Daily record

Day 1 (July 1): Preparation of Plasmids

Goal:

- Clone the synthetic **INP-PBP** and **PelB-PBP** sequences into the **pET23b plasmid** for further transformation into *E. coli* DH5α and BL21 strains.

Steps:

1. **Plasmid Preparation**: Obtain the **pET23b** plasmid, linearize it using **NdeI** and **XhoI** restriction enzymes.
2. **Gene Insert**: Insert the codon-optimized **INP-PBP** and **PelB-PBP** sequences into the linearized plasmid via **ligation**.
3. **Transformation into DH5α**:
   a. Transform the ligated plasmids into *E. coli* DH5α competent cells via **heat shock**.
   b. Plate the transformed cells on LB agar plates with **Ampicillin (50 μg/mL)** for selection.

Expected Results:

- Colonies should appear after overnight incubation, indicating successful transformation of DH5α with the recombinant plasmids.

Day 2 (July 2): Transformation and Colony Selection

Goal:

- Select and verify transformed *E. coli* DH5α colonies containing the recombinant **INP-PBP** and **PelB-PBP** plasmids.

Steps:

1. **Colony PCR**: Pick individual colonies from the LB agar plates and perform **colony PCR** to verify the presence of the recombinant plasmids.
2. **PCR Analysis**:
   a. Use specific primers for **INP-PBP** and **PelB-PBP** to amplify target sequences.
   b. Run the PCR products on an **agarose gel** and visualize bands to confirm successful cloning.

Expected Results:

- PCR results should show bands of expected size for **INP-PBP** and **PelB-PBP**, confirming the presence of the target genes in the selected colonies.

Day 3 (July 3): Plasmid Extraction and Sequencing

Goal:

- Extract plasmids from the positive colonies and send them for sequencing to verify the correct sequence of **INP-PBP** and **PelB-PBP** constructs.

Steps:

1. **Plasmid Extraction**:
   a. Use a plasmid extraction kit (e.g., Tiangen) to extract the plasmids from confirmed DH5α colonies.
   b. Quantify the plasmid concentration using a **Nanodrop spectrophotometer**.
2. **Sequencing**: Send the extracted plasmids for **Sanger sequencing** to confirm the correct gene insertion.

Expected Results:

- Plasmid yields should be sufficient (>100 ng/μL) for sequencing. Sequencing should confirm the correct integration of **INP-PBP** and **PelB-PBP** constructs in the plasmids.

Day 4 (July 4): Transformation into BL21

Goal:

- Transform the verified plasmids into *E. coli* **BL21** for protein expression.

Steps:

1. **Heat Shock Transformation**:
   a. Transform the confirmed **INP-PBP** and **PelB-PBP** plasmids into **BL21** competent cells.
   b. Incubate the transformed cells on LB agar plates with **Ampicillin** for selection.
2. **Incubation**: Allow colonies to grow overnight at **37°C**.

Expected Results:

- Colonies should grow on the Ampicillin plates, indicating successful transformation of BL21 with the recombinant plasmids.

Day 5 (July 5): Initial Culturing of BL21 Strains

Goal:

- Begin culturing *E. coli* BL21 strains containing **INP-PBP** and **PelB-PBP** to prepare for phosphate adsorption experiments.

Steps:

1. **Inoculation**:
   a. Pick individual colonies from the transformation plates and inoculate them into **LB liquid medium** containing Ampicillin (50 μg/mL).
2. **Incubation**: Grow the cultures overnight at **37°C**, shaking at **250 rpm** to ensure optimal growth.

Expected Results:

- The bacterial cultures should turn cloudy, indicating successful growth of the BL21 strains expressing **INP-PBP** and **PelB-PBP**.

Day 6 (July 6): Plasmid Extraction from BL21 Strains

Goal:

- Extract plasmids from the transformed **BL21** strains to verify the stability of the recombinant plasmids.

Steps:

1. **Plasmid Extraction**:
   a. Use a plasmid extraction kit to isolate the plasmids from the BL21 cultures.
   b. Measure the plasmid concentration using a **Nanodrop spectrophotometer**.
2. **Plasmid Check**: Perform **restriction enzyme digestion** (NdeI and XhoI) and run on an **agarose gel** to check the integrity of the plasmids.

