm (red line), pH at 6.5 (green line), and pO2 at 57.2% (blue line).

Figure 3 – Screen image of bioreactor software during the run as oxygen decreases

When the culture is in active growth phase, it consumes oxygen and the amount of oxygen in the reactor is decreased (blue line). By increasing the impeller speed, more oxygen can be added to the culture (red line).

Figure 4 – Screen image of bioreactor software showing pH change over time

The pH of the culture is monitored continuously and over time it decreases as lactic acid is produced and then increases as base is added to the bioreactor (blue line).

*Figure

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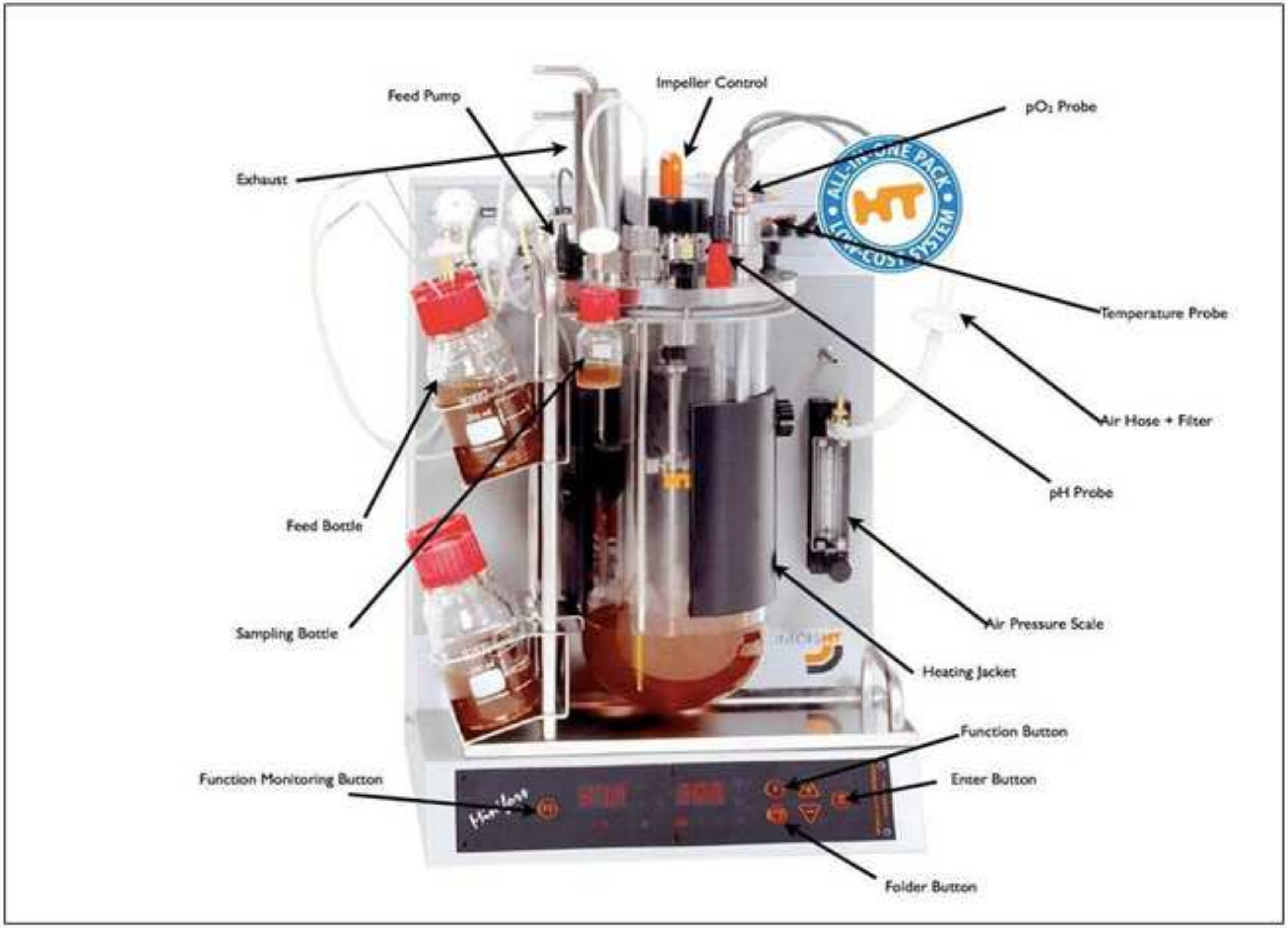

