Daily notebook

Phase 1: Detailed Day-by-Day Plan (Day 1 – Day 5)

Here is a **detailed breakdown of each day's tasks and procedures** for the first phase (Preparation and Learning). This will ensure that all necessary groundwork is covered before moving into the experimental stages.

Day 1: Study Experimental Procedures and Theory

Objective: Understand the full experimental design and the purpose behind each step.
Morning:

1. **Task: Read background literature** on glyphosate degradation and engineered *E. coli* strains.
    a.
        i. **Resources**: Articles or reviews on glyphosate absorption and degradation in bacteria.
        ii. **Outcome**: Understand the role of phnE1/E2 genes in glyphosate absorption and degradation.
Task: Review protocols for molecular cloning, transformation, and ELISA.
    a.
        i. **Resources**: Lab manuals and online resources for molecular biology techniques.
        ii. **Outcome**: Familiarize yourself with basic procedures such as PCR, restriction enzyme digestion, and transformation.

Afternoon:

1. **Task: Discuss the experimental design** with team members.
    a.
        i. **Steps**:
        ii. Identify key goals for each experiment (gene synthesis, cloning, absorption tests, etc.).
        iii. Break down the experimental process into smaller, manageable tasks for the next phases.
        iv. **Outcome**: Clear understanding of the overall project and experimental flow.
Task: Create a **detailed flowchart** of the experiment.
    a.
        i. **Tools**: Use paper or software like Lucidchart to outline each major step.
        ii. **Outcome**: Visual representation of the entire experiment, from gene cloning to ELISA measurements.

Day 2: Learn Basic Molecular Biology Techniques

Objective: Gain hands-on practice with the basic techniques required for the experiments.

Morning:

1. Task: PCR training session.
    a. i. Steps:
        ii. Prepare a practice PCR reaction (e.g., using a plasmid as the template).
        iii. Run the PCR using the thermocycler.
        iv. Outcome: Understand the steps of PCR and the purpose of each component (template, primers, polymerase, etc.).
2. Task: Gel electrophoresis practice.
    a. i. Steps:
        ii. Prepare an agarose gel.
        iii. Load the PCR product and run the gel.
        iv. Outcome: Gain experience visualizing DNA fragments after PCR.

Afternoon:

1. Task: Plasmid extraction session.
    a. i. Steps:
        ii. Extract plasmid DNA from *E. coli* using a commercial miniprep kit.
        iii. Outcome: Learn how to perform plasmid extraction and measure DNA concentration using a Nanodrop or spectrophotometer.
2. Task: Transformation training.
    a. i. Steps:
        ii. Practice transforming a plasmid into competent *E. coli* cells.
        iii. Plate on selective agar (e.g., LB + ampicillin) to observe the growth of transformed colonies.
        iv. Outcome: Understand the transformation process and antibiotic selection for transformed cells.

Day 3: Prepare Equipment and Materials

Objective: Ensure that all necessary equipment and reagents are ready for the full experiment.

Morning:

1. Task: Prepare LB medium.
    a. i. Steps:
        ii. Weigh out the components for LB medium (tryptone, yeast extract, NaCl) and dissolve in water.
        iii. Autoclave the solution to sterilize it.
        iv. Outcome: Sterile LB broth and agar plates for bacterial growth.

Task: Prepare antibiotics (ampicillin).

a.
   i. Steps:
   ii. Prepare a 50 mg/mL ampicillin stock solution and filter sterilize it.
   iii. Outcome: Ready-to-use antibiotic solution for selection in bacterial cultures.

Afternoon:

1.

Task: Check lab equipment.

a.
   i. Steps:
   ii. Test pipettes for accuracy.
   iii. Make sure the thermocycler, centrifuges, and spectrophotometer are functioning properly.
   iv. Outcome: All equipment is operational and calibrated for the experiments.

Task: Organize reagents.

a.
   i. Steps:
   ii. Label and arrange all reagents (e.g., restriction enzymes, PCR reagents, ELISA kit) in the lab for easy access.
   iii. Outcome: Organized workspace with all reagents in place.

Day 4: Pilot Experiment

Objective: Conduct a small pilot experiment to ensure that all experimental steps can be performed smoothly.

Morning:

1.

Task: Prepare LB agar plates.

a.
   i. Steps:
   ii. Pour sterile LB agar into Petri dishes and allow them to solidify.
   iii. Store plates for future use.
   iv. Outcome: Plates are ready for bacterial culture.

