Interview with Dr Whitham

WHO ARE WE INTERVIEWING? (job, studies…)

Dr. Jason M. Whitham is a post-doctoral fellow at North Carolina State University studying plant microbial interactions with the purpose of improving yields and tolerance of bioeconomy crops.

CONTEXT

After many unsuccessful attempts and ordering the strain twice to make *Clostridium ljungdahlii* grow, we decided to seek an expert. Dr. Whitham wrote a thesis about this bacterium. He notably is first author of the publication: "Metabolic Response of *Clostridium ljungdahlii* to Oxygen Exposure". We contacted him to better understand how to cultivate this bacterium.
We interviewed Dr. Whitham on September 1st and continued exchanging emails until the end of our last experiments at the beginning of October.

RATIONALE (What questions did we ask him? What answers did we want to have?)

1. <u>According to your experience, what is the recommended medium for *C. ljungdahlii*?</u>

So far, we only attempted growing *C. ljungdahlii* on (what we thought was) a rich medium containing yeast and beef extract. We always observed that the bacteria were just sporulating, which halted their growth and indicated that a crucial element was missing in the media.
We decided to switch to the recommended minimal medium from DSMZ (medium 879). To avoid having to buy all individual elements, we chose to use Yeast Nitrogen Base (YNB): a minimal medium for yeast that already contains most compounds *C. ljungdahlii* needs. To confirm this medium, we wanted Dr. Whitham's expertise and further recommendations.

2. <u>How should we handle the preparation of the media, vitamin solution, trace elements and reducing agents?</u>

So far, we had never used trace element solutions nor vitamins, and only Sodium Sulfide as a reducing agent. Also, we were unsure about how to degas all these solutions. We naturally wanted Dr. Whitham's expertise and further recommendations on the matter.

3. <u>Details about a proper cultivation: pH, temperature, agitation, growth behavior</u>

As none of our attempts were successful so far, we had no concrete information about the growth behavior *C. ljungdahlii*. Furthermore, we wanted to confirm the required pH, temperature and agitation for our strain.

4. <u>What is causing precipitation in our media?</u>

After setting up a successful *C. ljungdahlii* culture thanks to Dr Whitham's advice, we started noticing greyish precipitate in our vials that was not due to external contamination. As the precipitate was preventing us from collecting meaningful Optical Densities (OD) to attest the bacteria's growth, we wanted to know if this was a common problem and how to mitigate it for further work.

5. How should *C. ljungdahlii* be stored for future use?

As we were unsure about the protocol to store the strain for future uses at -80°C, we asked Dr Whitham his technique.

6. What do you think of our bioreactor design?

To attest *C. ljungdahlii* growth on a mixture of CO_2 and H_2, we designed and built a bioreactor with the available equipment in our laboratory. As we did not want to forget anything and because this bioreactor involved H_2, an explosive gas, we wanted Dr Whitham's expertise on the matter.

INTERVIEW (summary of the interview)

1. According to your experience, what is the recommended medium for *C. ljungdahlii*?

We showed Dr. Whitham the content of our newly designed medium (see *Experiments*) and he confirmed that the bacteria should grow in such conditions. He also confirmed that D-Fructose was preferable as a carbon source for anaerobic fermentation since D-Glucose would require an adaptation period for our strain. A mixture of CO_2 and H_2 should be perfect for growth on syngases. He also advised that we prepare trace elements with care as iron is a crucial element for *C. ljungdahlii* growth.
Na2S and L-Cysteine-HCL are crucial for starting a *C. ljungdahlii* culture as they depress and poise the redox potential at optimum levels.

2. How should we handle the preparation of the media, vitamin solution, trace elements and reducing agents?

For trace elements, Nitrilotriacetic acid (NTA) must be added first as it helps metallic compounds and ions to dissolve better. For every mol of metal, we should do one mol of NTA. If we want to wing it then we just do 1g. We need about 0.25g of KOH for every gram of NTA. The protocol is:
"In a beaker with a stir bar, add water (1 liter for example), add 1g of NTA, add 0.25g KOH, and let this stir for 15 minutes or more. Much of the NTA should dissolve. There will still be some undissolved NTA stirring around. Add in your Fe, Cu, Mn, Ca, Mg, Zn...salts (iron first). The trace elements solution should be somewhat acidic (pH 4 range) to prevent precipitation." After this and if needed, supplementary KOH must be added to reach pH 4.

