Resting cell experiments for measuring bacterial metabolism

A resting cell suspension contains cells with induced enzymes capable of doing metabolic reactions, but which are suspended in non-growth medium so they can't make new proteins or cells. Under these non-growth conditions, substrate depletion initially follows zero-order kinetics (linear), allowing simple calculation of apparent specific activities (*k*) from a straight line fit through the substrate depletion data. Resting cell suspensions are also useful because they allow concentration of cells from a large culture volume into a small volume, thus boosting the reaction rates, and sometimes making reactions measurable that can't be seen in growing cultures.

PROTOCOL

1) Choose a growth medium that you know will give you the enzyme activity of interest. e.g. if you were assaying the ethene monooxygenase in *Mycobacterium* NBB4 you would need to grow the cells in minimal medium on ethene as sole carbon source. e.g. if you wanted to see haloacid dehalogenase activity in *E.coli* cells carrying *dhlB* in a pET vector, you would need to induce with IPTG for 4 hours *after* culture growth to mid-log phase to ensure the gene is expressed.

2) Determine what volume of culture you need to grow to see the activity. If the activity is weak, you may need to grow 500 ml of cells to make a 10 ml resting cell suspension (50-fold concentration factor), but a strong enzyme activity may be visualised in the same volume of culture vs. resting cell suspension. If the activity of your assay is too high, the cells will consume the substrate too quickly for you to get good quantitative data (ie. a linear substrate depletion curve). If the activity is too low, a linear substrate depletion curve may not be apparent (or you may have to measure depletion over a very annoyingly-long period of time). If you incubate "resting cells" for too long, funny things can happen, e.g. the cells can uninduce, or other enzymes that you don't want may switch on.

3) Think about what controls you need. Will the negative control consist of the same strain grown in non-inducing medium? Or will it be an *E.coli* with vector-only, not the cloned gene of interest? Is there a positive control you can use?

4) Think about replicates. Do you need to grow multiple cultures on the same day? Or should you stagger these over different days? Is it OK to make replicate cell suspensions from the same parent culture…is that still a true 'replicate'?

5) Think about the need for sterility. Usually, once the culture has grown, we are in the realm of biochemistry rather than microbiology, so it's OK to use clean but non-sterile glassware and plasticware and solutions etc in a resting cell assay. However, if you want to keep the solutions for a long time, or if the assay runs for a long time (approx. >8 hr), and/or uses very rich ingredients (e.g. glucose), you may need to be more careful.

6) Plan the experimental protocol and timing. Make sure you have all the required media, consumables (esp. sterile centrifuge tubes/botttles!), and required solutions. Make sure you are familiar with the way the cultures grow and the analysis methods required before growing up lots of cultures or large volumes of cultures – a quick preliminary experiment is often invaluable to get the feel for an assay before committing a large amount of resources. How long do the cultures take to grow? How long does the assay take to run? Will you need to be in the lab at 3 am on Sunday morning? (if so, maybe rethink when you will inoculate the culture!)

7) Inoculate the cultures from a fresh inoculum (either plate or broth), and grow to mid-log phase. “Mid-log” (or mid-exponential) means different things for different bugs and different media, but this will typically be around OD_{600} = 0.5. If in doubt, let the cultures grow to full density, then assume that mid-exponential is about half of that OD_{600}. In some unusual cases, you may need to grow cultures into stationary phase or death phase or even lag phase, if the enzyme you are interested in is induced at those times, but for most common cellular enzymes, you will get best activity when the cells are happy and healthy ie mid-log.

8) If you are using wild-type cells which are induced by the growth conditions themselves, skip ahead to the next step. If you are using recombinant cells, where the inducer is artificial (e.g. IPTG, arabinose, tetracycline etc), add the inducer now at mid-log phase. Allow to induce for 4-48 hours depending on the strain, conditions, and enzyme of interest. For *E.coli* cells and standard induction systems, 4 hr is usually enough, but for mycobacteria or other slow-growers or for unusual enzymes or cold incubations, induction may require a lot longer. Monitoring OD600 during induction is a good idea to get a sense of how induction impacts on growth.

9) Harvest the cultures by centrifugation. Use the correct tubes/bottles and speeds. Bacteria can be pelleted at quite low g forces and times, e.g. 3000 *g* for 10 min is fine in most cases. Check the conversion from *g* to rpm if in doubt – these are usually in the same range (ie. 3000 g is about 4000 rpm in a ‘Centaur’ machine 50 ml rotor), but different rotors have different conversion factors. Some cells like mycobacteria may require additives (0.05% Tween-80) to centrifuge properly, if these aren’t already in the medium. (and these additives need to be maintained in both wash buffers and the assay buffer). Cells pellet easier at higher salt concentrations; pure water is quite difficult to pellet cells in.

10) WASH THE CELLS ! This is important, and often overlooked. If there are significant amounts of rich growth medium (e.g LB) present in your resting cell assay, the cells can grow, which will interfere with determining rates of reaction etc. Washing is done by centrifuging and then resuspending in a buffer like KP (20 mM K_2HPO_4, pH 7) or MOPS or PBS etc. To resuspend cells, its best to flick or vortex or shake the tube/bottle rather than using a pipette – clumpy cells in particular will stick to tips and be lost. You can shake vigorously, this won’t hurt most bacteria.