Task: Perform a test transformation.

a.
   i. Steps:
   ii. Use a control plasmid (e.g., pUC19) to transform *E. coli* cells.
   iii. Plate the cells on LB + ampicillin plates.
   iv. Outcome: Transformed colonies should grow on the plates, confirming that the transformation protocol works.

Afternoon:

1.

Task: Test plasmid extraction.

a.
   i. Steps:
   ii. Use a test culture of *E. coli* to extract plasmid DNA.
   iii. Measure the concentration and purity of the DNA.

iv. **Outcome**: Confirm that the plasmid extraction process yields sufficient DNA for future steps.

Task: Run a small PCR.

a.

i. **Steps**:
ii. Set up a PCR reaction with known primers and template.
iii. Run the PCR and analyze the product via gel electrophoresis.
iv. **Outcome**: Verify that the PCR conditions and equipment are working properly.

Day 5: Learn Data Analysis Software

Objective: Learn how to analyze experimental data, particularly for ELISA and enzyme kinetics.

Morning:

1.

Task: Introduction to ELISA data analysis.

a.

i. **Steps**:
ii. Review the steps for running an ELISA assay, including sample preparation, loading, incubation, and reading absorbance.
iii. Learn how to calculate concentration based on standard curves.
iv. **Outcome**: Students understand how to process raw absorbance data and calculate glyphosate concentration.

Task: Software setup and tutorial.

a.

i. **Steps**:
ii. Install and set up data analysis software such as GraphPad Prism or Excel.
iii. Walk through how to input data and generate standard curves.
iv. **Outcome**: Software is ready to use for later data analysis.

Afternoon:

1.

Task: Learn how to analyze enzyme kinetics.

a.

i. **Steps**:
ii. Review enzyme kinetic theory (Michaelis–Menten equation, Km, Vmax).
iii. Set up sample data in GraphPad Prism and fit the data to the Michaelis–Menten model.
iv. **Outcome**: Students can calculate kinetic parameters (Km, Vmax) from experimental data.

Task: Practice with sample data.

a.

i. **Steps**:
ii. Use sample data to practice generating graphs and calculating enzyme activity.
iii. **Outcome**: Students are confident in using the software to analyze real data in future experiments.

Summary of Phase 1:

- A solid understanding of the experimental procedures.
- Practical experience with key techniques such as PCR, plasmid extraction, and transformation.
- All necessary equipment and materials prepared.
- A pilot experiment completed to ensure smooth workflow.
- Basic data analysis skills, ready for later stages of the experiment.

Phase 2: Detailed Day-by-Day Plan (Day 6 – Day 15)

Day 6: Synthesize and Optimize phnE1/E2 Genes

Objective: Receive synthesized genes and prepare for cloning.
Morning:

1.
    a. **Task: Verify receipt of phnE1/E2 gene synthesis.**
        i. **Steps**:
        ii. Check that the synthesized phnE1 and phnE2 genes (ordered from a synthesis company) have been delivered.
        iii. Verify the sequence alignment of the synthesized genes against the reference sequence to confirm accuracy.
        iv. **Outcome**: Confirmed delivery and correct gene sequence for further cloning steps.

Afternoon:

1.
    a. **Task: Prepare plasmid vector (pSB1A3).**
        i. **Steps**:
        ii. Prepare the pSB1A3 vector for cloning by performing a restriction enzyme digest with appropriate enzymes (e.g., EcoRI, XbaI).
        iii. Set up the digestion reaction:
            1. 1 µg pSB1A3 plasmid DNA.
            2. 1 µL of each restriction enzyme.
            3. 5 µL of buffer (10x).
            4. Adjust to 50 µL with nuclease-free water.
        iv. Incubate at 37°C for 1 hour.
        v. **Outcome**: Digested pSB1A3 vector ready for ligation.

Day 7: Clone Genes into pSB1A3 Vector

Objective: Insert the phnE1/E2 genes into the pSB1A3 plasmid vector and prepare for transformation.
Morning:

1.
    a. **Task: Run agarose gel electrophoresis** to verify digestion of pSB1A3 vector.

i. Steps:
ii. Prepare a 1% agarose gel with TAE buffer.
iii. Mix 10 µL of digested vector with 2 µL of loading dye and load onto the gel.
iv. Run the gel at 100V for 30 minutes and visualize the bands under UV light.
v. Outcome: Confirmation of successful plasmid digestion (correct band size for linearized vector).

