April

6th April

Our team was officially established.

9th April

We held our first team meeting and, through brainstorming, preliminarily determined the project theme: Microbial Production of Colored Cellulose Fibers.

10th~15th April

We searched if any previous iGEM teams had completed similar projects, and if there were any projects we could reference. We conducted extensive research on literature related to microbial fibers and microbial dyes. We also explored existing commercial products related to microbial fibers. All of the above confirmed the feasibility and significance of our project.

16th April

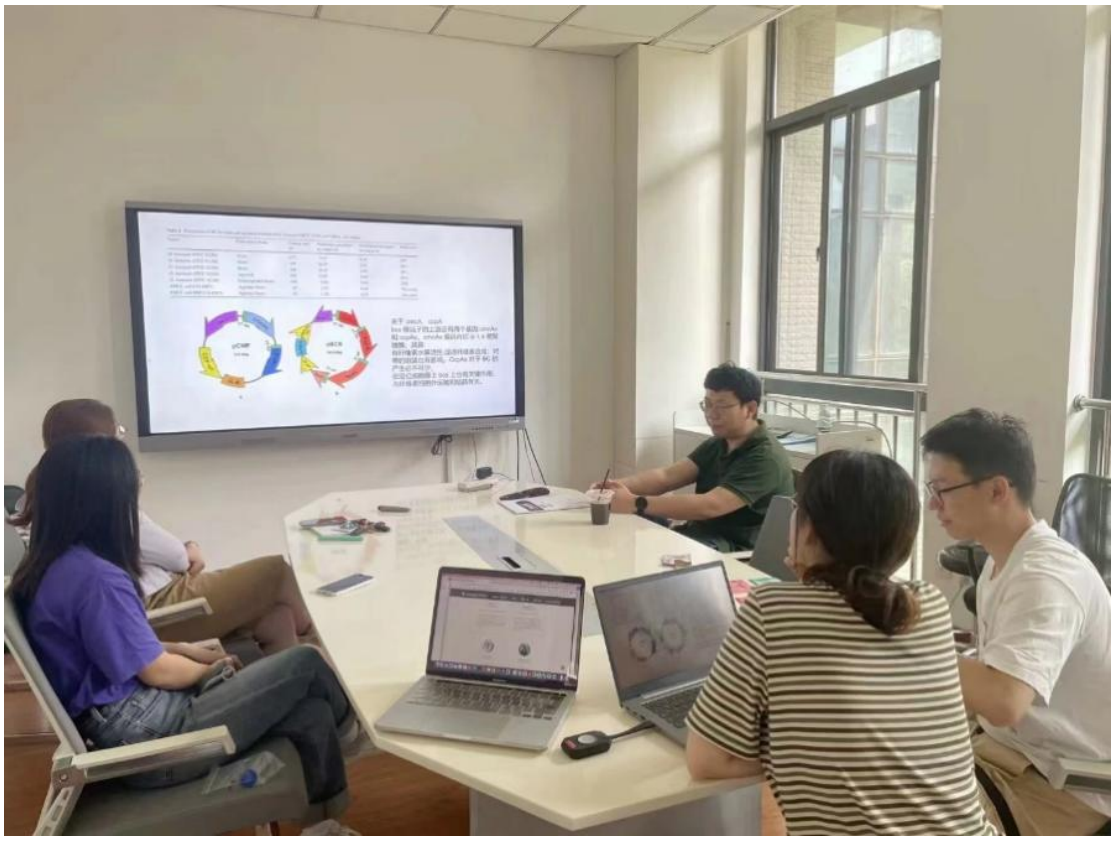
We held a team meeting to discuss the findings of our literature research, which deepened our understanding of the project theme.

17th~26th April

We continued our literature search, contemplating ways to enhance the properties of microbial cellulose, such as waterproofing and programmability. Simultaneously, we gathered reference sequences for Parts from previous iGEM projects related to our theme.

27th April

We had a discussion with our PI regarding the project's progress, and we outlined the next steps in our plan: researching plasmids for cellulose production and exploring suitable host cells.



May

3rd~14th May

We started learning some basic experiment techniques from senior students in the lab. Meanwhile, we conducted literature reviews to gain insights into bacterial cellulose yield, properties, cultivation methods, and engineering modifications. We also studied the crystallographic structure of cellulose enzymes, as well as the whole-genome sequencing results of cellulose-producing bacteria.

18th May

We held a meeting to discuss the strains and gene sequences, and the meeting confirmed the use of K. xylinus as the host cell.

19th~30th May

We conducted literature reviews and compiled information on genes related to cellulose synthesis and yield improvement. Our focus was on studying methods for enhancing cellulose production through genetic engineering.

June

2nd~4th June

We completed and submitted the safety form.

6th~24th June

We successfully constructed a plasmid system containing indigo synthase (bpsA) in *Corynebacterium glutamate*. After several days of cultivation, we obtained the expected indigoidine dye.

● Preparation of Linearized Vectors

We linearized the plasmid vector using a double enzyme digestion method.
Prepared according to the following enzyme digestion system.

Kpn1	1 μl
EcoR1	1 μl
pEKEX2	2 μg
10×Buffer	5 μl
ddH_2O	to 50 μl

● Preparation of Inserts

We performed codon optimization on EGFP(YFP) and bpsA, and then entrusted the synthesis to a company.
The subsequent PCR amplification of the target gene was prepared according to the following system.

Template	10ng
2×Phanta	25 μl
Primer F	2 μl
Primer R	2 μl
ddH2O	to 50μl

● Recombination

1. Dilute the vector and insert at an appropriate ratio to ensure the accuracy of pipetting before preparing the recombination reaction system, and the amount of each component is not less than 1 μl.
2. Prepare the following reaction on ice:

Components	Recombination	Negative control-1[b]	Negative control-2[c]	Positive control[d]
Linearized Vector[a]	X μl	X μl	0 μl	1 μl
Insert[a]	Y_1 - Y_n μl	0 μl	Y_1 - Y_n μl	1 μl
5 × CE MultiS Buffer	4 μl	0 μl	0 μl	4 μl
Exnase MultiS	2 μl	0 μl	0 μl	2 μl
ddH_2O	to 20 μl	to 20 μl	to 20 μl	to 20 μl

3. Gently pipette up and down for several times to mix thoroughly (DO NOT VORTEX!). Briefly centrifuge to collect the reaction solution to the bottom of the tube.
4. Incubate at 37℃ for 30 min and immediately chill the tube at 4℃ or on ice.