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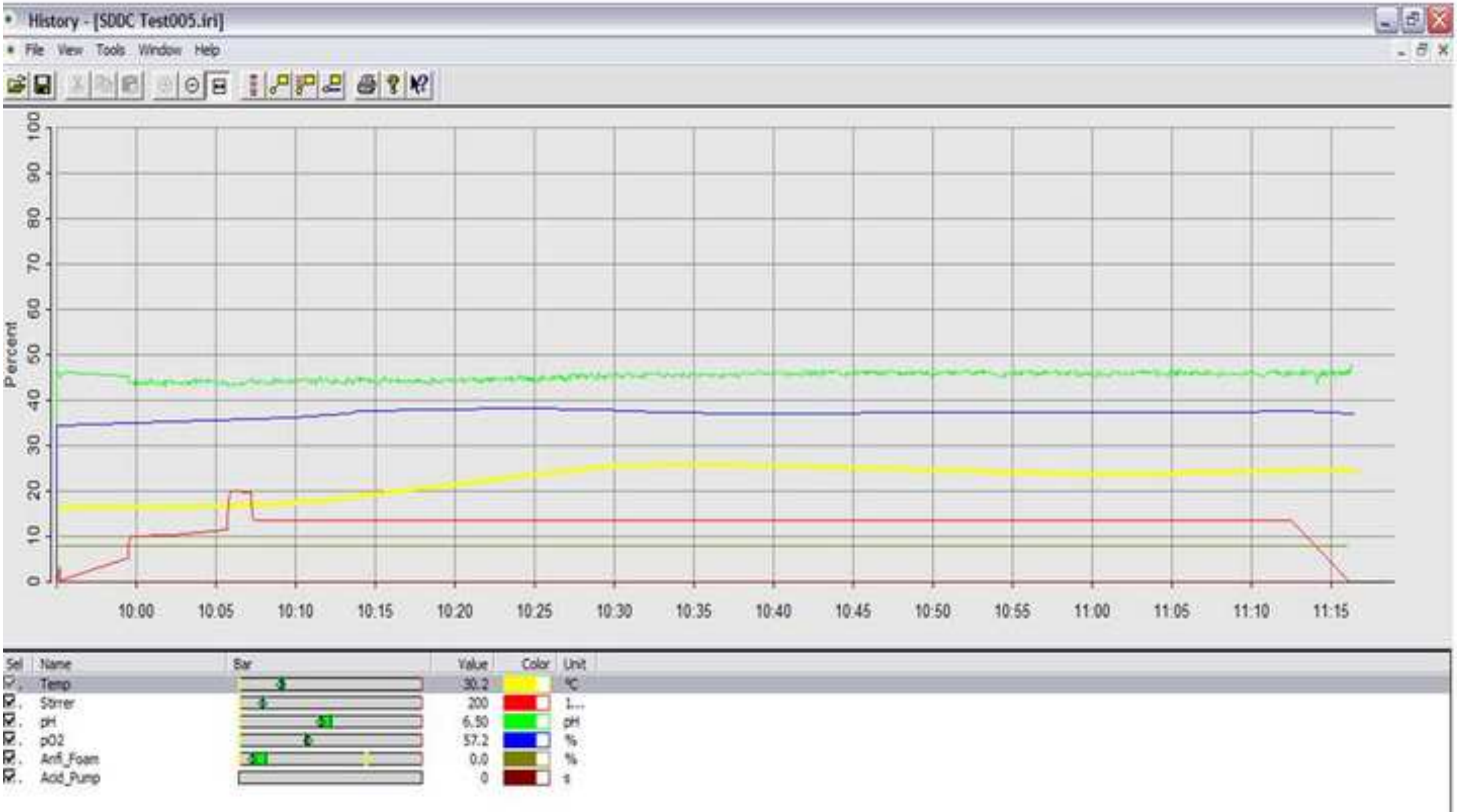