Afternoon:

1.
a. Task: Set up ligation reaction for phnE1/E2 into pSB1A3.
i. Steps:
ii. Mix the digested pSB1A3 vector and phnE1/E2 insert at a 1:3 molar ratio in a ligation reaction:
1. 50 ng digested vector.
2. 3–fold molar excess of phnE1/E2 insert.
3. 1 µL T4 DNA ligase.
4. 2 µL of ligase buffer (10x).
5. Adjust to 20 µL with water.
iii. Incubate the reaction at 16˚C overnight (or 1 hour at room temperature if time is constrained).
iv. Outcome: Ligation product containing phnE1/E2 genes in the pSB1A3 vector.

Day 8: Transform Plasmid

Objective: Transform the ligated phnE1/E2–pSB1A3 plasmid into *E. coli* BL21.

Morning:

1.
a. Task: Prepare competent cells for transformation.
i. Steps:
ii. Thaw chemically competent *E. coli* BL21 cells on ice.
iii. Mix 10 µL of the ligation product with 50 µL of competent cells in a cold 1.5 mL tube.
iv. Incubate on ice for 30 minutes.
v. Outcome: Cells ready for heat–shock transformation.

Afternoon:

1.
a. Task: Perform heat–shock transformation.
i. Steps:
ii. Heat–shock the cells at 42˚C for 45 seconds, then immediately place them back on ice for 2 minutes.
iii. Add 500 µL of SOC medium to the cells and incubate at 37˚C for 1 hour with shaking.
iv. Plate 100 µL of the transformation mix onto LB + 50 µg/mL ampicillin plates.
v. Incubate the plates overnight at 37˚C.
vi. Outcome: Transformed colonies should appear the next day, indicating successful plasmid uptake.

Day 9: Colony PCR Verification

Objective: Verify the presence of phnE1/E2 genes in transformed colonies through colony PCR.

Morning:

1.
    a. **Task: Pick colonies for PCR verification.**
        i. **Steps**:
        ii. Pick 4–6 colonies from the transformation plates and transfer each to 5 mL LB + ampicillin for overnight culture.
        iii. Use the same colonies to set up colony PCR.
        iv. Prepare PCR mix (per reaction):
            1. 1 µL forward primer (specific to phnE1).
            2. 1 µL reverse primer (specific to phnE2).
            3. 12.5 µL PCR master mix.
            4. 10.5 µL water.
            5. Use a toothpick or pipette tip to transfer a small amount of bacterial colony into the PCR tube.
        v. **Outcome**: PCR ready to verify the presence of phnE1/E2 genes.

Afternoon:

1.
    a. **Task: Run PCR and analyze results.**
        i. **Steps**:
        ii. Perform PCR using the following cycling conditions:
            1. Initial denaturation: 95°C for 3 minutes.
            2. 30 cycles of:
            3. 95°C for 30 seconds.
            4. 56°C for 30 seconds.
            5. 72°C for 1 minute.
            6. Final extension: 72°C for 5 minutes.
        iii. Run the PCR products on a 1% agarose gel and visualize the bands.
        iv. **Outcome**: Successful PCR amplification indicates that the colonies contain the correct phnE1/E2 insert.

Day 10: Plasmid Extraction

Objective: Extract the plasmid DNA from verified colonies for sequencing.

Morning:

1.
    a. **Task: Inoculate colonies for plasmid extraction.**
        i. **Steps**:
        ii. Pick 2–3 positive colonies based on colony PCR results and inoculate them into 5 mL LB + ampicillin.
        iii. Incubate the cultures overnight at 37°C with shaking (180 rpm).
        iv. **Outcome**: Liquid cultures of the positive colonies ready for plasmid extraction.

Afternoon:

1.

a. Task: Perform plasmid miniprep.
    i. Steps:
    ii. Use a commercial plasmid extraction kit to purify the plasmid DNA.
    iii. Measure the concentration and purity of the extracted DNA using a Nanodrop spectrophotometer.
    iv. Outcome: High-quality plasmid DNA (concentration ⩾100 ng/μL, 260/280 ratio ~1.8) for sequencing.

Day 11: Sequencing Verification

Objective: Confirm the correctness of the cloned phnE1/E2 genes through sequencing.
Morning:

1.
    a. Task: Prepare plasmid samples for sequencing.
        i. Steps:
        ii. Submit the purified plasmid samples to a sequencing facility.
        iii. Include forward and reverse sequencing primers for phnE1/E2.
        iv. Outcome: Sequencing samples submitted.

Afternoon:

1.
    a. Task: Analyze sequencing data.
        i. Steps:
        ii. Once sequencing results are received (usually takes 1–2 days), analyze the data by aligning the sequencing results with the reference phnE1/E2 sequences.
        iii. Outcome: Sequencing results confirm the correct insertion and sequence of the phnE1/E2 genes.

