How to conduct a *C. phytofermentans/S. cerevisiae* consortium fermentation

Trevor R. Zuroff

Updated: 6.12.14

Purpose

This protocol describes the method for conducting fermentation of cellulose or lignocellulosic biomass using a dual species consortium containing *Clostridium phytofermentans* and *Saccharomyces cerevisiae*. This protocol focuses on how to initiate a fermentation while the specific sampling schedule, analyses and treatments are based on each experiment.

Background

Consortia containing *C. phytofermentans* and various yeast have been demonstrated in both published [1] and unpublished studies. The obligate anaerobe, *C. phytofermentans*, provides hydrolytic capabilities to degrade insoluble substrates releasing soluble carbohydrates for consumption by both organisms to produce fuel. To achieve population control, oxygen is diffused into anaerobic culture medium through 10 cm of neoprene tubing at a rate of roughly 8 µmol/L hr (Figure 1). The yeast, *S. cerevisiae*, consumes oxygen, "protecting" *C. phytofermentans*, but only if provided soluble carbohydrates via *C. phytofermentans*-mediated hydrolysis. This interactive consortium model has been applied to improving the production of ethanol and botryococcene from purified cellulose. The consortium design is likely flexible in that an organism can be removed and replaced with another as long as it maintains the symbiotic role in the interaction (i.e., either hydrolysis or oxygen removal). This could enable adaptation of this protocol for various other microorganisms and process designs.

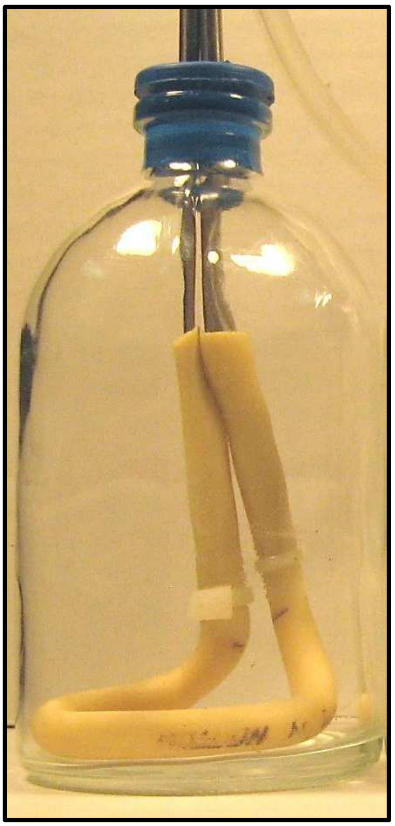

Figure 1. Anaerobic serum bottle fitted with 10 cm neoprene tubing for diffusive oxygen transfer. From [1].

Consortium fermentations are conducted in specifically designed rich medium that enables growth of both organisms under conditions that promote ethanol formation. Oxygen transfer is achieved via diffusion through neoprene tubing and has been demonstrated to scale from 50 mL to 500 mL. Population dynamics (and population control) are determined via selective plating where media and environmental conditions are used to select for each species. Microscopy can also be used to confirm the presence of species though quantitative determinations of populations should be interpreted with care as solid cellulose particles and biofilm formation may reduce accuracy of these results. Additional samples

can be removed from consortium fermentations over time to enable monitoring of substrate consumption and product formation, to determine rates and to calculate final yields. Typical samples are discussed below along with the volumes necessary.

The time required to initiate a consortium fermentation (following completion of a detailed experimental plan) should be roughly 6 days (Figure 2). To enable the most efficient use of time, this protocol should be started on Friday. The first day will be used to make media, prepare reactors with cellulose or biomass and start cultures from cryostock. Cultures should be grown after roughly 3 days of incubation and on the 4th day they can be restarted and media can be prepared in the reactors (this step should always be done 1-2 days before inoculating the experiment to "preserve" the medium while providing sufficient time to reduce the medium). The inocula will then grow during day 5 and 6 and when at the appropriate density can be used to inoculate. Inoculation will consume roughly ½ of the final day but depending on the level of experience, preparedness and the size of the experiment this could range from only a few hours to over 10 hours.