Expected Results:

- Sufficient plasmid yield and correct size bands on the gel should confirm the integrity of the plasmids in BL21 strains.

Day 7 (July 7): Initial Phosphate Adsorption Test for INP-PBP

Goal:

- Perform the first **phosphate adsorption test** for the **INP-PBP** engineering strain.

Steps:

1. **Culture Preparation**:
   a. Adjust the bacterial culture to an **OD600 of 1** in **Tris-HCl buffer** (pH 7.4).
   b. Add **10 mg/L KH2PO4** to the solution.
2. **Incubation**: Incubate the culture at **room temperature**, shaking at **250 rpm** for **3 hours**.
3. **Phosphate Detection**: After incubation, measure the remaining phosphate concentration in the supernatant using the **Malachite Green Phosphate Detection Kit**.

Expected Results:

- Phosphate adsorption should be observed, with a decrease in phosphate concentration in the solution.

Day 8 (July 8): Phosphate Adsorption Test for PelB-PBP

Goal:

- Perform the same phosphate adsorption experiment as the previous day, but for the **PelB-PBP** strain.

Steps:

1. **Culture Preparation**:
   a. Adjust the **PelB-PBP** bacterial culture to **OD600=1**.
   b. Add **10 mg/L KH2PO4** to the solution.
2. **Incubation**: Incubate at **room temperature** (250 rpm, 3 hours).
3. **Phosphate Detection**: Measure the phosphate concentration using the **Malachite Green Phosphate Detection Kit**.

Expected Results:

- Phosphate adsorption should be evident, allowing for comparison with the results of **INP-PBP**.

Day 9 (July 9): Initial Data Analysis

Goal:

- Analyze the phosphate adsorption data from both **INP-PBP** and **PelB-PBP** strains.

Steps:

1. **Data Entry**:
   a. Enter the phosphate concentration data into **GraphPad Prism** for analysis.
2. **Comparison**: Compare the adsorption capacities of both strains based on the change in phosphate concentration.
3. **Statistical Test**: Perform a **t-test** to check if there are significant differences between the adsorption capacities of the two strains.

Expected Results:

- A preliminary understanding of which strain shows better adsorption efficiency under standard conditions.

Day 10 (July 10): Optimization of Adsorption (pH Testing for INP-PBP)

Goal:

- Begin optimizing the phosphate adsorption for **INP-PBP** by testing its adsorption efficiency at different **pH levels**.

Steps:

1. **Buffer Preparation**:
   a. Prepare buffers of **pH 3, 5, 7, 8, and 10** (using acetic acid for pH 3 and 5, PBS for pH 7, and Tris-HCl for pH 8 and 10).
2. **Adsorption Experiment**:
   a. Adjust the **OD600** of the bacterial culture to 1 and suspend it in the different buffers.
   b. Add **10 mg/L KH2PO4** and incubate at room temperature, shaking at **250 rpm for 3 hours**.

3. **Phosphate Detection**: Measure phosphate concentrations after incubation using the **Malachite Green Kit**.

Expected Results:

- Identify the pH level at which **INP-PBP** exhibits the highest phosphate adsorption.

Day 11 (July 11): Optimization of Adsorption (pH Testing for PelB-PBP)

Goal:

- Perform the same pH optimization experiment for **PelB-PBP**, similar to the INP-PBP strain.

Steps:

1. **Buffer Preparation**:
   a. Prepare buffer solutions at different **pH levels (3, 5, 7, 8, 10)** using appropriate buffers (acetic acid for pH 3 and 5, PBS for pH 7, Tris-HCl for pH 8 and 10).
2. **Adsorption Experiment**:
   a. Adjust the **OD600** of **PelB-PBP** culture to 1.
   b. Add **10 mg/L KH2PO4** to the buffer solutions and incubate for **3 hours at 250 rpm** at room temperature.
3. **Phosphate Detection**: Measure the phosphate concentrations in the supernatant after incubation using the **Malachite Green Phosphate Detection Kit**.

Expected Results:

- Compare the phosphate adsorption capacities at different pH levels to identify the optimal pH for **PelB-PBP**.

Day 12 (July 12): Optimization of Adsorption (Temperature Testing for INP-PBP)

Goal:

- Optimize the adsorption efficiency of the **INP-PBP** strain by testing at different **temperatures**.