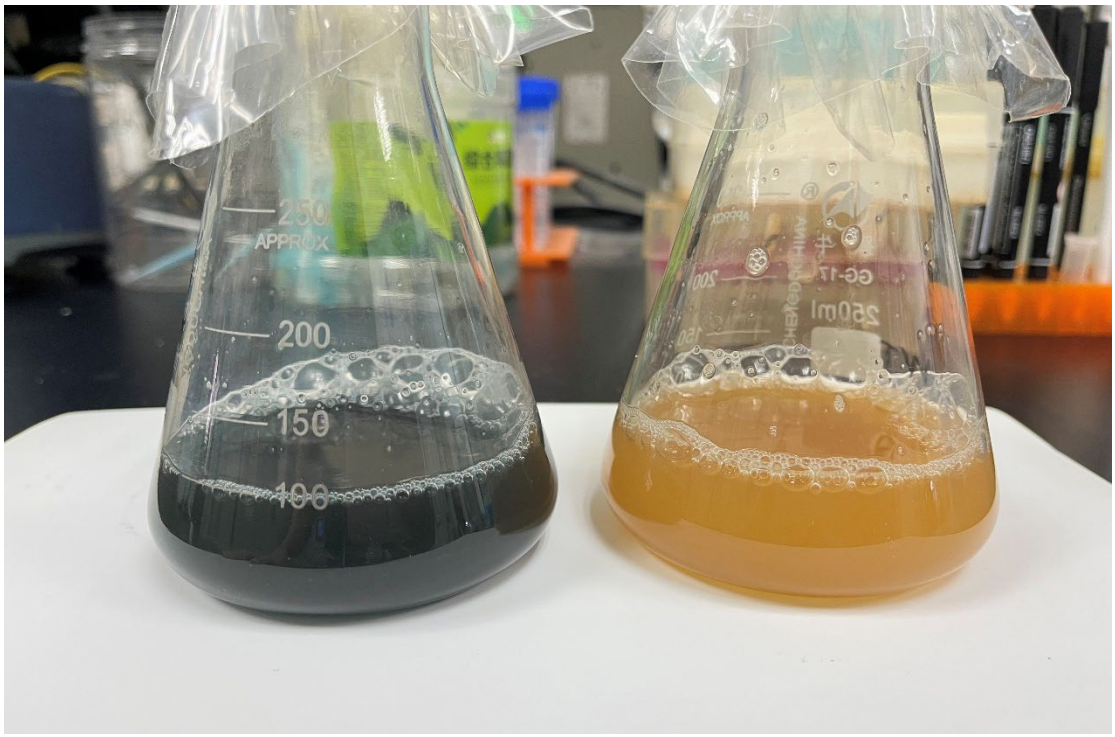
- **Transformation into competent cells**

1. Thaw the competent cells on ice (e.g., DH5α Competent Cell, Vazyme #C502).
2. Pipette 10 µl of the recombination products to 100 µl of competent cells, flick the tube wall to mix thoroughly (DO NOT VORTEX!), and then place the tube on ice for 30 min.
3. Heat shock at 42℃ water bath for 45 sec and then immediately place on ice for 2 - 3 min.
4. Add 900 µl of SOC or LB liquid medium (without antibiotics). Then, shake at 37℃ for 1 h at 200 - 250 rpm.
5. Preheat the corresponding resistant LB solid medium plates in a 37℃ incubator.
6. Centrifuge the culture at 5,000 rpm (2,400 × g) for 5 min, discard 900 µl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile bent glass rod to gently spread on the plate which contains the appropriate selection antibiotic.
7. Incubate at 37℃ for 12 - 16 h.

- **Extract the plasmid from bacteria culture.**

✧ Use FastPure Plasmid Mini Kit (DC201) to extract the plasmid.

1. Take out approximately 10ml of bacterial culture containing PSB plasmid, centrifuge at around 8000rpm for 3 minutes, and pour off the supernatant.
2. Add 500µl of Buffer P1 to the centrifuge tube containing the bacterial pellet. Vortex to mix.
3. Add 500µl of Buffer P2 to each tube and gently invert the tubes 8-10 times until the solution becomes viscous and clear, indicating complete bacterial lysis.
4. Add 700µl of Buffer P3 to each tube and gently invert the tubes 8-10 times to thoroughly neutralize P2. A white flocculent precipitate will form.
5. Centrifuge at approximately 9500rpm for 10 minutes.
6. Place the adsorption column in a 2ml collection tube. Pass the supernatant through the column in three equal portions, approximately 600µl each time. Centrifuge at 12000rpm for 30s, discard the waste liquid in the tube, and place the adsorption column back into the collection tube.
7. Add 600µl of Buffer PW2 to the column, centrifuge at 12000rpm for 30s, and discard the waste.
8. Repeat step 7.
9. Centrifuge at 12000rpm for 1 minute to remove any residual wash liquid from the adsorption column.
10. Dry the adsorption column in a 65°C metal bath for 10-15 minutes. Heat up the H_2O that will be used for elution at the same time.
11. Prepare the new 1.5ml EP tubes and label them. Place the adsorption column into each tube and add 50µl of H_2O to the center of the column membrane. Sit at room temperature for 2 minutes, then centrifuge at 12000rpm for 1 minute to elute the DNA.



Indigoidine secreted by IPTG-induced/pTac-ind. *C. glutamicum* was induced with 0.1 mM IPTG at 30 °C for 5 h. The indigoidine secreted into the culture shows a deep blue color.
As shown above, the right conical flask shows the fermentation results after introducing empty PEKEX2 into the *C. glutamicum*, whereas the left conical flask shows the fermentation results of indigoidine production after introducing bpsA plasmid into *C. glutamicum*.

27th~28th June

We drafted the project description and uploaded it to the wiki.

July

1st July

We obtained K. xylinus ATCC 700178 from Nanjing Tech University.

2nd~9th July

Based on literature, we identified the initial culture medium for K. xylinus as HS medium. Additionally, we actively reached out to Huazhong University of Science and Technology, Jiangnan University, and other iGEM teams for advice on culturing *K.*

xylinus. After one week of cultivation, we observed that the growth of *K. xylinus* was mediocre, indicating that there was significant room for improvement.

- **Prepare HS culture medium.**

1. Rinse the graduated cylinder and the large beaker three times with distilled water.
2. Prepare HS culture medium according to the following formula.

Glucose	20 g/L
Peptone	5 g/L
Yeast extract	5 g/L
Na_2HPO_4 (Sodium hydrogen phosphate)	2.7 g/L
Citric acid	1.15 g/L

Since the amount of glucose is relatively large, it can be directly weighed in a beaker; the rest should be weighed using weighing paper.