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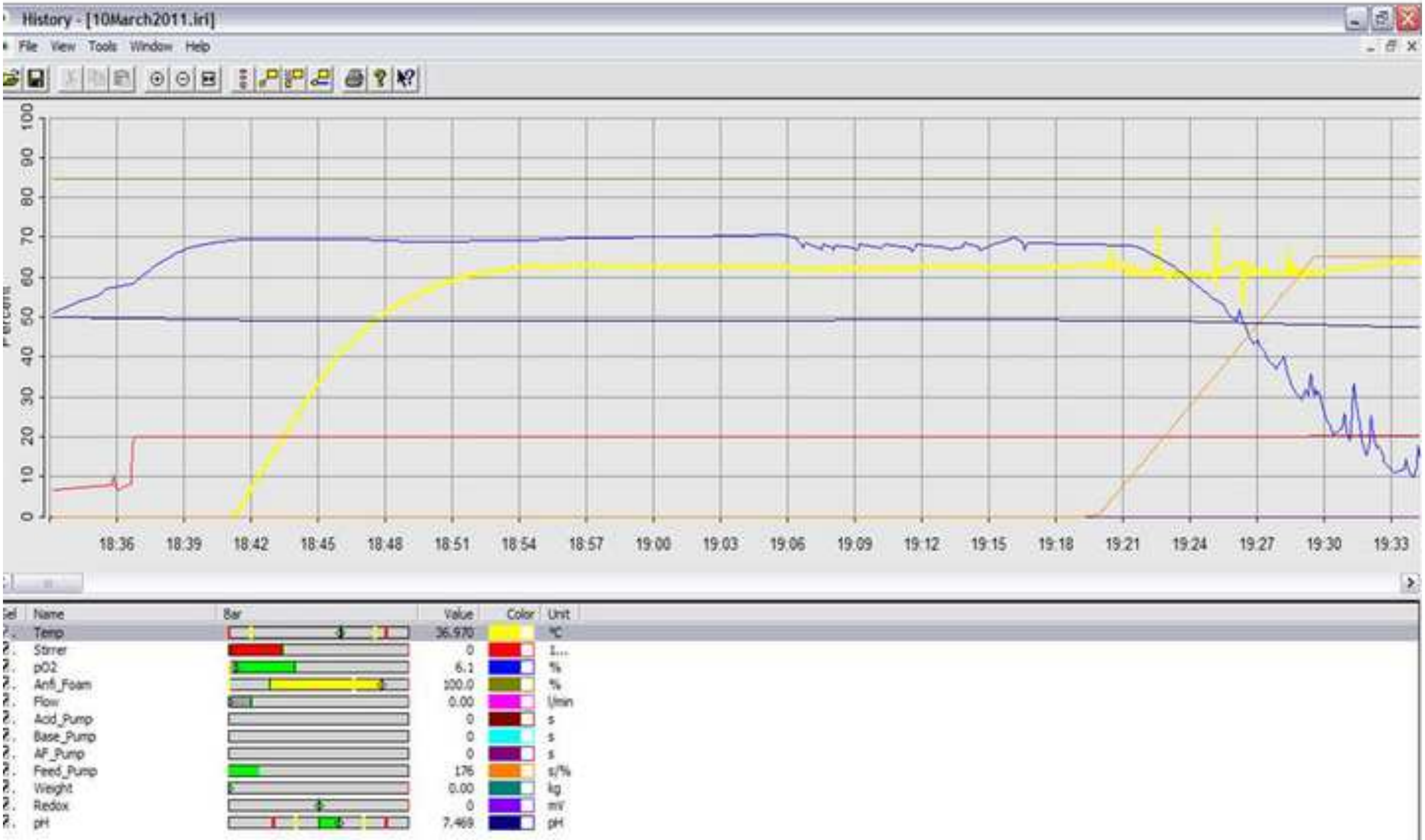