Steps:

1. **Temperature Adjustment**:
   a. Prepare **Tris-HCl buffer (pH 7.4)** and adjust the **reaction temperature** to **25°C, 35°C, and 45°C** using temperature-controlled shakers.
2. **Adsorption Experiment**:
   a. Adjust the **OD600** of the bacterial culture to 1, suspend it in the buffer, and add **10 mg/L KH2PO4**.
   b. Incubate the culture for **3 hours at each temperature** (shaking at **250 rpm**).
3. **Phosphate Detection**: Measure phosphate concentration in the supernatant using the **Malachite Green Phosphate Detection Kit**.

Expected Results:

- Determine the optimal temperature for phosphate adsorption in **INP-PBP**. Higher temperatures may enhance adsorption but could also cause denaturation.

Day 13 (July 13): Optimization of Adsorption (Temperature Testing for PelB-PBP)

Goal:

- Perform the same temperature optimization experiment for **PelB-PBP**, as done with INP-PBP.

Steps:

1. **Temperature Setup**:
   a. Prepare **Tris-HCl buffer (pH 7.4)** and adjust the temperature to **25°C, 35°C, and 45°C**.
2. **Adsorption Experiment**:
   a. Adjust the **OD600** of **PelB-PBP** culture to 1, add **10 mg/L KH2PO4**, and incubate for **3 hours** at each temperature (250 rpm).
3. **Phosphate Detection**: Use the **Malachite Green Kit** to measure the remaining phosphate in the solution.

Expected Results:

- Identify the temperature that provides the highest phosphate adsorption for **PelB-PBP**.

Day 14 (July 14): Initial Phosphate Desorption Study for INP-PBP

Goal:

- Evaluate the **phosphate desorption capacity** of the **INP-PBP** strain under different pH and temperature conditions.

Steps:

1. **Culture Preparation**:
   a. Culture **INP-PBP** strains overnight and adjust to **OD600=1** in **Tris-HCl buffer (pH 7.5)**.
   b. Saturate the bacteria with phosphate by incubating in **150 mg/L KH2PO4** for **3 hours**.
2. **Desorption Experiment**:
   a. Wash the bacteria twice with buffer (10 mM Tris-HCl, 1 mM MgCl2, pH 7.5) to remove unbound phosphate.
   b. Incubate the phosphate-saturated bacteria in desorption buffers of varying **pH (3, 5, 7, 8, 10)** and **temperatures (25°C, 35°C, 45°C)** for **3 hours at 250 rpm**.
3. **Phosphate Detection**: Use the **Malachite Green Kit** to measure the phosphate concentration in the supernatant after desorption.

Expected Results:

- Observe how pH and temperature affect phosphate release from the INP-PBP bacteria. Higher temperatures and more extreme pH values (e.g., pH 3) may promote desorption.

Day 15 (July 15): Phosphate Desorption Study for PelB-PBP

Goal:

- Perform the same phosphate desorption study for the **PelB-PBP** strain, similar to INP-PBP.

Steps:

1. **Culture Preparation**:
    a. Adjust the **PelB-PBP** culture to **OD600=1** and saturate with **150 mg/L KH_2PO_4** for 3 hours.
    b. Wash the bacteria to remove unbound phosphate.
2. **Desorption Experiment**:
    a. Incubate the phosphate-saturated cells in desorption buffers at different **pH levels (3, 5, 7, 8, 10)** and temperatures (25°C, 35°C, 45°C).
3. **Phosphate Detection**: Measure phosphate release with the **Malachite Green Kit**.

Expected Results:

- Evaluate the effect of different desorption conditions on **PelB-PBP**. Compare results with those from the **INP-PBP** strain.

Day 16 (July 16): Isolation of Cytoplasmic and Membrane Components of INP-PBP

Goal:

- Separate the **cytoplasmic** and **membrane** components of **INP-PBP** engineering bacteria and analyze their respective phosphate binding capacities.