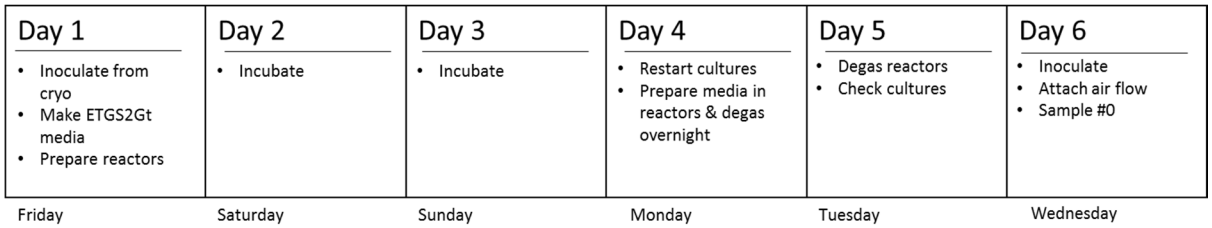

Figure 2. Approximate schedule for initiating a consortium fermentation.

Typical conditions

30˚C, 200 rpm, $pH_{initial}$ = 7, ETGS2Gt medium, 100 mL serum bottle with gas release valve (see pg. 98 of TRZ Notebook #3 and Figure 3 A).

Typical samples

1 mL samples taken approximately every 3 to 4 days using a sterile syringe and needle. The following analyses are most commonly completed:

- HPLC (using 200 µL) to measure fermentation products (e.g., ethanol, acetate, etc.)
- Anthrone assay (using 50 µL) to measure soluble carbohydrates
- CFU counts (using 20 µL) to monitor microbial populations
- Dry weight (using pellet) to determine cellulose conversion

Notes:

- There are numerous other analytical methods that would provide significant insight into the performance of the system. Additional sample volume may be necessary to allow for these analyses.

- For example, roughly 500 µL of sample is necessary to enable analysis of botryococcene concentraitons.

Initial Culture Preparation

Before you begin:

- Prepare medium components (see Media Preparation protocols)
  - GS2 for *C. phytofermentans*
  - YPC for *S. cerevisiae* cdt-1 or SCE for *S. cerevisiae* ZX17808
- Locate cryo-stocks for each organism

Steps:

1. Prepare 5 mL of GS2 medium in a screw cap tube using GS2 Medium Preparation Protocol.
2. Aliquot 5 mL of yeast medium into an aerobic culture tube.
3. Flame inoculation loop and remove a small amount of inoculum and place in each medium, respectively.
4. Place *S. cerevisiae* culture in 30°C, 200 RPM incubator under atmospheric conditions.
5. Place *C. phytofermentans* tube with cap loose in anaerobic chamber incubator at 32°C, verify the medium is reduced (after roughly 1-2 hour) by monitoring color change from pink to clear.
6. Let incubate for roughly 2-3 days until $OD_{600} \geq 0.2$ for *C. phytofermentans* and $OD_{600} \geq 0.5$ for *S. cerevisiae*.

Inoculum Preparation ("Restart Culture")

Before you begin:

- Prepare medium
  - 50 mL GS2 for *C. phytofermentans*
  - 250 mL YPC for *S. cerevisiae* cdt-1 or SCE for *S. cerevisiae* ZX17808
- Verify appropriate OD_{600} of initial cultures (as in step 6 above)

Note: You may need additional volume depending on the number of reactors to be inoculated. See Overview in Inoculation for details on necessary volumes.

Steps:

7. Place 50 mL GS2 medium in a 100 mL serum bottle for *C. phytofermentans*.
8. Place 250 mL of yeast medium into a 1 L Erlenmyer flask with sponge plug for *S. cerevisiae*.
9. Measure and record OD_{600} of initial cultures.
10. Add roughly 2 mL of *C. phytofermentans* initial culture to 50 mL GS2 medium.
11. Add all 5 mL of *S. cerevisiae* culture to 250 mL medium.
12. Place *S. cerevisiae* culture in 30°C, 200 RPM incubator under atmospheric conditions.

13. Place *C. phytofermentans* culture in anaerobic chamber incubator at 32°C, verify the medium is reduced (after roughly 1-2 hour) by monitoring color change from pink to clear.
14. Let incubate for roughly 1-2 days until $OD_{600} \geq 0.25$ for *C. phytofermentans* and $OD_{600} \geq 1.0$ for *S. cerevisiae*.