Day 12: Strain Growth for Absorption Test

Objective: Prepare the engineered *E. coli* strain for glyphosate absorption testing.
Morning:

1.
    a. Task: Inoculate a starter culture.
        i. Steps:
        ii. Inoculate a single positive colony from Day 9 (or frozen stock) into 50 mL of LB + 50 μg/mL ampicillin.
        iii. Grow the culture overnight at 37°C with shaking (180 rpm).
        iv. Outcome: A large culture of the engineered strain ready for glyphosate absorption testing.

Afternoon:

1.
    a. Task: Prepare media for absorption test.
        i. Steps:
        ii. Prepare LB media containing 80 mg/L glyphosate and 50 μg/mL ampicillin.

iii. Autoclave the media and store at room temperature for the next day's experiment.
iv. Outcome: Media ready for glyphosate absorption experiments.

Day 13: Prepare Media

Objective: Ensure all media and reagents are prepared for absorption and degradation tests.
Morning:

a. Task: Finalize LB + glyphosate media.
i. Steps:
ii. Confirm that all media prepared the previous day is sterile and ready for use.
iii. Prepare additional fresh LB + ampicillin for control

cultures.

1.
a. Outcome: All media is ready for use in the glyphosate absorption experiment.

Afternoon:

1.
a. Task: Prepare glycerol stocks of engineered strain.
i. Steps:
ii. Mix 500 µL of overnight culture with 500 µL of sterile 50% glycerol.
iii. Freeze at −80°C for long-term storage.
iv. Outcome: Frozen stocks of the engineered strain for future use.

Day 14: Initial Glyphosate Absorption Test

Objective: Perform an initial test to measure the engineered strain's ability to absorb glyphosate.
Morning:

1.
a. Task: Set up glyphosate absorption test.
i. Steps:
ii. Dilute the overnight culture 1:100 into 50 mL LB + glyphosate media.
iii. Incubate at 37°C with shaking for 3 hours.
iv. Outcome: Culture ready for sampling and analysis.

Afternoon:

1.
a. Task: Prepare samples for ELISA.
i. Steps:
ii. Take 1 mL samples at the start and after 3 hours.
iii. Centrifuge at 10,000 g for 10 minutes at 4°C.
iv. Collect the supernatant and store at −20°C for ELISA testing.
v. Outcome: Samples ready for glyphosate concentration measurement via ELISA.

Day 15: ELISA Testing

Objective: Measure the glyphosate concentration in the samples using an ELISA kit.
Morning:

1.
    a. Task: Set up ELISA test.
        i. Steps:
        ii. Thaw samples collected from Day 14.
        iii. Prepare glyphosate standards and samples according to the ELISA kit protocol.
        iv. Add standards and samples to the ELISA plate.
        v. Incubate and wash according to the manufacturer's instructions.
        vi. **Outcome**: ELISA reaction set up and incubation underway.

Afternoon:

1.
    a. Task: Analyze ELISA results.
        i. Steps:
        ii. After incubation, add the substrate solution and measure the absorbance at 450 nm.
        iii. Plot the standard curve and calculate glyphosate concentrations in the samples.
        iv. **Outcome**: Glyphosate absorption data obtained, confirming the strain's absorption efficiency.

Summary of Phase 2:

- Successfully cloned the phnE1/E2 genes into the pSB1A3 vector.
- Verified the gene sequence through colony PCR and sequencing.
- Prepared the engineered strain and tested its glyphosate absorption ability using ELISA.
- Collected initial absorption data for analysis.

Phase 3: Detailed Day-by-Day Plan (Day 16 – Day 25)

In this phase, we will conduct **time-course experiments**, optimize experimental conditions, and construct the **phnE1/E2-phnJ and phnO strains** for testing their glyphosate degradation and AMPA degradation capabilities. This phase involves deeper data analysis, adjustments, and troubleshooting to ensure reliable results.

Day 16: Glyphosate Absorption Time-Course Test

Objective: Measure the glyphosate absorption by the engineered strain over time to determine absorption kinetics.
Morning:

1.
    a. Task: Prepare culture for time-course experiment.
        i. Steps:

ii. Inoculate 50 mL of LB + 50 µg/mL ampicillin with the engineered strain (from the glycerol stock made earlier or from a fresh colony).
iii. Grow overnight at 37°C with shaking at 180 rpm to an OD600 of ~0.6.
iv. **Outcome**: Active culture ready for time-course sampling.