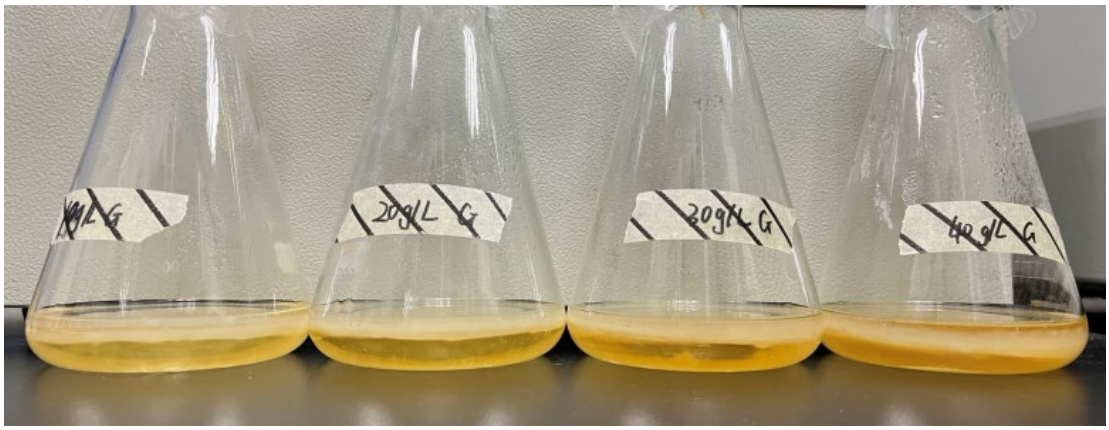
3. Add water to reach 800-900ml.
4. Place it on a magnetic stirrer, stir, then transfer it to a graduated cylinder. Add water to reach 1L and pour it back into the beaker.
5. Briefly sonicate the solution to make it clear.
6. Distribute into two conical flasks, each with 100ml, and store the remaining 800ml in a glass bottle.
7. Autoclave at 115°C (including glucose, so it cannot exceed 115°C, as it may caramelize) for 20 minutes.

10th~13rd July

The dry lab team explored bacterial cellulose production under various carbon source concentrations and ethanol supplementation, thus determining the optimal conditions for cultivating K. xylinus.

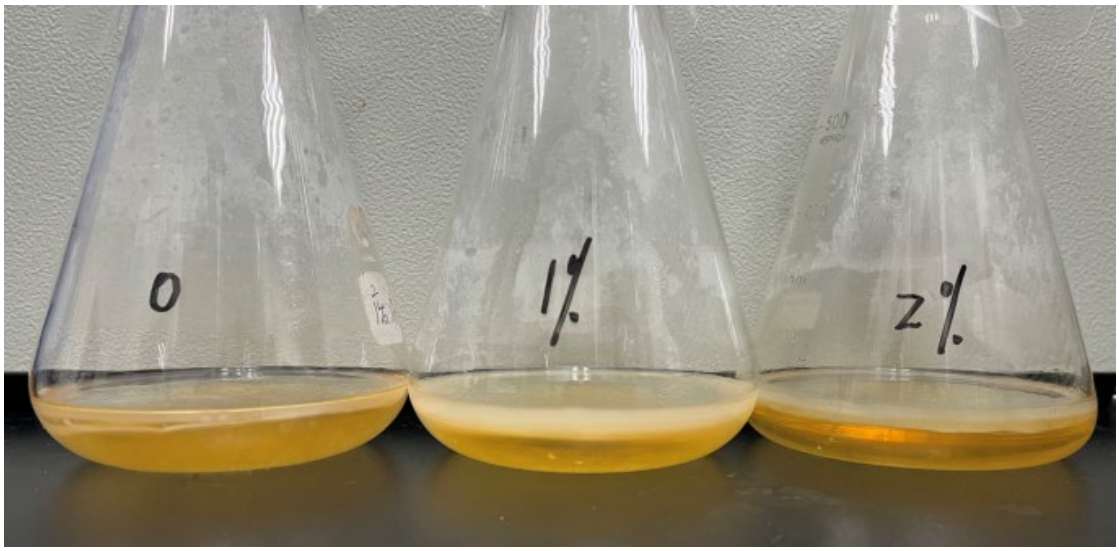
1. Carbon source (glucose) concentrations in the culture medium: Four concentrations, namely 10, 20, 30, and 40g/L, were selected. A 100 mL culture medium was inoculated, and static cultivation was conducted at 30°C for 6 days to obtain a complete BC membrane. After washing the membrane and removing the bacterial cells, it was dried to a constant weight and weighed.

Glucose concentration（g/L）	BC yield（g BC membrane/L culture medium）
10	2.4
20	9
30	8.3
40	6.2



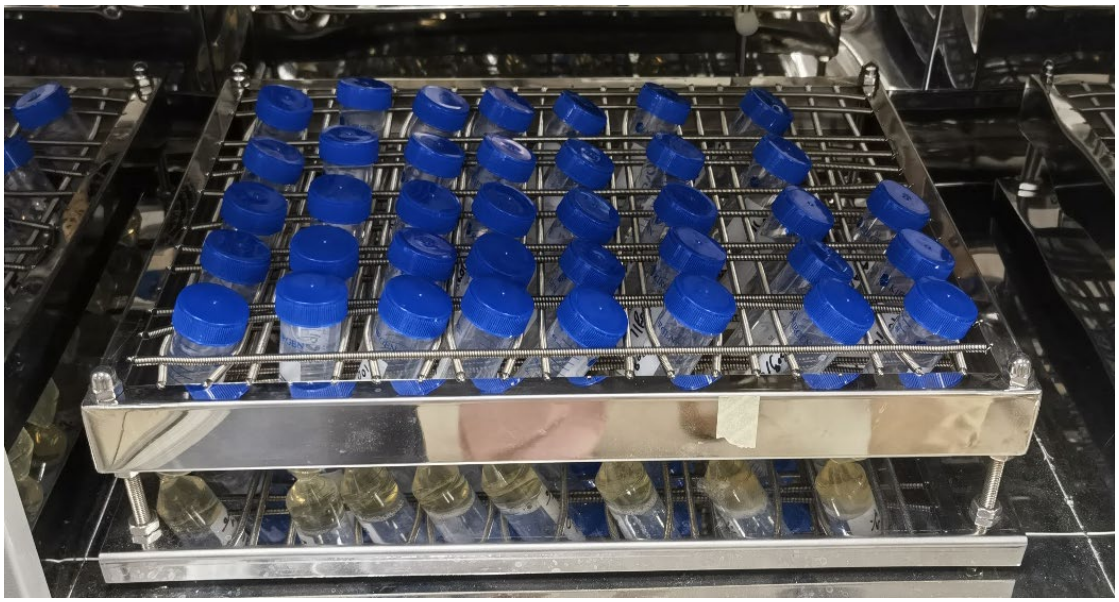
2. Ethanol supplementation in the culture medium: Three supplementation levels, namely 0%, 1%, and 2% (v/v), were selected. A 100 mL culture medium was inoculated, and static cultivation was conducted at 30°C for 6 days to obtain a complete BC membrane. After washing the membrane and removing the bacterial cells, it was dried to a constant weight and weighed.