Reactor Preparation

Following preparation of inoculum cultures, media can be added to reactors and degassed for use in the experiment.

To Do:

- Prepare ETGS2Gt medium for consortium fermentations and distribute to reactors (Note: media is prepared based on GS2 Medium Preparation protocol adjusted for ETGS2Gt base. The water volume can and should be adjusted to accommodate the addition of enzyme – or anything else - if desired).
- Place reactors in anaerobic chamber on bench at least overnight to degas.
- Prepare pre-weighed eppendorf tubes for dry weight sampling.
- Label all reactors with the appropriate labels based on the desired treatments

Inoculation

Overview: *C. phytofermentans* and *S. cerevisiae* should be inoculated at an initial OD_{600} of about 0.0125-0.025 and 0.5 respectively by adding 2.5 mL of inoculum. *C. phytofermentans* inocula will be used directly while *S. cerevisiae* cultures will be concentrated to $OD_{600} = 10$. Therefore, in theory, 50 mL of *C. phytofermentans* at $OD_{600} = 0.25$ can be used to inoculate 20 reactors and 250 mL of *S. cerevisiae* at $OD_{600} = 1.0$ can be used to inoculate 10 reactors. In reality it is difficult to remove the final roughly 5 mL from a sealed serum bottle and thus excess inoculum should be prepared. Mono-cultures should receive a suitable replacement of the organism that was left out (e.g., buffer, media, etc.).

Before you begin:

- Autoclave Sterilize
    - Gas release valve units (Figure 3 A)
    - ~ 5 butyl rubber stoppers (Figure 3 B)
    - ~ 5 anaerobic serum bottles (Figure 3 C)
    - 2 x 250 mL centrifuge bottles
- Prepare 32 mL of 1/32 Minncare by adding 1 mL Minncare to 31 mL DI H_2O.
- Add roughly 40 mL of 1x Phosphate Buffered Saline (PBS) to one serum bottle (retain another roughly 40 mL outside the anaerobic chamber).
- Add roughly 40 mL of sterile DI H_2O to another serum bottle.
- Add enzyme solution (if desired) to another serum bottle.
- Place PBS, DI H_2O and enzyme solution in anaerobic chamber near catalyst stack to degas.
- Verify 30˚C, 200 rpm incubator space for all reactors.

- Verify the availability of sufficient syringes, needles and eppi-tubes for inoculation and sampling.
- Prepare aquarium pumps with tubing for reactors needing O_2 transfer.

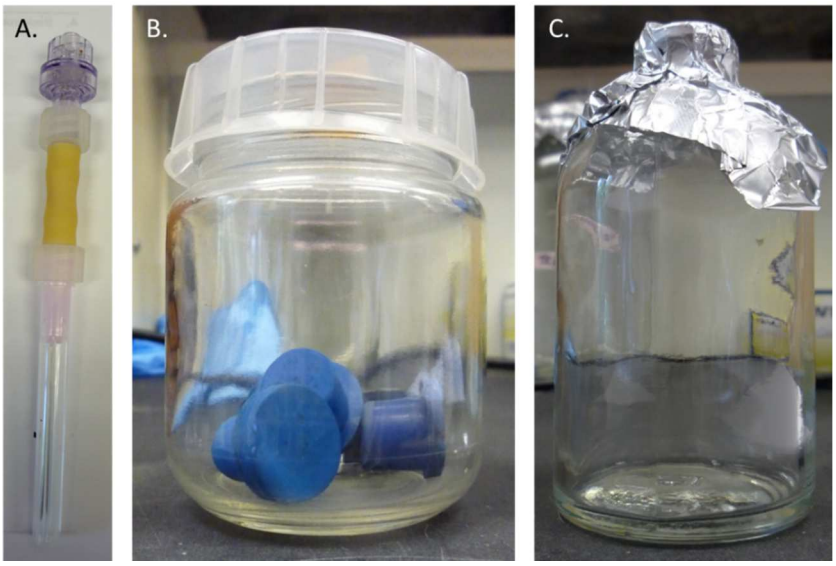

Figure 3. Supplies needed for inoculation. Gas release valves (A.), stoppers (B.) and serum bottles (C.).