Steps:

1. **Bacterial Culture**: Grow **50 mL of INP-PBP bacteria** overnight and harvest the cells via **centrifugation** at 10,000 rpm for 10 minutes.
2. **Cell Lysis**:
    a. Re-suspend the pellet in **10 mL PBS** and lyse the cells using **ultrasonication** (150W, 1 second on, 3 seconds off, for 20 minutes).
3. **Component Separation**:
    a. Centrifuge the lysate at **5,000 rpm for 10 minutes** to remove debris, and then at **39,000 rpm for 1 hour** to separate the membrane fraction (pellet) from the cytoplasmic fraction (supernatant).
    b. Collect both fractions separately for further testing.
4. **Phosphate Adsorption Test**: Test the adsorption capacity of the cytoplasmic and membrane components using **Malachite Green Kit**.

Expected Results:

- Determine whether the **membrane component** or **cytoplasmic component** contributes more to phosphate adsorption.

Day 17 (July 17): Isolation of Soluble and Periplasmic Components of PelB-PBP

Goal:

- Perform similar component isolation for **PelB-PBP**, focusing on **soluble** and **periplasmic** fractions.

Steps:

1. **Bacterial Culture**: Grow **50 mL of PelB-PBP bacteria** overnight and harvest cells by centrifugation.
2. **Periplasmic Extraction**:
   a. Re-suspend the bacterial pellet in **TES buffer** and incubate for 10 minutes.
   b. Centrifuge at 10,000×g and collect the **periplasmic component** (supernatant).
3. **Soluble Component Extraction**:
   a. Lyse the bacteria using **sonication** to collect the **soluble cytoplasmic component**.
4. **Phosphate Adsorption Test**: Test the phosphate adsorption ability of each component.

Expected Results:

- Compare phosphate adsorption between the **periplasmic** and **soluble components** of **PelB-PBP**. Expect the periplasmic fraction to have higher adsorption, as the PelB signal directs proteins to this region.

Day 18 (July 18): Data Analysis for Adsorption and Desorption Tests

Goal:

- Analyze data from the **adsorption** and **desorption** experiments for both **INP-PBP** and **PelB-PBP** strains.

Steps:

1. **Data Entry**: Enter all phosphate concentration data from adsorption and desorption experiments into **GraphPad Prism.**

Statistical Tests:

1.
   a. Use **one-way ANOVA** to determine the significance of pH and temperature effects on adsorption/desorption.
   b. Perform **post hoc Tukey's tests** to compare specific groups.

Graph Plotting: Plot graphs for **phosphate adsorption** as a

1. function of **pH** and **temperature** for both strains.

Expected Results:

- Significant differences between different conditions (pH and temperature), with optimal conditions clearly identified.

Day 19 (July 19): Replication of Key Experiments

Goal:

- Repeat key adsorption and desorption experiments to verify reproducibility of results under optimal conditions.

Steps:

1. **Adsorption**: Perform additional adsorption experiments for both strains at **pH 7** and **35°C** (optimal conditions identified).
2. **Desorption**: Repeat desorption at **pH 7.5** and **45°C** for both strains.
3. **Phosphate Detection**: Measure phosphate adsorption/desorption using **Malachite Green Kit**.

Expected Results:

- Reproducibility of the previously identified optimal conditions.

Day 20 (July 20): Final Analysis and Experimental Adjustments

Goal:

- Conduct a final analysis of all experiments and make any necessary adjustments for ongoing or future experiments.

Steps:

1. **Final Data Analysis**: Complete the statistical analysis and confirm the key findings.
2. **Troubleshooting**: If certain experiments (e.g., desorption) did not yield consistent results, plan for **troubleshooting steps** or method adjustments.
3. **Planning**: Prepare for the next set of experiments based on the data gathered.

Expected Results:

- Finalized understanding of the optimal conditions for phosphate adsorption and desorption, with clear next steps for any further experiments.

Day 21 (July 21): Optimization of Phosphate Adsorption and Desorption for INP-PBP

Goal:

- Fine-tune and verify the phosphate adsorption and desorption properties of **INP-PBP** under optimal conditions.

Steps:

1. **Culture Preparation**:
   a. Grow **INP-PBP** strains in LB + Amp (50 μg/mL) at **37°C, 250 rpm** overnight.

   b. Adjust the culture to **OD600=1** using **Tris-HCl buffer (pH 7.5)**.
2. **Adsorption Experiment**:
   a. Add **10 mg/L KH2PO4** to the culture and incubate for **3 hours** at the optimized temperature (**35°C**).
3. **Desorption Experiment**:
   a. After adsorption, perform the desorption step by suspending phosphate-saturated bacteria in buffer at **45°C** and **pH 7.5** for **3 hours**.
4. **Phosphate Measurement**: Use **Malachite Green Phosphate Detection Kit** to measure phosphate levels after adsorption and desorption.