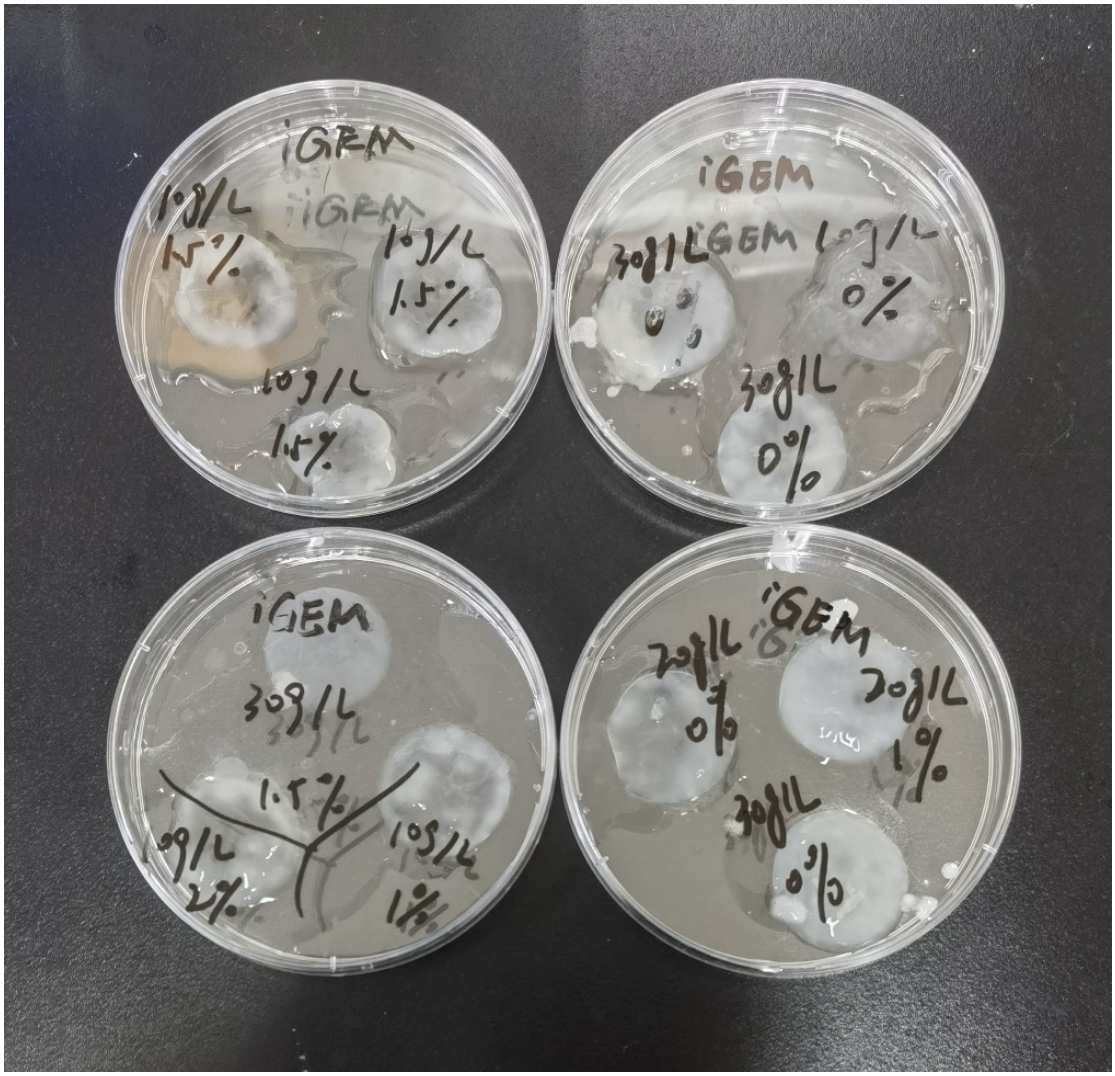
Ethanol supplementation（v/v）	BC yield（g BC membrane/L culture medium）
0%	0.5
1%	10.4
2%	4.8



14th~23rd July

The dry experiment group needs to obtain more BC yield data under different concentrations of glucose and ethanol to simulate the optimal conditions for K. xylinus more accurately. Therefore, the wet experiment group inoculated K. xylinus at different concentrations of glucose (10%, 20%, 30%, 40%) and ethanol (0%, 1%, 1.5%, 2%) respectively. The BC dry weight was measured after 48 hours, 72 hours, and 96 hours of cultivation.





24th~31st July

Based on the analysis of the BC dry weight data collected by the dry experiment group, the optimal conditions for acetobacter xylinum cultivation were determined to be a glucose concentration of 30-35% and an ethanol concentration of 0-0.5%.

August

1st~7th August

We consulted Synmetabio company online for their expertise in *K. xylinus* cultivation. According to the results obtained by the dry lab team, we improved the cultivation conditions for *K. xylinus*. This led to a significant enhancement in the cultivation performance, and *K. xylinus* exhibited robust growth.