Inoculation Steps:

1. Check the optical density of *C. phytofermentans* (50 mL) and *S. cerevisiae* cdt-1 (250 mL) restarts and record. *S. cerevisiae* OD_{600} must be at least 1 and *C. phytofermentans* OD_{600} must be at least 0.25 to have sufficient inoculum for typical experiments. If OD's are sufficiently high, proceed to step 2 but place *C. phytofermentans* culture back under anaerobic conditions during steps 2 through 8. Note: The *C. phytofermentans* OD_{600} must be ≥ 0.15 to ensure log phase culture.
2. Pour 250 mL of *S. cerevisiae* into a pre-sterilized ultra-centrifuge bottle rated to at least 10,000 rpm.
3. Using the Avanti JE centrifuge, pellet the *S. cerevisiae* culture by centrifugation at 7,500 rpm for 30 minutes at 20˚C.
4. Pour the supernatant off the *S. cerevisiae* culture and use a serological pipette to remove any remaining cell-free liquid.
5. Based on the OD_{600} measured in step 1, calculate the volume of 1 x PBS needed to reach a *S. cerevisiae* OD_{600} of 10.
6. Add 1 x PBS (volume calculated in step 5) to the centrifuge bottle and resuspend the pellet by pipetting up and down and vortexing.
7. Pipette the solution from step 6 into a pre-sterilized anaerobic serum bottle and cover with sterile aluminum foil cap.
8. Place *S. cerevisiae* resuspended culture into anaerobic chamber to degas for at least 1 hour. Mix periodically to assist gas transfer.
9. After at least 1 hour degassing, place butyl rubber stoppers in the *S. cerevisiae*, *C. phytofermentans,* water, PBS and enzyme solution serum bottles (See Appendix 1).

10. Place butyl rubber stoppers in the experimental reactors (See Appendix 1).
11. With everything sealed with a stopper, remove cultures, solutions and reactors from the anaerobic chamber and place in the biocontainment hood.
12. Clean the surface of all serum bottles using 1/32 Minncare on a fresh Kimwipe. You should already be wearing gloves but this is especially critical at this step as Minncare will burn your skin and leave an unpleasant odor.
13. Record time of inoculation initiation.
14. Inoculate the reactors by adding 2.5 mL of each culture (for co-cultures) or 2.5 mL of a culture and 2.5 mL 1x PBS (in place of yeast or *C. phytofermentans*). Use a fresh needle and syringe for each reactor to avoid cross contamination.
15. Add desired volume of enzyme solution to each reactor designated "+ Enzyme" and add equal volume DI H_2O in place of enzymes to those designated "No Enzyme". Use a fresh needle and syringe for each reactor to avoid cross contamination.
16. Record time of inoculation completion.
17. Mix the reactors by hand and immediately take the first sample (see Sampling below) with a 1 mL syringe and needle (Note: 18 g needles are useful to remove cellulose for DW samples, if removal is not desired, e.g. for biomass fermentations, use a smaller gauge needle to act as a filter).
18. Place one-way valves (Figure 2 A) through the stopper of the reactors.
19. Place the reactors in the incubator and attach the air flow to initiate gas transfer. Reactors can be placed in series with ≤ 5 reactors per aquarium pump.
20. Verify air flow through the reactors by touch with a wetted palm.
21. Record time of incubation initiation.
22. Clean up. This procedure is not finished until this step is conducted.

Sampling

Before Starting:

1. Verify that eppendorf tubes have been prepared – 1 pre-weighed tube for the cellulose pellet and 1 sterile sample tube for storing the supernatant for each culture. Label appropriately.

Sampling Steps:

1. Clean the surface of all serum bottles using 1/32 minncare on a fresh Kimwipe.
2. Remove a 1 mL sample from each reactor using a 1 mL syringe and 18 g needle and place in a pre-labeled, pre-weighed eppi-tube.
3. Repeat step 2 for each reactor.
4. Once all samples are taken, clean the stoppers with 1/32 minncare and immediately place the reactors back in the incubator and attach air flow.
5. Centrifuge the 1 mL samples at 13,000 rpm for 10 minutes to pellet cells/cellulose.
6. Remove the supernatant and place in pre-labeled, sterile eppi-tube.
7. Add 750 µL DI H_2O to cell/cellulose pellet and vortex to wash.

