Ecto- and Extracellular Enzyme Assay

In this experiment, we investigate the activity of four bacterial ecto- and extracellular enzymes. We introduce a substrate analog for a particular enzyme that has a fluorescent label (4-methylumbelliferyl (MUF) or 7-amido-4-methylcoumarin (AMC)) associated with it at a concentration of 100 μM. In the presence of active enzymes, the substrate is cleaved (hydrolyzed) which liberates MUF or AMC. Using the fluorometer, the MUF/AMC compound is excited at 364 nm, while the fluorescence is read at 445 nm. The fluorometers have previously been calibrated using MUF dissolved in 200 mM glycine buffer (pH of 10.5) at concentrations of 0 through 1000 nM. The readings are linear up to ~4000 nM, and are reported in units of μM, not nM (and fluorometer displays it incorrectly as "ug/L"). Because there may be slight differences between the two fluorometers, always use the same fluorometer throughout your study.

1. Remove ***more*** than 16 ml from the ***bottom port*** on your Winogradsky column (gloves are recommended; otherwise, you'll have "smelly hands").
2. Aliquot 4 ml of sample into each of four 15 mL Falcon tubes that are labeled accordingly with the substrate being added (see table below).
3. Add 400 μL of enzyme substrate (one substrate per tube, see table). Mix tubes by swirling. Note exact time of addition of substrate; this is time zero.
4. Into 4 borosilicate tubes aliquot 4 ml of 200 mM glycine buffer (pH 10.5), each.
5. Transfer 1 ml of enzyme-sample solution into borosilicate tubes. Note exact time of addition. Exposure to buffer stops the reaction because of high pH.
6. Measure fluorescence on fluorometer and record value. Fluorometer calibrated output is in μM (but incorrectly displays as ug/L units).
7. Repeat steps 4, 5 and 6 at least two more times at approximately 30-60 min. intervals. You may need to dilute the sample more than 1:5 if fluorometer reading goes off scale or above 4 μM. Be sure to maintain final volume of sample plus buffer at 5 ml if a higher dilution is required. Borosilicate tubes must have ~ 5 mL or more to get a reading. Also, be sure to record dilution factor, which initially starts at 5:1.
8. For the problem set, plot MUF concentration (nM) versus time and use linear regression to calculate enzyme activity in units of nmole l^{-1} h^{-1}. Make sure to account for dilution in steps 4+ 5, or step 7 if needed.

Fluorescence Substrates:

Tube	Substrate	Enzyme Assayed	Conc. (mM)*
A	MUF-N-acetyl-β-D-glucosaminide (M-2133†)	Chitobiase (a chitinase)	1
P	MUF-phosphate (M-8883)	Phosphatase	1
B	L-Leucine-7-amido-4-methylcoumarin HCl (L-2145)	Endopeptidase (a protease)	1
G	MUF-β-D-glucoside (M-3633) (or -β-D-glucopyranoside)	Cellobiase (a cellulase) (β-1,4-glucosidase)	1

† Sigma Chemical Co. catalog numbers.

* Concentration of substrate being added.