- **Prepare a cellulose stock solution and sterilize it by filtration.**

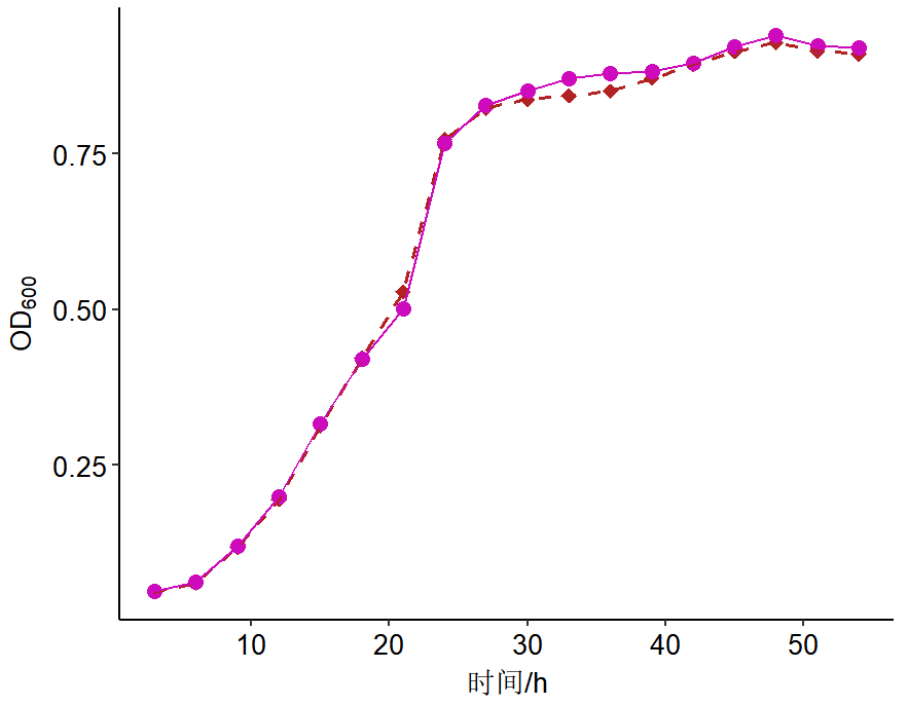
The original cellulase we bought from the company has a concentration of 115 U/mg. Based on previous experiments, a concentration of 3 U/ml is suitable for culturing K. xylinus. However, it is generally necessary to prepare a concentrated solution first and then dilute it in a 1000:1 ratio for use.
Therefore, this time we need to prepare a solution with a concentration of 3000 U/ml, which, when converted to "mg/ml" units, is:
3000 U/ml * (1 mg/115 U) = approximately 26.09 mg/ml
For this preparation of 5 ml of stock solution, we need to weigh 130.5 mg (0.1305 g) of cellulase enzyme.

After sterilization by filtration, divide the solution into 5 tubes, with each tube containing 1ml. Since cellulase requires protection from light, wrap aluminum foil around the EP tubes. Store 4 tubes at -20°C for freezing, and keep 1 tube at 4°C for immediate use.

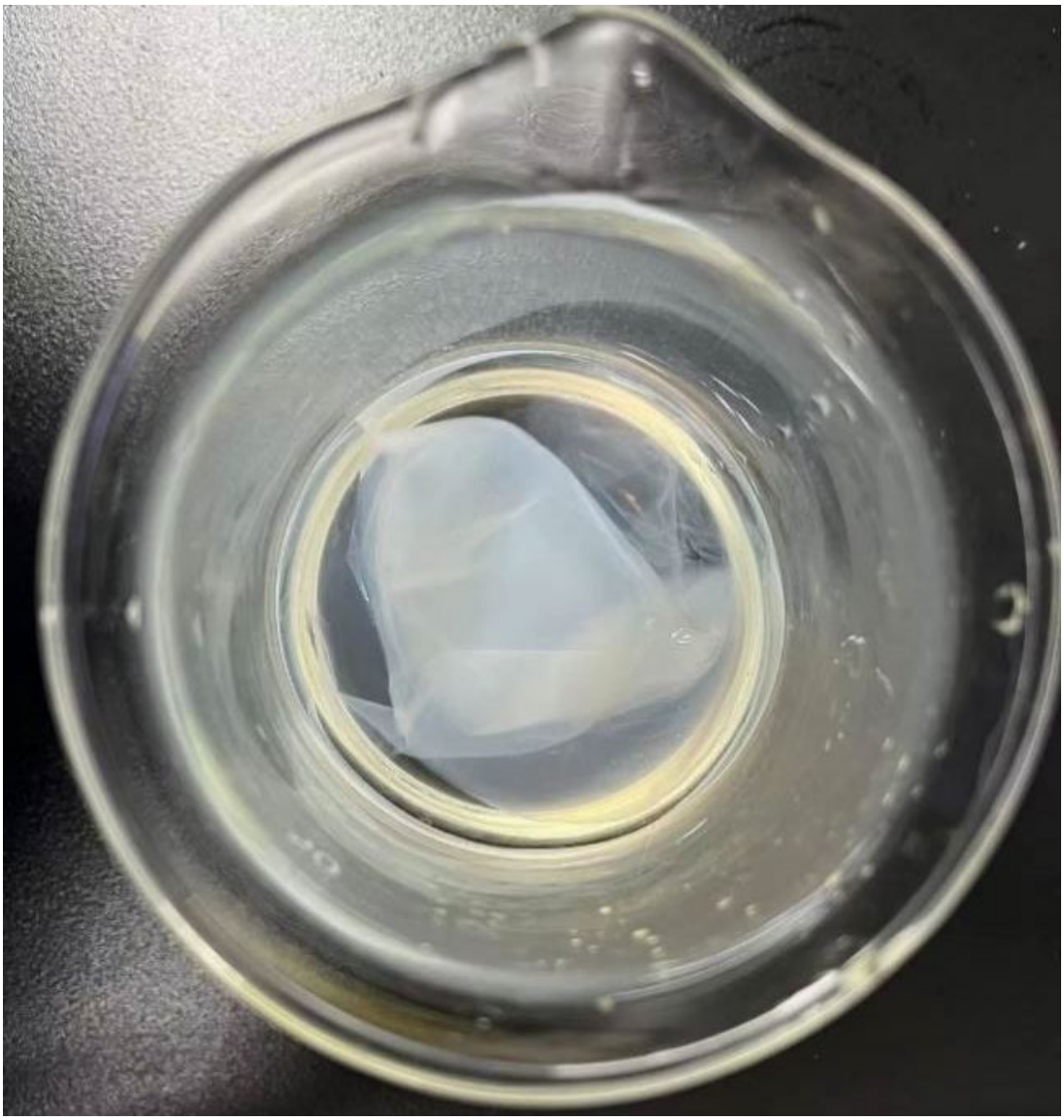
- **Measure the *K. xylinus* growth curve**

1. Take 3 sterilized 250 ml Erlenmeyer flasks, labeled 1, 2, and 3, and add 50 ml of HS culture solution respectively
2. Set the initial OD value of the bacterial solution to 0.02.
3. Add cellulase in the bottle at 1:1000, respectively
4. Seal the Erlenmeyer flask, put it into a shaker for culture, and take out 3-4ml of bacterial solution every 3 hours to measure the OD value.

The growth curve of K. xylinus is as follows.