Expected Results:

- Final verification of phosphate adsorption and desorption capacities at optimized conditions, confirming **pH 7.5** and **35-45°C** as ideal for adsorption and desorption.

Day 22 (July 22): Optimization of Phosphate Adsorption and Desorption for PelB-PBP

Goal:

- Repeat and verify the phosphate adsorption and desorption experiments for **PelB-PBP** at optimal conditions.

Steps:

1. **Culture Preparation**: Grow **PelB-PBP** strains overnight at **37°C, 250 rpm** in LB + Amp (50 µg/mL).
2. **Adsorption**: Adjust the culture to **OD600=1** and suspend in **Tris-HCl buffer (pH 7.5)**. Add **10 mg/L KH2PO4** and incubate at **35°C** for **3 hours**.
3. **Desorption**: Transfer the phosphate-saturated bacteria to a **desorption buffer (pH 7.5)** and incubate at **45°C** for **3 hours**.
4. **Phosphate Measurement**: Measure phosphate adsorption and desorption using the **Malachite Green Kit**.

Expected Results:

- Similar to INP-PBP, optimal adsorption and desorption conditions should be confirmed at **pH 7.5** and temperatures between **35°C - 45°C**.

Day 23 (July 23): Immobilization of INP-PBP on Sepharose Beads

Goal:

- Perform **immobilization** of INP-PBP onto **NHS-Activated Sepharose beads** to investigate the possibility of creating a reusable phosphate-capturing system.

Steps:

1. **Immobilization Setup**:
   a. Prepare fresh **NHS-Activated Sepharose 4 Fast Flow beads** by washing them with **1 mM HCl**.

   b. Mix **INP-PBP enzyme solution** (20 mL) with the washed NHS beads and incubate for **16 hours at 4°C, 30 rpm**.
2. **Bead Washing**: Wash the immobilized beads three times with **Tris-HCl (pH 7.5)** to remove unbound protein.
3. **Phosphate Adsorption Test**:
   a. Test the adsorption capacity of the **INP-PBP immobilized beads** by incubating them in a **10 mg/L KH2PO4 solution** at **room temperature (25°C)** for **10 minutes**.
4. **Phosphate Measurement**: Use **Malachite Green Kit** to measure the phosphate concentration in the supernatant.

Expected Results:

- The immobilized INP-PBP should retain its phosphate adsorption capacity, enabling the beads to act as a reusable phosphate-capturing system.

Day 24 (July 24): Immobilization of PelB-PBP on Sepharose Beads

Goal:

- Perform the same **immobilization experiment** for **PelB-PBP** on Sepharose beads as conducted for INP-PBP.

Steps:

1. **Immobilization**: Mix **PelB-PBP enzyme solution** (20 mL) with washed **NHS-Activated Sepharose beads** and incubate for **16 hours at 4°C, 30 rpm**.
2. **Washing**: Wash the immobilized beads three times with **Tris-HCl (pH 7.5)**.
3. **Phosphate Adsorption Test**:
   a. Incubate **PelB-PBP beads** in **10 mg/L KH2PO4** at **25°C** for **10 minutes** to test phosphate adsorption.
4. **Phosphate Measurement**: Measure phosphate concentration in the supernatant using the **Malachite Green Kit**.

Expected Results:

- **PelB-PBP immobilized beads** should demonstrate effective phosphate adsorption, providing a comparison with the INP-PBP system.

Day 25 (July 25): Phosphate Desorption Test for Immobilized INP-PBP

Goal:

- Test the ability of the **INP-PBP immobilized beads** to release phosphate under different desorption conditions.

Steps:

1. **Saturation and Washing**:
   a. Saturate **INP-PBP beads** with phosphate by incubating them in **150 mg/L KH2PO4** at **25°C**.

    b. Wash the beads three times with **Tris-HCl buffer (pH 7.5)** to remove unbound phosphate.
2. **Desorption Experiment**:
    a. Incubate the beads in buffers of varying **pH (3, 5, 7, 8, 10)** and **temperatures (25°C, 35°C, 45°C)** for **10 minutes** to assess phosphate release.
3. **Phosphate Measurement**: Measure the amount of phosphate released from the beads using the **Malachite Green Kit**.