Afternoon:

1.
    a. **Task: Start time-course absorption test.**
        i. **Steps:**
        ii. Dilute the overnight culture 1:100 into fresh LB + 80 mg/L glyphosate and 50 µg/mL ampicillin (total 50 mL).
        iii. Incubate at 37°C with shaking.
        iv. Take 1 mL samples every hour for 5 hours.
        v. Centrifuge the samples at 12,000 g for 10 minutes at 4°C and collect the supernatant.
        vi. Store the supernatant at −20°C for ELISA.
        vii. **Outcome**: Time-point samples are ready for ELISA analysis.

Day 17: Data Analysis and Optimization

Objective: Analyze the results from the time-course experiment and adjust experimental conditions to improve glyphosate absorption.

Morning:

1.
    a. **Task: Perform ELISA for time-course samples.**
        i. **Steps:**
        ii. Thaw the collected supernatant samples from Day 16.
        iii. Set up the ELISA test according to the kit instructions.
        iv. Measure absorbance at 450 nm after the colorimetric reaction.
        v. **Outcome**: Absorbance values for each time point are recorded.

Afternoon:

1.
    a. **Task: Analyze time-course data.**
        i. **Steps:**
        ii. Plot the glyphosate concentration over time using GraphPad Prism or Excel.
        iii. Fit the data to an absorption curve to determine the maximum absorption rate and the time to reach saturation.
        iv. Identify areas for optimization (e.g., shaking speed, temperature, concentration of glyphosate).
        v. **Outcome**: Clear analysis of glyphosate absorption over time and identification of key factors for optimization.

Day 18: Repeat Absorption Experiment

Objective: Confirm the absorption results under optimized conditions and ensure reproducibility.

Morning:

1.
    a. Task: Set up optimized absorption experiment.
        i. Steps:
        ii. Based on the Day 17 analysis, adjust the experimental conditions:
            1. Optimize shaking speed (e.g., increase to 200 rpm if the absorption rate was slow).
            2. Adjust temperature if needed (e.g., lower to 30°C if high temperature affected absorption).
            3. Test a slightly higher concentration of glyphosate if the absorption reached saturation too quickly.
        iii. Repeat the absorption test over 5 hours, taking samples hourly.
        iv. Outcome: Samples are prepared for a second round of analysis with optimized conditions.

Afternoon:

1.
    a. Task: Perform ELISA and compare results.
        i. Steps:
        ii. Repeat the ELISA test for the new samples.
        iii. Compare the results with the initial experiment to see if the optimized conditions improved glyphosate absorption.
        iv. Outcome: Data confirms whether optimization was successful and if the absorption efficiency has improved.

Day 19: Construct phnE1/E2-phnJ Strain

Objective: Clone the phnJ gene into the engineered strain to build the phnE1/E2-phnJ strain, which should have enhanced glyphosate degradation ability.

Morning:

1.
    a. Task: Digest phnJ gene and pSB1A3 vector for cloning.
        i. Steps:
        ii. Use restriction enzymes (e.g., EcoRI and SpeI) to digest the phnJ gene fragment and pSB1A3 vector.
        iii. Set up the digestion reaction:
            1. 1 µg of phnJ insert and pSB1A3 plasmid.
            2. 1 µL of each restriction enzyme.
            3. 5 µL of digestion buffer.
            4. Add water to make up a 50 µL reaction.
        iv. Incubate at 37°C for 1 hour.
        v. Outcome: Both the insert and vector are prepared for ligation.

Afternoon:

1.
    a. Task: Ligation and transformation.
        i. Steps:
        ii. Perform ligation of the phnJ gene into the digested pSB1A3 vector:
            1. 50 ng of digested vector.
            2. 3-fold molar excess of the phnJ insert.

3. 1 µL of T4 DNA ligase.
4. 2 µL of ligation buffer.
5. Add water to make a 20 µL reaction.

iii. Incubate at room temperature for 1 hour.
iv. Transform the ligation product into competent *E. coli* BL21 cells.
v. Plate on LB + 50 µg/mL ampicillin and incubate overnight at 37°C.
vi. Outcome: Transformed colonies containing the phnE1/E2–phnJ plasmid should appear the next day.

Day 20: Test Glyphosate Degradation Ability

Objective: Test whether the phnE1/E2–phnJ strain has enhanced glyphosate degradation capability.

Morning:

1.
   a. Task: Verify presence of phnJ via colony PCR.
      i. Steps:
      ii. Pick several colonies from the transformation plate and perform colony PCR using primers specific to phnJ.
      iii. Run PCR with the following cycling conditions:
         1. 95°C for 3 minutes.
         2. 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute.
         3. Final extension at 72°C for 5 minutes.
      iv. Run the PCR products on an agarose gel.
      v. Outcome: PCR bands confirm the presence of the phnJ gene in selected colonies.

