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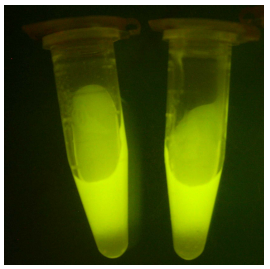
Fluorescein diacetate assay - for plastic degrading enzymes in algae



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We use this protocol and it's working

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Abstract

Fluorescein diacetate (FDA) hydrolysis assays can be used to measure the enzyme activity of cells in a sample. A bright yellow-green glow is produced and is strongest when enzymatic activity is greatest. This can be quantified using a spectrofluorometer or a spectrophotometer (**REF**).
In this protocol, we describe a method for assessing the enzymatic activity of plastic-degrading enzymes in algal cultures using Fluorescein Diacetate (FDA) hydrolysis. The assay involves sampling algal cultures, centrifuging to obtain clear supernatants, and performing a fluorescence-based activity assay. Key steps include ensuring no cell contamination in the samples, preparing the assay with phosphate buffer at optimal pH, and conducting kinetic readings using a plate reader. This protocol is crucial for identifying efficient plastic-degrading enzymes in microalgae, with applications in environmental biotechnology and plastic waste management

Guidelines

Objective: This assay measures the enzymatic activity of plastic-degrading enzymes in algal cultures using fluorescein diacetate (FDA) hydrolysis.
Note: Esterases, including native enzymes from cells, can show activity in this assay. Avoid cell or lysed cell contamination in the sample.

Safety warnings

1. **Chemical Handling:** Always handle chemicals, including fluorescein diacetate and acetone, in a well-ventilated area while wearing appropriate personal protective equipment (PPE) such as gloves and safety goggles.
2. **Biological Material:** Treat algal cultures as potential biohazards. Follow your laboratory's biosafety procedures when handling and disposing of biological samples.
3. **Centrifuge Safety:** Ensure the centrifuge is balanced before use to avoid equipment damage or injury.
4. **Instrument Safety:** Operate the plate reader and other instruments according to the manufacturer's guidelines to prevent damage and ensure accurate readings.
5. **Waste Disposal:** Dispose of chemical and biological wastes according to your laboratory's waste disposal protocols.

Before start

1. **Algal Culture Sample:** For measuring enzyme activity.
2. **96-Well Clear Plate:** For sample measurement.
3. **Centrifuge:** To separate supernatant from cells.
4. **100 mM Phosphate Buffer (pH 8.0):** For assay preparation.
5. **Fluorescein Diacetate (FDA):** 2 mM solution in acetone.
6. **Plate Reader:** Capable of measuring absorbance at 750 nm, chlorophyll fluorescence at 440/680 nm, and FDA fluorescence at 490/yyy nm.
7. **Acetone:** For FDA solution preparation.
8. **Pipettes and Tips:** For accurate liquid transfer.
9. **Excel Software:** For data analysis.
10. **FITC Standard Curve:** For calculating activity per mol.

Checking cell density

1 Sample 160 µL of culture in a 96-well-clear plate.

2 Measure the absorbance at 750 nm and Chlorophyll fluorescence at 440/680nm. For example, in Infinite® M200 PRO plate reader, set the gain to 100, sensor distance 18000um, and top reading.

Sample preparation 13m

3 Spin down 1 mL of a sample at max speed (e.g., 20000 g) for at least 00:03:00 . 3m

4 Transfer the supernatant to a clean tube or use it quickly (e.g., less than a few minutes ~ 00:10:00) to avoid any cells getting back into the solution. Also, handle samples with care to avoid mixing. 10m

Assay preparation 10m

5 Add 89 µL of Phosphate buffer [M] 100 millimolar (mM) , pH 8 to a well. *The buffer can vary and depends on the enzyme being assayed. PHL7 has max activity at pH 8 and can be assayed even in highly concentrated buffer solutions such as* [M] 1 Molarity (M) *Also, any buffering system works as long as the pH is buffered around pH 8, as Tris. Each enzyme has it is one particularity.*

6 Add 10 µL of sample to the well (e.g., supernatant)

7 Measure the absorbance at 750 nm and Chlorophyll fluorescence at 440/680nm. Step to check cell carried over, RFU - Relative Fluorescence units of >100 in Chlorophyll reading indicates cell or lysate present, which interferes with the activity assay. Values close to 100 can still be informative but need to be checked with care in the analysis step

8 Incubate plate @ 37 °C for 00:10:00 . 10m

Assay performance

9 Set the plate reader to 37 °C prior to the reading activity. Enzymes display different activities in different temperatures, so we need to standardize one and keep it constant. PHL7 actually has an optimum temperature of 70 °C. Typically, plate readers can't handle that temperature.

10 Add 1 µL of FDA [M] 2 millimolar (mM) in acetone to each well. Use multichannel for several wells. FDA concentration can be used in different values. For our assay, it just needs to be in excess so the substrate does not limit the reaction speed. We can be sure it was not by the shape of the kinetic curve. If a plateau is reached, it means the substrate was consumed, and at the steps before the plateau, the reaction was limited by the substrate reducing the apparent enzyme activity in that region of the graph. Acetone's final concentration shouldn't be higher than [M] 1 % (v/v), ideally.

11 Immediately after adding FDA, start reading the activity kinetics RFU FDA . In an Infinite® M200 PRO plate reader set ex/em to 490/520 nm, gain 40, sensor distance 18000um, top reading Kinetic cycle: 1 read every minute for 30 cycles

12

Data Analysis

13 Convert the FDA RFU to √RFU to all data points. The square root is used to transform the data to make it linear. RFU increase in a reaction of order 2, meaning a quadratic function. Probably due to 2 bonds needing to be broken by the enzyme to restore FITC fluorescence.

14 Add the sample name to the Excel file for the specific well

15 Discard results in which a chlorophyll signal higher than 100 was detected in the activity experiment well. Cells containing esterase also generate signal in the experimental setup, interfering with the measurement.

16 Calculate "=Linest" to each sample in Excel

17 Calculate activity per mol by using the FITC standard curve

18 Save the result