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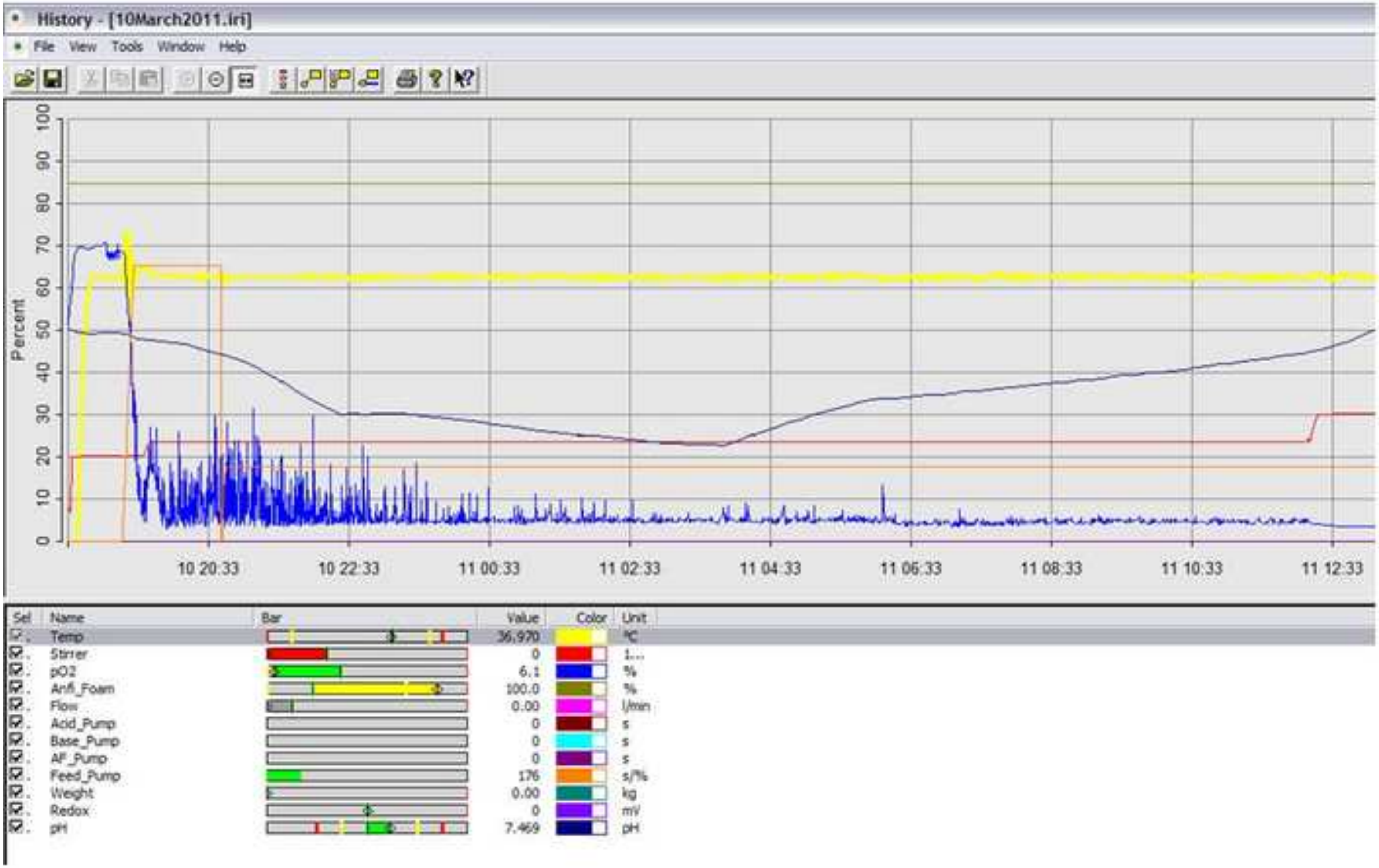