The cellulose membrane produced by K. xylinus is as follows.



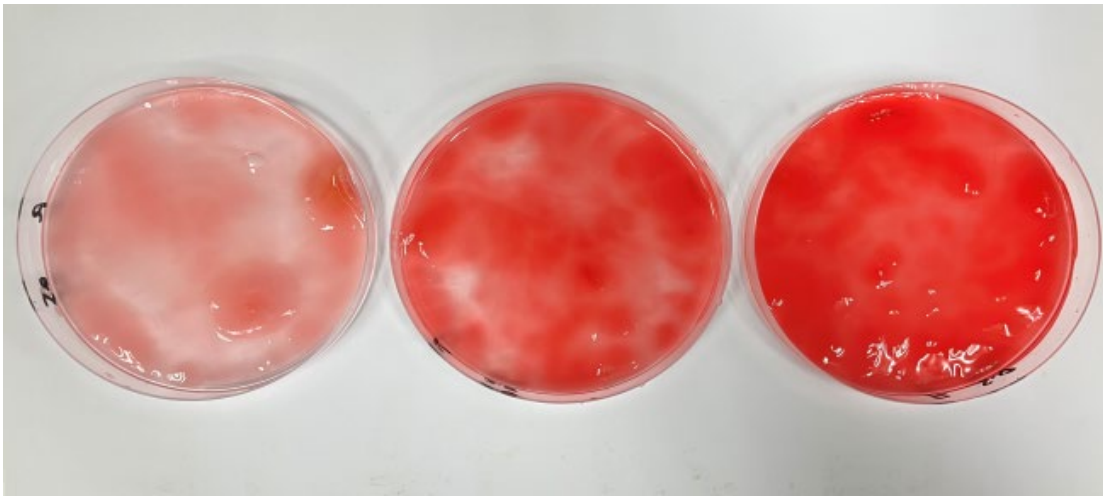
8th~15th August

We investigated the capability of bacterial cellulose to bind water-soluble dyes and found that the binding effect was quite satisfactory.

1. Binding of water-soluble dye, carmine, with BC membrane: Different concentrations of carmine dye were added to a 20 mL culture medium containing BC membrane. The mixture was statically cultured for 6 days. Afterward, the BC membrane was removed and washed repeatedly with water until it no longer released color. A 0.5g piece of the membrane was cut and placed in 3 mL of water. Then, 30 μL of cellulase solution with a concentration of 3000 U/mL was added to degrade the membrane, resulting in a suspension. The suspension obtained after BC degradation without dye served as the blank control. Absorbance at 508nm (the maximum absorption wavelength of carmine) was measured.

Carmine concentration（mg/mL）	A508
0.5	0.698
2	1.115
5	1.435
10	Complete BC membrane did not grow.

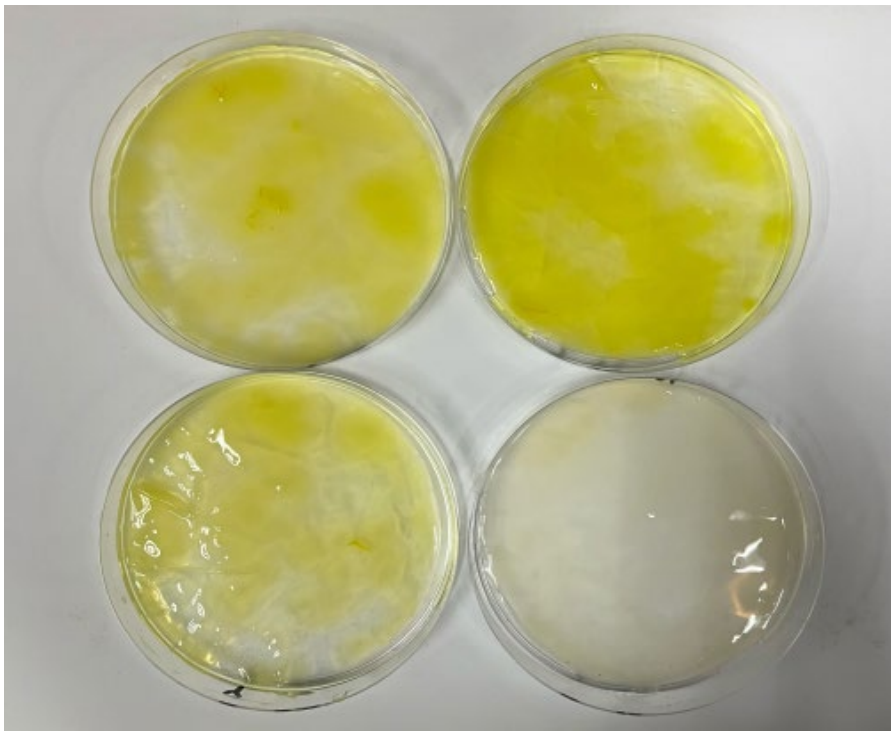
Reference data: A508=1.326 for a 0.05mg/mL carmine aqueous solution.



2. Binding of water-soluble dye, Tartrazine, with BC membrane: Different concentrations of Tartrazine dye were added to a 20 mL culture medium. The mixture was statically cultured for 6 days. Afterward, the BC membrane was removed and washed repeatedly with water until it no longer released color. A 0.5g piece of the membrane was cut and placed in 3 mL of water. Then, 30 μL of cellulase solution with a concentration of 3000 U/mL was added to degrade the membrane, resulting in a suspension. The suspension obtained after BC degradation without dye served as the blank control. Absorbance at 428nm (the maximum absorption wavelength of Tartrazine) was measured.

