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Protocol

Chemostat Culture for Yeast Physiology

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The use of chemostat culture facilitates the careful comparison of different yeast strains growing in well-defined conditions. Variations in physiology can be measured by examining gene expression, metabolite levels, protein content, and cell morphology. In this protocol, we show how a combination of sample types can be collected during harvest from a single 20-mL chemostat in a ministat array, with special attention to coordinating the handling of the most time-sensitive sample types.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPE: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Ammonium bicarbonate (50 mM, pH 7.8), freshly prepared and maintained on ice (for harvesting samples for protein analyses only)

Chemostat medium

Examples of media are provided in Protocol: ***Assembly of a Mini-Chemostat Array*** *(Miller et al. 2015).*

Ethanolamine (10 mM in 0.1 M potassium phosphate buffer [pH 6.7]), freshly prepared (for harvesting samples for microscopy only)

Addition of ethanolamine will change the pH of the solution from pH 6.5 to pH 6.7.

Formaldehyde (37%) (for harvesting samples for microscopy only)

Liquid nitrogen (for harvesting samples for RNA and protein analyses only)

Methanol (for harvesting samples for metabolite analyses only)

Potassium phosphate buffer (1 M, pH 6.5) (for harvesting samples for microscopy only) <R>

Yeast strain of interest

Equipment

Chemostat array

Assemble the apparatus as described in Miller et al. (2013) and Protocol: ***Assembly of a Mini-Chemostat Array*** *(Miller et al. 2015).*

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Dewar flask (1-L)
Filter holder (25 mm; stainless steel support) (VWR 26316-692)
Forceps
MAGNA nylon membrane filters (0.45-µm pore size; 25-mm diameter)
Ring stand with clamp
Serological pipettes (10-mL)
Silicone tubing, medium (1/4-inch × 3/8-inch)
Silicone tubing, small (3/32-inch × 7/32-inch)
Stopper, black rubber (no. 1 with two holes)
Stopper, silicone (no. 8 with 3/8-inch hole)
Tongs
Tubes, conical (15- and 50-mL)
Tubes, with locking lid (2-mL)
Tubing connector, 1/4- to 1/8-inch
Tubing connector, male luer slip, 1/8-inch barb
Vacuum flask (1-L, with sidearm)
Vacuum tubing

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METHOD

This method is adapted from Skelly et al. (2013).

Establishing Steady State in the Chemostats

1. Start the ministat array as follows:
   i. Turn on the pump to fill the ministats, and then turn it off when the medium reaches the 20-mL mark.
      Assess the volume (marked on the tubes) without bubbling.
   ii. Inoculate each ministat with 0.1 mL of overnight culture grown in appropriate chemostat medium.
   iii. Allow the cultures to grow for 30 h at 30°C.
      They should look dense.
   iv. Turn on the pump at a speed of ~6 rpm (if using the Watson-Marlow pump suggested in Protocol: **Assembly of a Mini-Chemostat Array** [Miller et al. 2015]). Ensure that it is pumping medium from the carboy to the ministats and not the other way around.
   v. Adjust the ministat volumes (with the air off) by adjusting the heights of the effluent needles.
   vi. When there is overflow from all of the effluent lines, empty the effluent bottles and note the time.
2. Determine when the cultures reach steady state by collecting and analyzing samples as follows:
   i. Record the time.
   ii. Move the effluent corks from the bottles to sample collection tubes.
   iii. Record the volume of effluent (V_{eff}) from each ministat. Use this and the time elapsed to calculate the dilution rate. Adjust the pump or individual pump cartridges as needed to achieve a dilution rate of 0.17 (±0.01) vol/h.
   iv. Once a sufficient sample volume is collected, replace the effluent corks in the rinsed effluent bottles.

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v. Assay the cell density by two measurements using a spectrophotometer, colorimeter, and/or flow cytometer. Dilute the samples as needed to ensure that they are in the linear range for each instrument.

vi. Harvest the cells when the cultures have reached steady state, that is, when the cell density readings are within 5%–10% of each other on two consecutive days.

Steady state is typically attained 3–4 d after inoculation, at approximately generation 10–20. Samples up to generation 35 are generally free of detectable de novo mutation accumulation.

Harvesting the Samples

Physiological variations can be assessed by collecting samples to examine gene expression (Steps 3–15), cell morphology (Steps 16–26), metabolite levels (Steps 27–33), and protein content (Steps 34–38).