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Component	Specifications
Infors Minifors Fermentation System	Agitation via impeller, 0-1200 rpm pH control from 2-12 pO2 measurement from 1-150% pO2 control via agitation, gas sparge and O2 Supplementation Temperature control ambient +5 C to 60C 3.5 L working volume, 5 L total volume
Air Admiral Air Compressor	20" Hg max pressure (0.667 bar) 0.37 cfm flow rate
VWR 1175 PD Recirculating Chiller	1700 W of cooling at 20 C 20-100 psi (0-5 bar) pressure 3.8L/min Flow Rate 4.1 bath volume
Broadley James pH probe	Gel filled probe, range 2-12 Autoclavable
Broadley James pO2 probe	Poloragraphic probe Range 1-150%
Laboratory Autoclave	Used to sterilize Minifors vessel and culture growth media

Comments
Used for growth of microbial or animal cell cultures depending upon configuration
Provides air source for Minifors sparge
Needed for maintaining temperature in the Minifors as well as servicing the exhaust gas condenser
For measurement of pH
Measurement of liquid dissolved oxygen
Liquid Cycle Operation, 20-25 minutes, 121 C

*Table of Reagents/ Materials Used

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Reagent	**company**	**catalog number**
LB broth	Sigma	L3022
ampicillin	Sigma	A1593-25G
L-arabinose	Sigma	A3256
antifoam 204	Sigma	A8311
pGLO bacterial transformation Kit	Bio_Rad	166-0003EDU

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Institution: Johns Hopkins University

Article Title: Operation and Maintenance of a Benchtop Bioreactor

Signature: Patrick J. Cummings Date: 12/3/2012

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Dear Dr. Kinahan:

Thank you for the opportunity to resubmit the article “"Operation and Maintenance of a Benchtop Bioreactor (JoVE50582)” for consideration to be published in the Journal of Visualized Experiments. The authors have added our responses to the editorial and reviewer comments below.

Editorial comments:
* Please re-write your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) For example, "The vessel and headplate must be cleaned" should be re-written as "Clean the vessel and headplate."

Author Response: Corrected as recommended

* After being reformatted to comply with JoVE specifications (font size, spacing) your protocol exceeds our 3 page limit. Many short steps could possibly be combined to fit within 3 pages. Please remember to leave a space between each individual numbered step. Alternatively, instead of re-writing the protocol you may select which steps you would like to film and highlight these steps (up to 2.75 pages). Steps that you choose not to film will remain in the written protocol and can be referred to in the video. Lists of equipment, notes or asides that won't be filmed should not be highlighted. Please either shorten OR highlight your protocol.

Author Response: Corrected as recommended.

* Please update your figure captions. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

Author Response: Corrected as recommended.

* Please revise your discussion section. The discussion should cover the following in detail: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Author Response: Discussion was rewritten to include the recommended topics.

* JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your

manuscript. For example, the Wave from GE healthcare. If you would like to refer to alternative equipment, please do so more generally.

Author Response: Company brand names have been removed from the text and replaced with generic descriptions of the other Bioprocessing technologies.

Please modify your references section to comply with JoVE instructions for authors. Examples of how to cite various sources can be found in the instructions. Please ensure that each listed reference is cited within the manuscript text.

Author Comments: Corrected as recommended.

Reviewer #1 Comments:
Summary:
In this article, the authors describe the operation and maintenance of a benchtop bioreactor. This article will be very useful for scientists who would like to use this technology. The article clearly lays out the rationale, and the step by step protocol for operation of the instrument. This reviewer found this article to be catering to the needs of both the novice and experts in fermentation technology.

Major Concerns:
Some major comments: There is no description of post operational harvest and processing of the culture and clean up and maintenance of the instrument between runs.

Author Response: An addition step (#46) was added to describe the method for harvesting and decontaminating the bioreactor vessel contents post run.

The title clearly states the maintenance part but is not dealt with in the article.

