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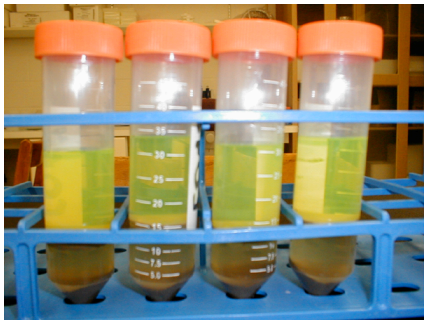
Fluorescein diacetate hydrolysis assay

Background information

The fluorescein diacetate (FDA) hydrolysis assay measures the enzyme activity of microbial populations and can provide an estimate of overall microbial activity in an environmental sample. The assay is considered non-specific because it is sensitive to the activity of several enzyme classes including lipases, esterases, and proteases. Activity of these enzymes results in the hydrolytic cleavage of FDA (colorless) into fluorescein (fluorescent yellow-green).



The fluorescent yellow color is produced as microbial enzymatic activity hydrolyzes FDA (colorless) into fluorescein (yellow). Note the soil in the bottom of the tubes.

In the FDA assay, the environmental sample is mixed with FDA and buffer and incubated with shaking for 1 – 2 hours. The intensity of the resulting yellow-green color is indicative of the amount of enzymatic cleavage of the FDA molecule and the overall enzymatic activity in the sample. Quantification of enzyme activity is performed by assessing the intensity of color formation using spectrophotometry.

Materials

60 mM phosphate buffer (pH 7.6, sterile)
2000 μg ml^{-1} FDA in acetone (this is a working solution for the activity assay made fresh from an overall stock solution of 20 mg ml^{-1})
5 mg ml^{-1} fluorescein sodium salt in buffer (this is the solution for standards, see below)
Chloroform/methanol (mixed 2:1)
Five - 50 ml falcon tubes for each sample (sterile)

Five - 50 ml falcon tubes as catch-flasks for each sample (non-sterile, but clean)
One - 50 ml falcon tube for each standard (n = 5).
200 μl pipette with tips
Metal spatula
Round filter paper (10 cm diameter)
Water bath shaker
Spectrophotometer

- Wear gloves throughout the entire protocol.
- Do not cross-contaminate your samples or the solutions. Be aware of your pipette tip.
- Work clean, either on fresh blue bench paper, in the hood, or on a freshly ethanol treated bench top.
- Perform all steps involving chloroform in the fume hood.

The protocol in brief

You will incubate your sample with buffer and FDA for 1 – 2 hours. The amount of fluorescent color formation during the incubation is indicative of the enzymatic activity of the microbial community in the sample. The intensity of the color will be measured by a spectrophotometer and compared to a standard curve to determine the relative microbial activity in your sample.

A. FDA assay

Soil, plant material or sediment (for water samples, see below)

1. For each sample measure 2 g of material into each of three - 50 ml falcon tubes. Add 2 g of material into a fourth tube that will serve as the "no FDA" control. This is control "1" that will contain soil, but no FDA.

2. Add 20 ml of 60 mM phosphate buffer to each tube. Prepare an additional tube that contains buffer only. This will be a second control ("no sample", but containing FDA).

3. Briefly vortex the solutions to suspend the sample.

4. Add 100 μl of 2000 μg ml^{-1} stock solution FDA to each of the tubes except for the "no FDA" control. The resulting concentration of FDA will be 10 μg ml^{-1}.

5. Shake again briefly, by hand.

6. Place the falcon tubes at an angle in a water bath shaker (200 rpm). Incubate for 30 – 60 minutes at 30 °C.

7. Following the incubation, quickly and carefully add 20 ml of 2:1 chloroform/methanol to each tube. Make sure the shaking action keeps the material in suspension to stop the reaction.

8. Shake the tubes briefly, by hand.

9. Spin the falcon tubes at 5000 x g for 5 min to settle the soil and separate the aqueous (buffer) and organic (chloroform/methanol) phases.

10. Remove approximately 5 ml of the aqueous phase (top phase) and filter into a clean falcon tube.

11. Measure the intensity of the fluorescent coloration of your samples, including both controls) on a spectrophotometer (wavelength 490 nm).

Water samples

The FDA assay is not commonly performed on water samples, however the method can be modified to facilitate the analysis of water. The following steps outline a filtration procedure that traps bacteria on a membrane thereby concentrating them from a large volume of water. The membrane containing the bacteria is then subjected to the assay in a similar manner to soil or other solid matrix.

Additional materials needed

Milipore filtration unit
Vacuum manifold
Sterile forceps
47 mm (0.45 μm pore size) polycarbonate membrane filter with absorbent pad

1. Sterilize forceps by soaking in 70 % ethanol and passing through a flame. The alcohol will kill any bacteria on the glass surface and the flame will burn-off the alcohol. With sterile forceps, remove a filter membrane (0.45 μm) and absorbent pad from plastic wrapping and place it onto the base of a Millipore disposable filtration unit. Be sure to place the pad-side down with the filter facing up. Snap the filter cup onto the base.

    1a. Prepare three filters for each sample.

2. Place the filtration unit onto a vacuum manifold. Open all of the valves that control flow to vacuum ports that have filtration units attached. Close all others.

3. Place an appropriate volume of sample into the cup. The filter will hold 100 ml of water, but continue to add water following each filtration (below) in order to filter as much water as possible. Be sure to note exactly how much water was filtered.

4. Connect the tubing from the vacuum pump to the manifold via the back-flow preventor. Turn the pump on. The water will be sucked through the filter membrane while cells present in the water will be trapped on the membrane.