Harvesting for RNA

Because of how quickly gene expression changes, the goal here is to filter and freeze the cells within 30 sec of disturbing the chemostat. Prepare and gather everything before you start, and leave the pump on until all the sampling is finished. Plan to do a test run first, to make sure you are not missing something critical.

3. Label 2-mL locking-lid tubes and 15-mL conical filtrate collection tubes so that there is one for each sample plus one extra conical tube for rinses. Gather the 25-mm nylon filters, 10-mL serological pipettes (and pipette controller), forceps for handling filters, and tongs for retrieving frozen samples from liquid nitrogen. Also set aside an extra chemostat vessel to house the dripping stopper/needle assembly during sampling.

4. Assemble the vacuum apparatus (Fig. 1) as follows, and perform a test run with water to be sure the apparatus is correctly assembled and rinsed.

   i. Arrange a ring stand with clamp to hold the filter apparatus.

   ii. Push a one-hole stopper onto the bottom of the funnel half of the apparatus, followed by a piece of tubing. Ensure that the ring stand clamp holds the stopper so that the apparatus is fairly upright.

   iii. Connect the tubing to a two-hole stopper that fits into the top of the filtrate collection tube, and connect the vacuum trap to tubing from the other hole in that stopper.

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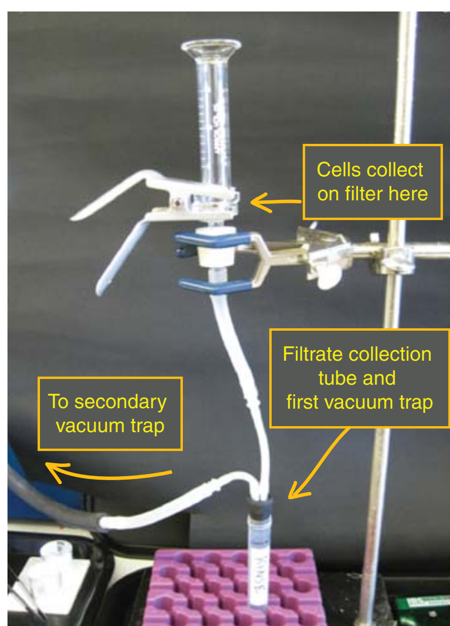

FIGURE 1. Harvesting cultures for RNA assessment. The filter apparatus for RNA sampling.

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Alternatively, you could just use a vacuum flask, but this setup allows you to collect and save the filtrate from each sample.

iv. Fit the stopper in the filtrate collection tube marked "rinse," and turn on the vacuum.

v. Use forceps to put a filter on the metal screen in the apparatus. Listen for hissing or whistling, which indicate a leak. Reposition or replace whistling filters.

vi. Clamp the cylinder into place, and pipette 20 mL of ddH_2O into the top of the apparatus, checking for leaks. If there are leaks, try adjusting the junction of the two glass pieces or the height of the metal screen. If there are no leaks, reset the apparatus by replacing the filtrate tube and filter.

vii. Fill a 1-L Dewar flask about half full with liquid nitrogen.

Now is the time to do a test run of the actual harvest as described below. Keep in mind that if you are also going to sample for microscopy, protein, or metabolites, you should have those sample tubes ready and waiting.

5. Make sure the vacuum is on, the filter apparatus is properly assembled with the filtrate collection tube for the first sample, and a fresh filter is in place.
6. Open the 2-mL tube for the first sample and drop it into the liquid nitrogen in the open Dewar flask.
7. Being careful of the exposed needles, take the stopper assembly off the first ministat and place it in an empty/dummy chemostat vessel in a neighboring well of the heat block.
8. Pick up the chemostat and pipette 5 mL of the culture onto the filter.

   If you are also taking metabolite or protein samples, pipette these into the specified tubes while the RNA sample is collecting on the filter.

9. As soon as the sample has completely filtered, remove the clamp and top section from the apparatus.
10. Using tongs, dump the liquid nitrogen of the 2-mL tube and put it open in a tube rack.
11. Use the forceps to roll the filter onto itself, so that the cells are to the inside, and put it in the frozen tube.

    You may want to use a gloved finger to hold the back of the filter loosely in place while you manipulate it with the forceps.

12. Immediately close the tube as it sits in the rack, and drop it back into the liquid nitrogen.

    Now that the samples are frozen, they can be stored at −80°C until further processing.

13. Cap the filtrate sample to preserve the concentration of ethanol or other volatiles of interest, and store at −20°C. Be sure to leave enough headroom in the tube to accommodate sample expansion on freezing.

    If also harvesting for microscopy (Steps 16–26), metabolites (Steps 27–33), or protein (Steps 34–38), proceed with that sample handling at this time.