Author Response: Yes, we agree that maintenance was not addressed, therefore, the title should be edited to be "Operation of a Benchtop Bioreactor"

The type of fermentor depicted in figure 1 and manufacturer are not spelled out. It would be useful for some who would like to adopt this work. The same is true for many other parts and reagents used in this instrument. It is usual practice to mention the manufacturer and location and in some cases part/ catalog number so that the reader can benefit from that information unless it is the policy of this journal not to furnish such information. Also, the manuscript can benefit if colloquial terms are avoided. Some examples are given below.

Author Response: The manufacturer and reagent information is included in the tables and not in the narrative as required by JoVE.

Minor Concerns:

Abstract:

1) Line 1: I would change "Fermentation systems have been used" to "Fermentation systems are used"

2) The viewer will be familiarized with the sterilization process and shown how to inoculate vessel with culture- you do not inoculate the vessel but the medium in the vessel. I suggest changing it to "how to inoculate the growth medium in the vessel"

3) Basic concepts of operation, sampling and harvesting will also be shown.. will be demonstrated sounds better.

Long abstract: Line 13: Their ability to carefully control..may want to change to "the ability afforded by fermentors to carefully control"

Line 19: how to inoculate vessel- see comment above

Line 23: ..is an extension of the simple shake flask. Suggestion: is an extension of the simple shake flask technique for growing cultures.

Line 34: "consume other chemicals"- suggestion "consume other metabolites"

Line 44: this sentence is unclear; do you mean to say" feedback control of heating and cooling of this jacket by a sensor inserted into the vessel usually results in temperature control +/- 0.1C around setpoint?

Line 48: "addition of air" suggestion: "infusion of air"

Line 51: "not normally available to growth" suggestion: "not normally available for use in shake flask cultures"

Line 61: "and precise than with a shake flask" Suggestion: "and precise than with a shake flask method"

Line 67: Would help if brand name, company and location of the company are added in parentheses after the "standard vessel"

Line 79: "gas addition" suggestion "gas infusion or pumping"

Line 96: Please add the usual vessel volume and the working volume. For example for a 5 or 10 lit vessel, what is the working volume?

Line 148: Modify: Autoclave the vessel for 25 to minutes at 121 C, liquid cycle(add the pressure info).

Line 148-149: the vessel does not come out of the autoclave. Change the sentence to something like:

Be careful for the vessel will be very hot when removing from the autoclave.

Line 162: Remove "Put" suggestion "install"

Line 209: Specify inoculum: bacteria or cell culture and at what concentration: overnight? (CFU/ml) and volume?

Line 225: change to "Sampling interval is culture dependent"

Line 231: Refer to figure 1 first somewhere in the text prior to Figure 2. Figure 1 is not

mentioned anywhere.
Line 241: adding a caption or text box above the lines- of what each line represents in the figure would help.
Line 248: It may also have an important role in development of individualized medicine: in what way?
Line 259: that mixing occurs due to a rocking motion. "to" is missing

Author Response: The revised text has incorporated the suggested edits.

References: Only 4 references are quoted in the body of the manuscript. What about references 5-12. Where are they referenced in the text?

Author Response: Additional references added and referenced in the text.

Figures: Please add a text box above the lines in the graphs to indicate what that line represents, pH or DOC etc..Probably the color legend in the bottom of graph indicates it. But it is not obvious.

Author Response: Figure legends have been edited to better explain the data presented.

Table1: add manufacturer and location of the manufacturer

Author Response: NA

Reviewer #2:
Minor Concerns:
Discussion might include some insight or direction into how to establish the initial bioreactor conditions, since shake flask growth is not a predictor of successful production in the bioreactor.

Author Response: Additional information provided as recommended.

Reviewer #3:

Minor Concerns:
Missing seems to be any discussion regarding possible trouble spots and how to best troubleshoot common problems. Obviously, contamination could be a major issue, and while precautions are listed to avoid contamination, no discussion of how best to detect contamination and resolve it should it become a problem are discussed. Also missing seems to be a discussion on the best means for harvesting the culture.

Author Response: Additional information provided as recommended.

Regards

Dr. Patrick Cummings

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