Afternoon:

1.
   a. Task: Set up glyphosate degradation experiment.
      i. Steps:
      ii. Inoculate the verified phnE1/E2–phnJ strain into LB + 50 µg/mL ampicillin.
      iii. Grow the culture overnight.
      iv. Dilute 1:100 into LB + glyphosate (80 mg/L) and incubate for 5 hours.
      v. Collect samples at the start and end of the incubation period.
      vi. Prepare samples for ELISA to measure glyphosate degradation.
      vii. Outcome: Samples are collected for analysis of glyphosate degradation.

Day 21: Construct phnO Strain

Objective: Clone the phnO gene into *E. coli* BL21 to construct the phnO strain for testing AMPA degradation.

Morning:

1.
   a. Task: Clone phnO into the pET23b vector.
      i. Steps:

ii. Digest the phnO gene and pET23b vector using EcoRI and XhoI restriction enzymes.
iii. Set up the digestion reactions as done previously (Day 19).
iv. Perform a ligation reaction with the digested phnO insert and pET23b vector.
v. Transform the ligated product into competent *E. coli* BL21 cells.
vi. Plate on LB + 50 µg/mL ampicillin.
vii. Incubate overnight at 37°C.
viii. Outcome: Transformed colonies containing phnO should grow on selective plates.

Afternoon:

1.
    a. Task: Prepare for phnO AMPA degradation test.
        i. Steps:
        ii. Inoculate a starter culture of the phnO strain from one of the colonies.
        iii. Grow overnight at 37°C with shaking.
        iv. Outcome: A culture of the phnO strain is ready for testing its AMPA degradation activity.

Day 22: AMPA Degradation Test

Objective: Test whether the phnO strain can degrade AMPA by measuring enzyme activity.

Morning:

a. Task: Prepare crude enzyme extract from phnO strain.
    i. Steps:
    ii. Grow the phnO strain in LB + ampicillin until OD600 reaches ~0.6.
    iii. Harvest the cells by centrifugation at 10,000 g for 10 minutes at 4°C.
    iv. Resuspend the cell pellet in 25 mM Tris–HCl buffer (pH 8.0) with 0.3 M NaCl, 10 mM imidazole, and 1 mM EDTA.
    v. Lyse the cells by

sonication (1 second on, 3 seconds off, for 10 minutes).

1.
    a. Centrifuge at 13,000 g for 20 minutes to collect the supernatant containing the crude enzyme extract.
        i. Outcome: Crude enzyme extract is ready for the AMPA degradation assay.

Afternoon:

1.
    a. Task: Set up AMPA degradation reaction.
        i. Steps:
        ii. Prepare a reaction mixture containing:
            1. 1 mM AMPA.
            2. 3 mM acetyl–CoA.
            3. 3 mM magnesium chloride.
            4. 100 µL of the crude enzyme extract.
            5. Incubate the reaction at 37°C for 3 hours.

iii. After incubation, add DTDP solution (to measure CoA production) and incubate for 30 minutes at room temperature.
iv. Measure absorbance at 324 nm.
v. **Outcome**: Absorbance data will indicate AMPA degradation activity by the phnO enzyme.

Day 23: Repeat AMPA Degradation Test

Objective: Ensure reproducibility of the AMPA degradation results.

Morning:

1.
   a. **Task: Prepare new enzyme extracts.**
      i. **Steps**:
      ii. Grow another culture of the phnO strain to ensure fresh enzyme activity.
      iii. Repeat the steps from Day 22 to prepare the crude enzyme extract.
      iv. **Outcome**: Fresh enzyme extracts prepared for the degradation assay.

Afternoon:

1.
   a. **Task: Perform another AMPA degradation assay.**
      i. **Steps**:
      ii. Set up the same reaction as on Day 22, using fresh enzyme extracts.
      iii. Measure CoA production using the DTDP method and absorbance at 324 nm.
      iv. **Outcome**: Confirm the reproducibility of AMPA degradation activity by comparing data from both assays.

Day 24: Enzyme Activity Test

Objective: Measure the kinetic parameters (Km and Vmax) of the phnO enzyme for AMPA degradation.