14. Thoroughly rinse the apparatus with ddH_2O between samples.
15. Once all samples are taken, put the spent vessel back in place to house the stopper assembly, which will continue to drip media, and move the dummy from position to position.

Harvesting for Microscopy

Prepare fresh solutions before beginning.

16. Pipette 1.2 mL of cells into microcentrifuge tubes containing 0.15 mL of formaldehyde and 0.15 mL of 1 M potassium phosphate buffer (resulting in 3.7% formaldehyde in 0.1 M potassium phosphate solution).
17. Let the samples sit for 10 min at room temperature.

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18. Centrifuge the samples in a microcentrifuge at 4500*g* for 7 min with slow acceleration to pellet the cells.
19. Resuspend the pellets in 1.5 mL of 0.1 M potassium phosphate containing 3.7% formaldehyde.
20. Incubate for 60 min at room temperature.
21. Centrifuge at 4500*g* for 7 min with slow acceleration to pellet the cells.
22. Resuspend the pellet in 1.5 mL of 0.1 M potassium phosphate containing 10 mM ethanolamine.
23. Incubate for 10 min at room temperature.

    This step deactivates the formaldehyde.
24. Centrifuge at 4500*g* for 7 min with slow acceleration to pellet the cells.
25. Resuspend the cells in 1 mL of 0.1 M potassium phosphate.
26. Store at 4°C until further processing.

Harvesting for Metabolites

If harvesting for metabolites and protein, the samples can be handled in parallel.

27. Pipette a 10-mL sample into a 50-mL conical tube on ice.
28. Centrifuge at 3000*g* for 3 min at 4°C and wash the pellet with 10 mL of cold water.
29. Centrifuge again at 3000*g* for 3 min at 4°C.
30. Immediately resuspend the pellet in 0.5 mL of cold water and quickly add 0.5 mL of cold methanol.
31. Invert to mix, and incubate the samples for 30 min in a dry ice–ethanol bath at approximately −40°C.
32. Thaw the frozen mixture on ice for 10 min, and centrifuge at 3000*g* for 5 min at 4°C.
33. Freeze the supernatant at −80°C until the time of analysis.

Harvesting for Protein

Note that the full 20-mL culture volume must be used to recover adequate protein for mass spectrometry, leaving none for other sample types. Consider running larger-volume chemostats to allow collection of multiple sample types.

34. Pipette or pour the 20-mL culture into a 50-mL conical tube on ice.
35. Centrifuge the sample at 3000*g* for 3 min at 4°C.
36. Wash the pellet in cold 50 mM ammonium bicarbonate (pH 7.8).
37. Centrifuge again at 3000*g* for 3 min at 4°C and freeze the sample using liquid nitrogen.
38. Store at −80°C until the time of analysis.

DISCUSSION

Allowing yeast culture to reach and maintain steady state is a key feature of the chemostat. Before a chemostat culture has stabilized, fluctuations in cell density show that the culture has not fully adjusted to the growth rate imposed by the system. Once the density stabilizes, however, the experimenter can be assured that the doubling time is being regulated by the dilution rate, and that the cells have reached a steady state. In this condition, the cells are coping with the experimental conditions imposed on them, and their physiology can be examined by microscopy, as well as by profiling RNA, metabolites, and protein.

The most time-sensitive part of the protocol is the harvest of cells for RNA analysis. The slightest changes in environment can change gene expression, so great care must be taken to avoid any

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perturbation of the culture within 24 h before taking the RNA sample. Therefore, it is recommended that only passive sampling via the effluent line be taken before opening the chemostat to take the culture for RNA. Immediately after the cell sample is frozen, and during the initial fixation step for microscopy, cells for protein and metabolites can be handled together, because they both require immediate chilling in an ice bath and the same centrifugation steps at 4°C.

A 20-mL chemostat may not have enough volume to accommodate all of the described sample types from a single vessel. Although the small culture size of these "mini" chemostats may seem to limit the sampling possibilities, the highly adjustable nature of the ministats allows the user to increase the volume with a simple adjustment of the effluent needle. Technological advances will also likely reduce the importance of large sample sizes in the near future.

RECIPE

Potassium Phosphate Buffer (1 M, pH 6.5)

Reagent	Quantity (for 100 mL)
KH_2PO_4	9.5 g
K_2HPO_4	5.25 g

Dissolve the reagents in 80 mL of ddH_2O. Adjust the pH to 6.5. Make the volume up to 100 mL with ddH_2O. Filter sterilize and store at room temperature.

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