11) More cell washing hints: Don’t forget to add Tween to wash solutions for clumpy cells like mycobacteria (add Tween 80 after to wash solutions *after* autoclaving). Don’t use plain water as a wash buffer, and ideally use a wash buffer that is the same as the base components in your assay buffer in the later steps. Typically we would do <u>two</u> wash steps in a convenient volume, e.g. 20 ml buffer in a 50 ml Falcon tube for culture pellets from a few hundred ml of culture, or 0.5 ml buffer in an Eppi tube for pellets from a few ml of culture.

12) What temperature to use for centrifugation and wash steps? This is a bit controversial. As a starting point, it is best to use a cold centrifuge for both these steps, since this should “stop the cells in their tracks”, and minimise any changes their physiology. However in some cases, the cold treatment may shock the cells, and they may respond better to room temperature centrifugation and storage during setup. In all cases though, avoid them getting hot! (>30 C)

13) After washing, resuspend the cells in a <u>small volume</u> of the same buffer and keep on ice while you are preparing the assays. (though see above, sometimes room temp might be better). By “small volume” here, we mean like 100 x less than the original culture volume. This gives us the greatest flexibility for assay setup. Its easy to dilute cells further, but annoying to concentrate them. Measure the OD600 of the cell suspension, using an appropriate dilution (e.g. 20 µl into 980 µl of buffer). Make sure the spectro reading is <1, otherwise dilute further. Calculate back to figure out the actual OD600 of the suspension.

14) Set up the assay reactions in appropriate types and sizes of tubes/bottles. If the substrate volatile (e.g. ethene, dichloroethane), you will need gas-tight bottles (serum bottles), which are crimp-sealed. Otherwise, you can use McCartney bottles or Eppi's or Falcon tubes. If using McCartney bottles or other recycled glassware, wash these with RO water before using, to remove traces of detergent. If the cells need aeration, ensure sufficient headspace (say 75% of sealed bottle volume), or incubate loosely-capped. What is your target OD_{600}? (how much activity are you expecting?) – this will determine the volume of the assay, or more specifically, the ratio of the assay volume to the original culture volume. How many samples do you need to take, and what volume are these? (this also impacts the assay volume)

15) As an example, a typical resting cell assay using wild type mycobacteria with ethene as a substrate might use cells from a 30 ml culture, combined and resuspended in 3 x 4 ml aliquots in 16 ml serum bottles. This gives three suspensions with $OD_{600's}$ of ~2 (some losses occur during harvest and washing), and gives nice measurable rates of ethene metabolism, with convenient equilibration times and sample intervals.

16) Set up a master mix, which contains all the ingredients for your assay (buffer and substrate, maybe cofactors or stabilisers). Aliquot this mix out into your individual tubes/bottles, leaving room to add cells to bring the assays to their full volume. e.g. if your total assay volume is 4 ml, you want to get a final OD_{600} of 15, and your washed cells are at an OD_{600} of 60, you need to add 1 ml of cells, thus make up aliquots of 3 ml of master mix. Pre-incubate the master mix at the assay temperature for at least 10 min before starting, then add cells.

17) Immediately take a time zero sample. If this is a liquid sample, centrifuge this immediately in a cold centrifuge, and transfer the supernatant to a fresh tube for immediate assay, or for freezing and later analysis. Label tubes first! Keep in mind that the sampling process itself can interfere with the assay procedure… e.g. if you are too slow, the temperature of the incubating shaker won't be maintained in the cultures during sampling. e.g. if you take too much of the initial liquid volume out via sampling, this will change the way volatiles partition in the bottle etc etc.

18) Incubate the tubes with shaking at the required temperature. Keep in mind the need for good mixing! For smaller tubes/bottles, it is often better to incubate them horizontally to get good mixing action. This is even more important when assaying volatile substrates, where movement between liquid and gas phases happens. Sample at appropriate intervals – this may be between 1 minute to 1 hour, depending on activity levels.

19) At end of assay, discard cell suspension to autoclave waste, and if using glassware, rinse this out with water straight away (also discard to autoclave waste), so that cells don't dry onto the glass. If using volatile substrates, you can leave bottles open in fume hood overnight before discarding culture, to allow volatiles to disperse.

20) After doing your assays (GC, spectro, colorimetric etc), calculate the rate of substrate disappearance or product appearance; this will usually be in nanomoles per minute. This can then be converted into an "apparent specific activity" (k) by dividing by the protein content of the assay (mg). We can use previous standard curves to convert OD_{600} to amount of protein:

For ***E.coli*** **grown in LB:** protein (g) = 0.21 x wet weight of biomass, and biomass (g/L)= OD_{600}/0.534, therefore protein (g/L) = 0.393 x OD_{600} or **[protein (µg/ml) = 393 x OD_{600}]** (Kangwa et al 2015)

For ***Pseudomonas putida*** **grown in LB: [protein (µg/ml) = 69 x OD_{600}]**
(Mai Anh Ly, PhD thesis)

For ***Mycobacterium smegmatis*** **grown in MSM-glucose: [protein (µg/ml) = 99 x OD_{600}]**
(Mai Anh Ly, PhD thesis)

For ***Mycobacterium chubuense*** **grown in MSM-acetate: [[protein (µg/ml) = 112 x OD_{600}]**
(Laura Nolan, Hons thesis)