Vitamins cannot be autoclaved and must be filter-sterilized inside an already degassed vial.

We can add fructose and the trace elements directly to our base medium and autoclave. Na2S and L-Cysteine-HCl are autoclaved separately and added after.

Reducing agents should be prepared carefully to prevent as much oxygen from entering the vial. They should not be kept more than a week.

Vitamins and trace elements should be kept in the refrigerator with foil over the bottles to block out light. The autoclaved base media does not need to be refrigerated, but it should be kept in the dark, like in a cabinet. Once all of the components are added together, the complete media should also be kept in the dark.

3. Details about a proper cultivation: pH, temperature, agitation, growth behavior

pH: It is always better if we have access to a pH probe. We need to do the following test. When autoclaved, the pH of the media acidifies by about 0.5, so before applying the stopper and flushing the vial headspace, autoclave and add mix all the media components together, we should adjust the pH to 7.5. To be sure, we should give a couple of other pHs a try- 7.7, 8, 8.3. Hopefully one of these

will become pH 7-7.2. That's the range we want when we inoculate. *C. ljungdahlii* generally stops growing around pH 6 or 5.5, depending on the media. They would still twitch under the microscope, just wouldn't grow. pH seems to be a crucial factor for growth. Jason : "I wrote a paper on the effect of lower pH on C. thermocellum. In the case of that microbe, metabolism was inhibited in a variety of ways as indicated by slowing of growth, hundreds fold accumulation of some metabolites, and downregulation of critical central pathway genes. I never investigated the effect of acidic pH on C. ljungdahlii and I don't think anyone else has to a systems depth."

Agitation: Agitation is absolutely preferable, especially when feeding gas substrates. 150 or 200 rpm is standard. We might want to try a little higher. We should also lay serum bottles on their side to maximize surface area and gas transfer. We should put them in a box with paper towels so they are tightly packed and there is no clinking. Any clinking will cause differential growth though I can only guess why.

Temperature: 37°C is the optimal temperature to cultivate *C. ljungdahlii*.

4. What is causing precipitation in our media?

"I think the problem may be that when the pyrite forms, the iron/sulfur become inaccessible. Those growth factors are important for the bacteria and yeast.

In the case of the yeast culture, you could purposely sparge your reactor with oxygen and precipitate the reducing agents with iron in the media (making more pyrite) and then add even more iron so that some is in a non-precipitated form. Make sure that the iron you add is chelated with NTA like we discussed, otherwise it will precipitate rapidly. The pyrite makes it challenging to monitor OD but at least you could still measure fermentation products."

5. How should *C. ljungdahlii* be stored for future use?

Procedure:

1) Add 50 ml of water to a beaker with a stir bar.
2) Add 50 g of glycerol to the beaker slowly while stirring. I just pour being careful not to contaminate the stock.
3) Distribute 2.5 ml into a 10 ml serum bottle.
4) Stopper and crimp seal all your serum bottles.
5) Exchange headspace gas for 30 seconds. Depressurize to atmospheric pressure.
6) Autoclave all serum bottles. And allow them to cool to room temp.
7) Add 2.5 ml of active culture (ideally log phase) to your serum bottles using needle and syringe.
8) Store in - 80°C freezer.
9) Add 1ml of Freezer Stock (FS) to 50ml cultures.

Note: If you prefer 10 ml FS than just make and distribute double into 25ml serum bottles.

6. What do you think of our bioreactor design?

We scrupulously followed Dr. Whitham's recommendations when experimenting with the bioreactor.

It should work out fine as long as we stir the media. We can use the same media as for anaerobic fermentation, we just need to switch fructose for CO_2/H_2. The headspace should also be as large as you can get it in a batch mode (pv=nrt) to prevent overpressure.

We need to be careful when manipulating CO_2 and H_2 gasses. CO_2 can cause anoxia and H_2 can be flammable and explosive in presence of oxygen. We have to make sure that our bioreactor is hermetic and that the gas flows are correctly redirected outside the building. We should experiment

in a large and well vented room. Generating H_2 from a commercial electrolyzer should be fine as it does not produce too large quantities of H_2.

To start the culture, we should add 10% (4 - 20mL) of active culture (OD600nm 0.6 - 1.0) to the medium. we should go on the lower side (4mL) if we want to monitor more of the development; on the higher side (20mL) if we just want to get your reactor going.