5. Close the valves after each water sample has completely passed through each membrane to increase the vacuum suction to the remaining samples. Turn off the pump when each of the water samples has passed through the membranes.

6. Open the valves to release any remaining suction. Remove the filtration units from the vacuum manifold and remove the filtration cup from that base without disturbing the membrane.

7. With sterile forceps, carefully separate each membrane from the pad/base and place into a sterile 50 ml falcon tube and proceed with step 2 above (p. 2).

    7a. It is also possible to pre-incubate the filter to stimulate microbial activity by placing it onto a general solid media (TSA, R2A, etc.) for a few hours.

B. Preparing fluorescein standards

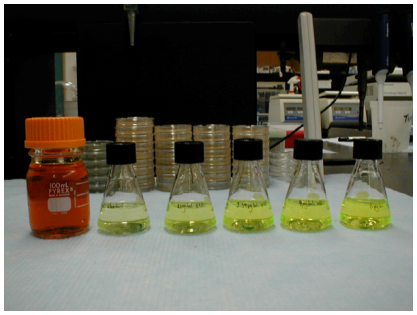
In order to determine the amount of FDA that was hydrolyzed by enzymatic activity in your samples, you must prepare a standard series of fluorescein dilutions from which a standard curve is generated. Using this curve, you will be able to convert your optical density measurement to μg fluorescein produced g^{-1} soil. In this lab exercise, you will be given a predetermined standard curve to which you can compare your sample results. However, you should be familiar with construction of the standard curve.

To prepare the standards, dilute a standard stock solution (5 mg ml^{-1} in acetone) of fluorescein sodium salt according to the following table.

Fluorescein standard (mg ml^{-1})	Dilution of fluorscein standard necessary to achieve each standard	Volume added to each "standard sample" (μl)	Resulting fluorescein concentration in "standard sample" (μg ml^{-1})
5	none	100	12.5*
2.5	1:2	100	5.0
1.0	1:5 (1:2.5 of previous dilution)	100	2.5
0.5	1:10 (1:2)	100	0.5
0.1	1:50 (1:5)	100	0.25
0.05	1:100 (1:2)	100	0.125

*This is the resulting concentration after adding 20 ml of the 2:1 chloroform:methanol

Since methanol is soluble in aqueous solutions, the resulting volume of the aqueous phase following the addition of chloroform/methanol to your samples (step 7, p. 2) will be greater than 20 ml. Therefore, to account for concentration differences that arise from the solubility of methanol, the standards are treated identically to the actual samples, i.e. 100 ml of each fluorescein standard is added to a falcon tube containing 20 ml of buffer (no sample), called the "standard sample", and incubated. By doing this, the resulting aqueous phase will contain a slightly diluted, but relatively accurate concentration of fluorescein that can be compared to that of the samples.



A series of fluorescein standards (yellow) as well as the stock solution of fluorescein (orange).

Since there is no environmental sample in the standards, there is no need to filter them. They can be analyzed in the spectrophotometer directly after centrifugation to determine the standard curve.

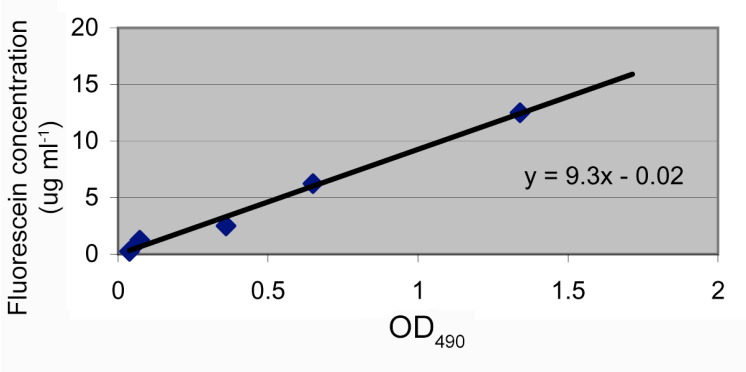
C. Calculating the enzyme activity in your sample

You will be given predetermined standard OD_{490} measurements from which you will determine a standard curve. This was done, as described above, by running "standard samples" along with actual samples and controls. Following the incubation, the OD_{490} of the "standard samples" was measured to generate a standard curve.

For example, measuring the OD_{490} of "standard samples" generated the following table:

Fluorescein concentration ($\mu g\ ml^{-1}$)	OD_{590}
12.5	2.5
5.0	1.2
2.5	0.7
1.0	0.28
0.5	0.14
0.25	0.06
0.125	0.02

Graphing the OD_{490} vs. fluorescein concentration generates a standard curve (and an equation describing the curve), against which the OD_{490} of the actual samples can be compared.



Assume that one of your samples resulted in an OD_{490} of 0.900. To determine the amount of fluorescein produced by that sample, first subtract the OD_{490} of the "no FDA" control and the "no sample" control (assume they sum to 0.050). This will account for any color formation that might have occurred that was not associated with your sample.

Use the resulting OD_{490} value (0.850) in the equation of the standard curve, i.e.,

$$\text{Fluorescein produced} = (9.3 \times OD_{490}) - 0.02$$

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$$\text{Fluorescein produced} = 7.88\ \mu g\ ml^{-1} \text{ of solution.}$$

Depending on the characteristics of your sample, determine the amount of fluorescein produced ml^{-1} (water sample) or g^{-1} dry weight (solid material).

Further reading:

Adam, G. and H. Duncan. 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. Soil Biol. Biochem. 33:943-951.

Schnurer, J. and T. Rosswall. 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Appl. Environ. Microbiol. 43:1256-1261.