Tartrazine concentration（mg/mL）	A428
0.5	0.325
2	1.064
5	0.853
10	0.303

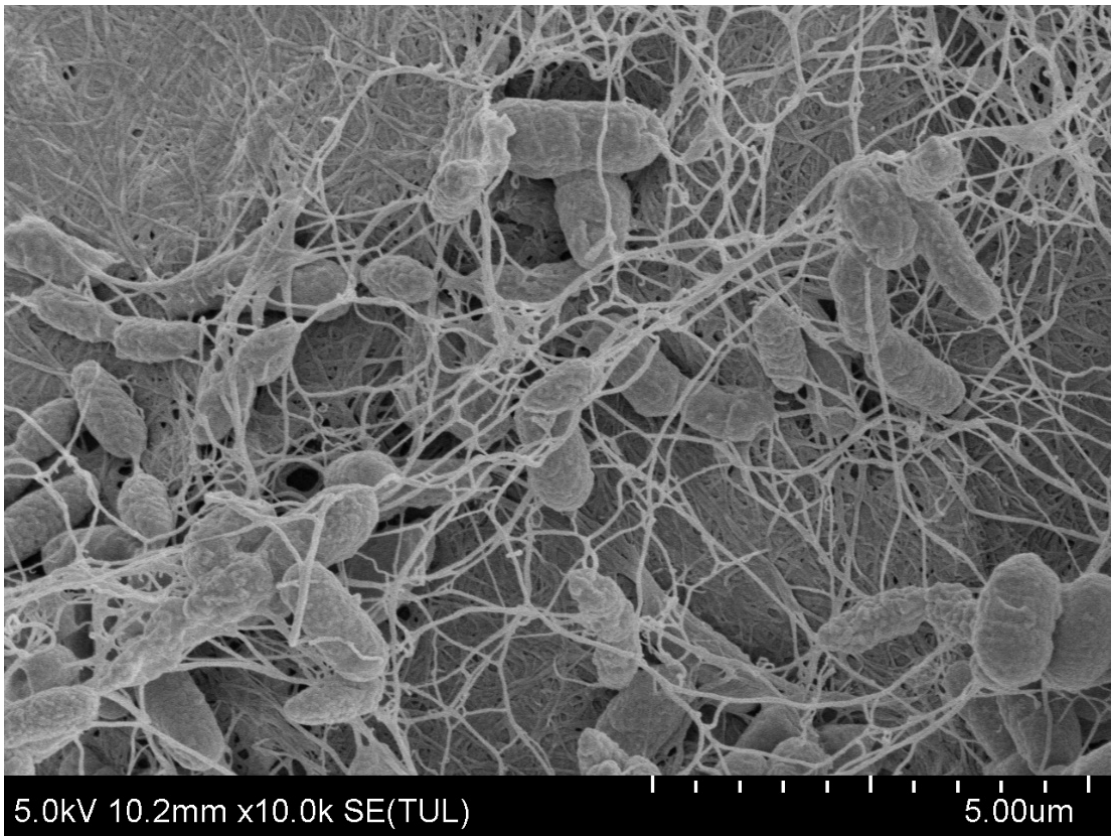
Reference data: A508=1.413 for a 0.05mg/mL Tartrazine aqueous solution.

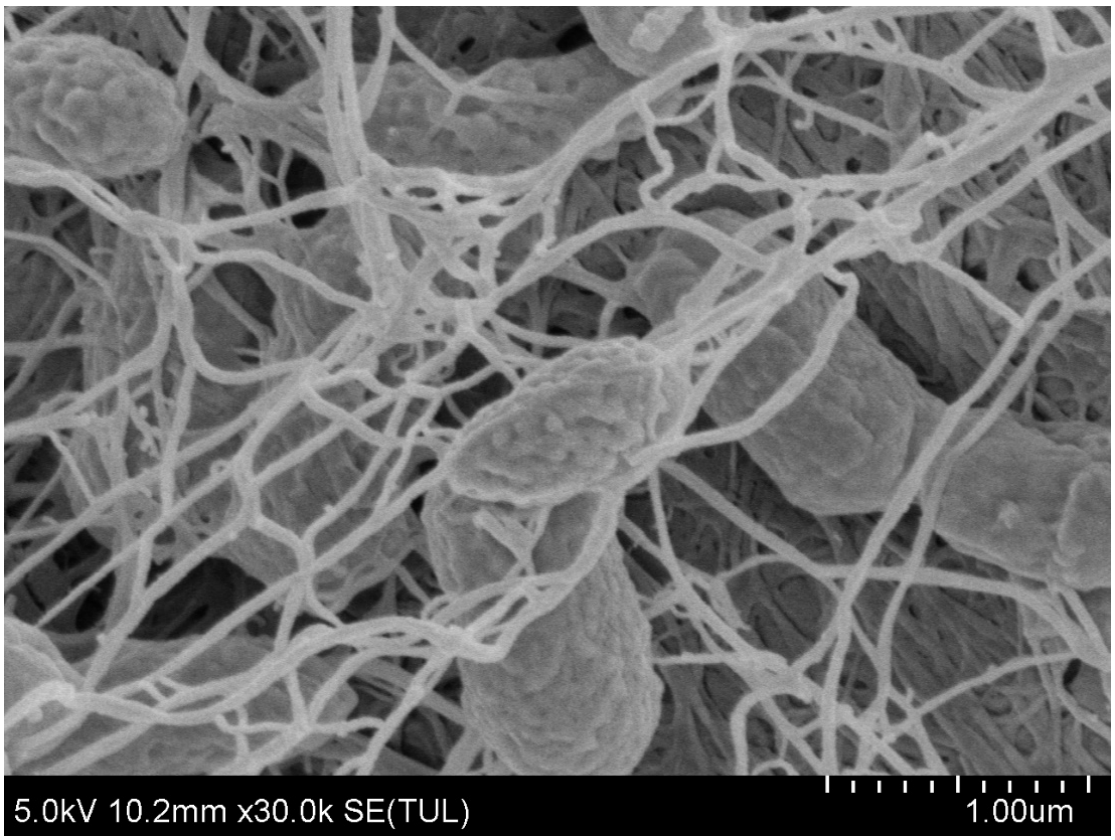


16th~23rd August

We directly used the obtained indigoidine to dye the bacterial cellulose membrane, and the results are as shown in the figure below.

We also captured electron microscopy images of the cellulose membrane dyed with indigoidine to figure out combination mode.





24th~31st August

We co-cultivated the cellulose-producing strain *K. xylinus* with the indigo-producing strain *C. glutamate* using shaking cultivation. We observed that the cellulose had a granular appearance and did not form a biofilm.

We conducted a literature search for similar cases to explore the causes and solutions, and found that shaking cultivation produces shear force and was the reason for the cellulose not forming a film. However, when using static cultivation, the cellulose membrane forms on the surface of the culture medium, while the dye forms beneath the membrane, preventing them from binding and coloring together. If we only reduce the shaking speed, the dyeing is uneven.

So, we started exploring whether it was possible to compress the dyed cellulose particles into a membrane through post-processing, but our attempts were unsuccessful. Additionally, we found that the colorfastness of the colored cellulose membrane produced in the co-culture system was not high, and it was challenging to achieve optimal cultivation conditions for both types of bacteria. Therefore, we decided to

change our approach.