Expected Results:

- Optimal desorption conditions should be identified, with **higher temperatures** and **extreme pH values** likely promoting phosphate release.

Day 26 (July 26): Phosphate Desorption Test for Immobilized PelB-PBP

Goal:

- Perform the same **phosphate desorption test** for **PelB-PBP immobilized beads** as conducted for INP-PBP.

Steps:

1. **Saturation and Washing**:
    a. Saturate **PelB-PBP beads** with phosphate and wash them to remove unbound phosphate.
2. **Desorption**: Incubate the beads in different **pH** and **temperature** conditions to test phosphate release.
3. **Phosphate Measurement**: Use the **Malachite Green Kit** to quantify phosphate released from the beads.

Expected Results:

- **PelB-PBP beads** should show effective phosphate release, allowing for comparison with **INP-PBP beads**.

Day 27 (July 27): Comparison of Adsorption Efficiency Between Free and Immobilized PBPs

Goal:

- Compare the phosphate adsorption efficiency of **free** INP-PBP and PelB-PBP in solution with the **immobilized PBPs** on Sepharose beads.

Steps:

1. **Adsorption Comparison**:
    a. Test the adsorption of **free** INP-PBP and PelB-PBP in solution using **10 mg/L KH_2PO_4** under optimal conditions (pH 7.5, 35°C).
    b. Repeat the adsorption test with **immobilized PBPs** under the same conditions.
2. **Phosphate Measurement**: Measure the remaining phosphate in solution after adsorption for both free and immobilized forms using the **Malachite Green Kit**.

Expected Results:

- **Immobilized PBPs** may exhibit slightly reduced adsorption compared to free PBPs due to steric hindrance, but immobilization offers the advantage of reusability.

Day 28 (July 28): Statistical Analysis and Data Visualization

Goal:

- Conduct comprehensive statistical analysis and visualize the results from adsorption, desorption, and immobilization experiments.

Steps:

1. **Data Entry**: Organize all phosphate concentration data from adsorption, desorption, and immobilization tests.
2. **Statistical Tests**:
    a. Perform **ANOVA** to assess the impact of different conditions (pH, temperature) on adsorption and desorption efficiency.
    b. Use **Tukey's post hoc test** for pairwise comparison of groups.
3. **Data Visualization**: Plot adsorption/desorption curves and bar graphs comparing free and immobilized PBP efficiency.

Expected Results:

- Significant differences between conditions should be evident, with clear identification of optimal conditions for both free and immobilized PBP systems.

**Day

29 (July 29)**: *Experimental Review and Troubleshooting*

Goal:

- Review the experimental results, troubleshoot any issues, and plan for potential reruns or method adjustments.

Steps:

1. **Review Results**: Analyze the overall findings from the phosphate adsorption, desorption, and immobilization experiments.
2. **Troubleshooting**: If any data appears inconsistent or unclear, plan for **replicating experiments** under controlled conditions.
3. **Method Adjustments**: Identify any procedural weaknesses and make adjustments for future experiments if necessary.

Expected Results:

- Clarification of any experimental inconsistencies and a clear plan for potential reruns.

Day 30 (July 30): Final Report Preparation and Conclusion

Goal:

- Summarize the experimental findings, prepare a report, and finalize the results for presentation to the evaluation committee.

Steps:

1. **Summary of Findings**:
   a. Prepare a comprehensive summary of the phosphate adsorption and desorption capacities of both **INP-PBP** and **PelB-PBP**, along with the results from the immobilization experiments.
2. **Conclusions**:
   a. Conclude which conditions (pH, temperature) are optimal for phosphate capture and release for each strain.
   b. Discuss the advantages and limitations of **immobilized PBP** systems.
3. **Report Preparation**: Compile all data, analysis, and conclusions into a formal report for presentation.

Expected Results:

- A clear, well-organized report that presents the experimental data and conclusions, highlighting the success of the **INP-PBP** and **PelB-PBP** engineering systems for phosphate adsorption.