Morning:

1.
   a. **Task: Set up enzyme kinetics experiment.**
      i. **Steps**:
      ii. Prepare a series of AMPA concentrations (e.g., 0.1 mM, 0.5 mM, 1 mM, 2 mM, 5 mM).
      iii. Set up reactions as before, using crude enzyme extract from the phnO strain.
      iv. Incubate the reactions at 37°C for 3 hours.
      v. Measure CoA production using the DTDP method.
      vi. **Outcome**: Absorbance data for various concentrations of AMPA.

Afternoon:

1.
   a. **Task: Analyze enzyme kinetics data.**
      i. **Steps**:
      ii. Plot the reaction rate (absorbance at 324 nm) against the AMPA concentration.
      iii. Use software like GraphPad Prism to fit the data to the Michaelis–Menten model and calculate Km and Vmax.

iv. Outcome: Kinetic parameters (Km and Vmax) are determined, providing insight into the enzyme's efficiency.

Day 25: Data Analysis and Report Writing

Objective: Analyze the full set of data from glyphosate and AMPA degradation tests and begin writing the experimental report.

Morning:

1.
    a. Task: Analyze all data collected.
        i. Steps:
        ii. Compile all data from the glyphosate absorption and degradation tests.
        iii. Organize the results of the enzyme kinetics experiments for phnO.
        iv. Plot graphs and calculate statistical significance where necessary.
        v. Outcome: Organized and analyzed data ready for the report.

Afternoon:

1.
    a. Task: Begin report writing.
        i. Steps:
        ii. Start writing the "Results" section of the report, including key findings from the absorption and degradation tests.
        iii. Discuss any challenges encountered and the optimizations that were made.
        iv. Outcome: A draft of the results section is completed, along with preliminary discussions of the data.

Summary of Phase 3:

- Conducted time-course experiments and optimized glyphosate absorption conditions.
- Constructed the phnE1/E2-phnJ and phnO strains.
- Tested both strains for glyphosate and AMPA degradation.
- Measured enzyme kinetics for the phnO enzyme.
- Compiled and analyzed the experimental data, ready for reporting.

Phase 4: Detailed Day-by-Day Plan (Day 26 – Day 30)

Day 26: Construct Cold-Inducible Reporter Strain

Objective: Clone the cold-inducible PcspA promoter and mRFP gene into the pSB1A3 plasmid to construct a temperature-sensitive reporter strain.

Morning:

1.
    a. Task: Digest PcspA promoter and mRFP gene.
        i. Steps:

ii. Digest the PcspA promoter and mRFP gene with appropriate restriction enzymes (e.g., XbaI and SpeI).
iii. Prepare the digestion reactions:
1. 1 µg of the PcspA promoter and mRFP insert.
2. 1 µL of each restriction enzyme.
3. 5 µL of digestion buffer.
4. Add water to make up a 50 µL reaction.
iv. Incubate at 37°C for 1 hour.
v. **Outcome**: Digested PcspA and mRFP are ready for ligation.

Afternoon:

1.

Task: Ligation of PcspA–mRFP into pSB1A3 vector.

a.
i. **Steps**:
ii. Set up a ligation reaction:
1. 50 ng of digested pSB1A3 vector.
2. 3-fold molar excess of the PcspA–mRFP insert.
3. 1 µL of T4 DNA ligase.
4. 2 µL of ligation buffer (10x).
5. Add water to make a 20 µL reaction.
iii. Incubate at room temperature for 1 hour.
iv. **Outcome**: Ligation reaction is completed, preparing for transformation.

Task: Transform the ligation product into competent *E. coli* BL21.

a.
i. **Steps**:
ii. Transform the ligated plasmid into competent *E. coli* BL21 cells.
iii. Plate on LB + ampicillin plates (50 µg/mL) and incubate overnight at 37°C.
iv. **Outcome**: Transformed colonies containing the cold-inducible reporter plasmid should appear the next day.

Day 27: Cold-Inducible Reporter Test

Objective: Test the cold-inducible PcspA–mRFP system by measuring mRFP expression at different temperatures.

Morning:

1.

Task: Verify the presence of PcspA–mRFP via colony PCR.

a.
i. **Steps**:
ii. Pick several colonies from the transformation plates and perform colony PCR using primers specific to PcspA and mRFP.
iii. Set up the PCR reaction and run the program as done previously.
iv. Analyze the PCR products on an agarose gel.
v. **Outcome**: PCR verification confirms the presence of the PcspA–mRFP construct in selected colonies.

Task: Inoculate positive colonies into LB media for fluorescence testing.

a.

i. Steps:
ii. Pick 2–3 positive colonies and inoculate them into 5 mL of LB + 50 μg/mL ampicillin.
iii. Incubate overnight at 37°C with shaking at 180 rpm.
iv. Outcome: Cultures are ready for fluorescence testing.