8. Centrifuge the samples at 13,000 rpm for 10 minutes to pellet cells/cellulose.
9. Remove the supernatant and discard.
10. Repeat steps 7-9 to perform a second wash.
11. Place all eppi-tubes in a labeled Ziploc bag and freeze at -20˚C until the completion of the experiment when they will be analyzed. Alternatively, pre-weighed tubes can immediately be placed open in 70°C oven to dry for ≥ 4days then cooled and weighed.

Appendix 1 – Placing sterilized stoppers on anaerobic serum bottles

Butyl rubber stoppers should be autoclaved in bulk in a baby food jar and can be individually removed and placed on serum bottles as needed. The method for removing stoppers and placing them in serum bottles is described below. Figure 4 shows this process on the bench for demonstration while this should be conducted in the anaerobic chamber in reality.

Steps:

1. Tilt baby food jar toward hand and place index finger and thumb on cap.
2. Use thumb and index finger to remove cap.
3. Shake stoppers until one sits on baby food jar rim with top of stopper pointed out.
4. Remove cap from baby food jar completely, balancing stopper on rim of jar.
5. Use opposite thumb and index finger to remove stopper from jar only touching the top of the stopper.
6. Close jar and set on bench.
7. Loosen aluminum foil on serum bottle, lift and place stopper in top.
8. Push and twist stopper to get into jar, try not to touch the bottom of the stopper.
9. Verify that stopper is sealed tightly into the serum bottle.

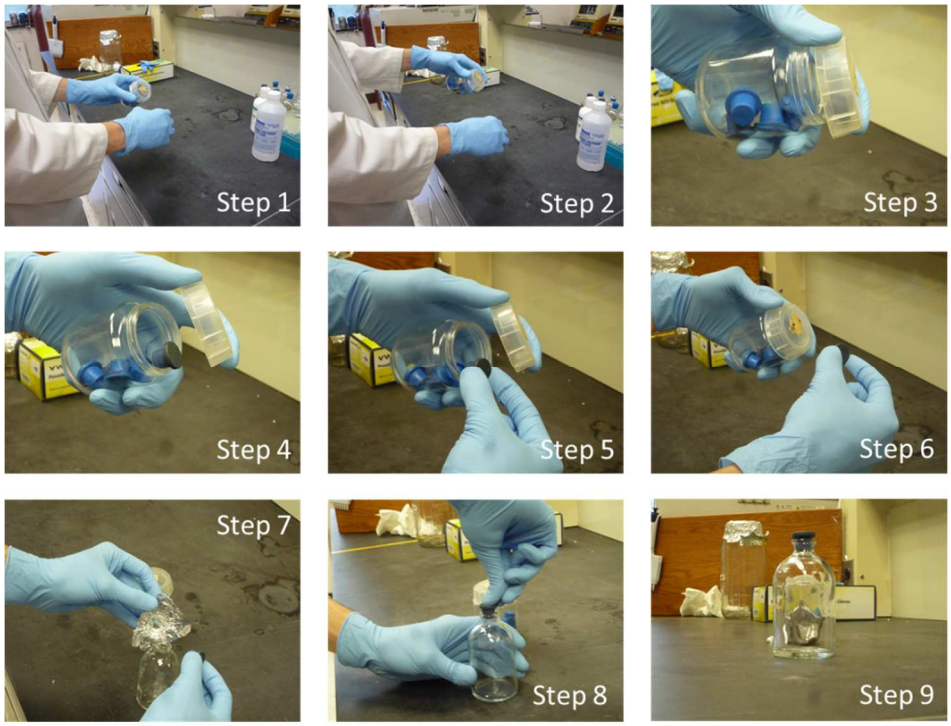

Figure 4. Procedure for placing stopper on anaerobic serum bottle.

References

[1] T. R. Zuroff, S. Barri Xiques, and W. R. Curtis, “Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic Clostridium phytofermentans/yeast co-culture,” *Biotechnol. Biofuels*, vol. 6, no. 1, p. 59, Apr. 2013.