Afternoon:

1.
   a. Task: Perform cold-inducible reporter test at different temperatures.
      i. Steps:
      ii. Dilute the overnight culture 1:100 into fresh LB media and incubate at three different temperatures (16°C, 25°C, and 37°C).
      iii. Take 200 μL samples at 0 hours and after 12 hours of incubation.
      iv. Measure the fluorescence intensity (excitation at 584 nm, emission at 607 nm) using a microplate reader.
      v. Measure OD600 to normalize the fluorescence data (Fluorescence/OD600).
      vi. Outcome: Fluorescence data for the PcspA-mRFP system at different temperatures is recorded.

Day 28: Construct Cold-Inducible Suicide System

Objective: Clone the cold-inducible PcspA promoter and mazF toxin gene into the pSB1A3 plasmid to create a cold-inducible suicide system.

Morning:

1.
   a. Task: Digest PcspA promoter and mazF gene.
      i. Steps:
      ii. Digest the PcspA promoter and mazF gene with appropriate restriction enzymes (e.g., XbaI and SpeI).
      iii. Prepare the digestion reactions similarly to previous cloning steps.
      iv. Incubate the reaction at 37°C for 1 hour.
      v. Outcome: Digested PcspA and mazF are ready for ligation.

Afternoon:

1.
   a. Task: Ligation and transformation of PcspA-mazF.
      i. Steps:
      ii. Perform the ligation reaction using the digested PcspA promoter and mazF insert.
      iii. Transform the ligated product into competent *E. coli* BL21 cells.
      iv. Plate on LB + 50 μg/mL ampicillin and incubate overnight at 37°C.
      v. Outcome: Colonies containing the PcspA-mazF construct should grow on selective media.

Day 29: Suicide System Test

Objective: Test the functionality of the cold-inducible suicide system by measuring cell survival at low temperatures.

Morning:

1.

Task: Verify the presence of PcspA–mazF via colony PCR.

a.

i. Steps:

ii. Pick several colonies from the transformation plate and perform colony PCR using primers specific to PcspA and mazF.

iii. Run the PCR and analyze the products on an agarose gel.

iv. Outcome: Confirmation of the PcspA–mazF construct in selected colonies.

Task: Set up cultures for suicide system testing.

a.

i. Steps:

ii. Pick 2–3 positive colonies and inoculate them into 5 mL of LB + ampicillin.

iii. Incubate overnight at 37°C with shaking.

iv. Outcome: Cultures are ready for testing the cold–induced suicide mechanism.

Afternoon:

1.

a. Task: Perform the cold–inducible suicide test.

i. Steps:

ii. Dilute the overnight culture 1:100 into 5 mL LB media containing ampicillin.

iii. Incubate one set of cultures at 37°C (control) and another at 16°C (induced condition).

iv. After 12 hours of incubation, take 200 µL samples and measure OD600 to assess cell growth and survival.

v. Outcome: Data on cell survival under cold temperatures, which should indicate a decrease in OD600 at 16°C due to the expression of the mazF toxin.

Day 30: Final Report Writing and Presentation Preparation

Objective: Compile all experimental data, write the final report, and prepare presentation materials for showcasing the project.

Morning:

1.

a. Task: Compile and analyze final data.

i. Steps:

ii. Gather all the experimental results from the previous phases (glyphosate absorption, AMPA degradation, cold–inducible reporter and suicide tests).

iii. Analyze and summarize the key findings from each test, including graphs, statistical analyses, and conclusions.

iv. Outcome: All data is compiled and ready for inclusion in the report.

Afternoon:

1.

Task: Finalize the experimental report.

a.

i. Steps:

ii. Complete the ”Discussion” and ”Conclusion” sections of the report, interpreting the significance of the results.

iii. Highlight any experimental challenges, optimizations, and future directions for research.
iv. Outcome: The final version of the report is completed.

Task: Prepare presentation materials.

a.
i. Steps:
ii. Create slides for a PowerPoint or Google Slides presentation, summarizing the project's goals, methods, results, and conclusions.
iii. Include key data visuals (graphs, images of gels, ELISA results, etc.) to support the findings.
iv. Rehearse the presentation to ensure clarity and flow.
v. Outcome: Presentation slides and report are ready for the final project presentation.

Summary of Phase 4:

- Constructed and tested the cold-inducible PcspA-mRFP reporter system and the cold-inducible PcspA-mazF suicide system.
- Collected data on the functionality of these systems under different temperature conditions.