September

1st September

We decided to transfer the indigoidine synthesis plasmid into *K. xylinus* to directly produce colored cellulose. First, we read literature reviews to understand the metabolic pathways of *K. xylinus*, including nitrogen metabolism and amino acid metabolism. Then, using KEGG pathway analysis, we discovered that *K. xylinus* can naturally synthesize glutamine using the nitrogen and carbon sources in the culture medium. Therefore, there was no need to introduce the glnA gene, and only the bpsA gene (encoding indigiodine synthase) and the pcpS gene (encoding PPTase) needed to be inserted.

2nd~12th September

We constructed two plasmids using homologous recombination, one containing the EGFP(YFP) gene and the other containing the bpsA& pcpS gene. The plasmid backbone pSB1A2 for the bpsA gene, based on literature and iGEM requirements, was selected, and we referred to the 2014 iGEM Imperial project to choose the promoters.

- **Preparation of Linearized Vectors**

We linearized the plasmid vector using a double enzyme digestion method.
Prepared according to the following enzyme digestion system.

EcoR I	1 μl
Spe I	1 μl
PSB1A2	2 μg
10×Buffer	5 μl
ddH_2O	to 50 μl

- **Preparation of Inserts**

We performed codon optimization on EGFP(YFP) and bpsA, and then entrusted the synthesis to a company.
The subsequent PCR amplification of the target gene was prepared according to the following system.

Template	10ng
2×Phanta	25 μl
Primer F	25 μl
Primer R	25 μl
ddH2O	to 50μl

Recombination

1. Dilute the vector and insert at an appropriate ratio to ensure the accuracy of pipetting before preparing the recombination reaction system, and the amount of each component is not less than 1 µl.
2. Prepare the following reaction on ice:

Components	Recombination	Negative control-1[b]	Negative control-2[c]	Positive control[d]
Linearized Vector[a]	X µl	X µl	0 µl	1 µl
Insert[a]	Y_1 - Y_n µl	0 µl	Y_1 - Y_n µl	1 µl
5 × CE MultiS Buffer	4 µl	0 µl	0 µl	4 µl
Exnase MultiS	2 µl	0 µl	0 µl	2 µl
ddH_2O	to 20 µl	to 20 µl	to 20 µl	to 20 µl

3. Gently pipette up and down for several times to mix thoroughly (DO NOT VORTEX!). Briefly centrifuge to collect the reaction solution to the bottom of the tube.
4. Incubate at 37℃ for 30 min and immediately chill the tube at 4℃ or on ice.

Transformation

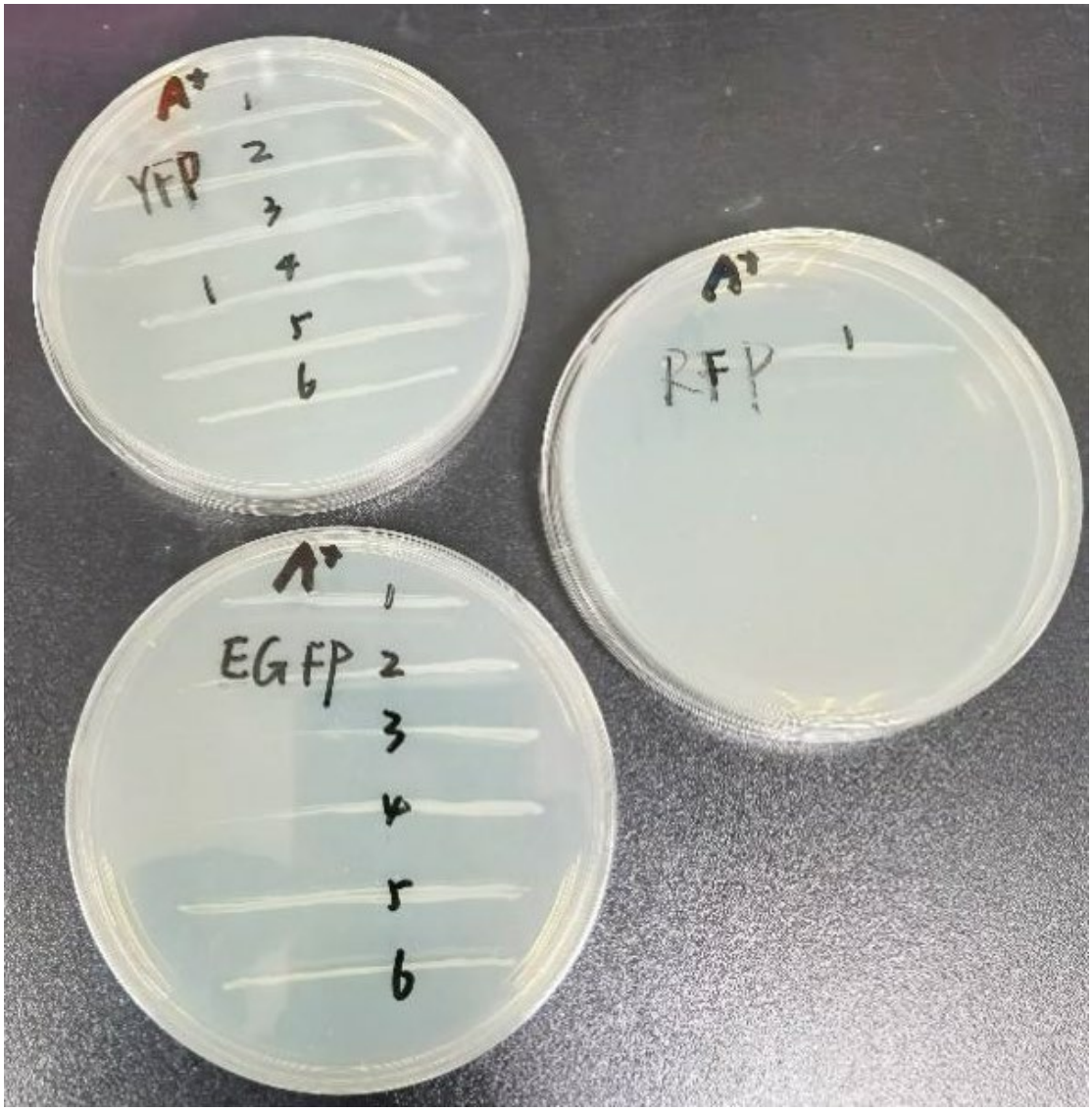
1. Thaw the competent cells on ice (e.g., DH5α Competent Cell, Vazyme #C502).
2. Pipette 10 µl of the recombination products to 100 µl of competent cells, flick the tube wall to mix thoroughly (DO NOT VORTEX!), and then place the tube on ice for 30 min.
3. Heat shock at 42℃ water bath for 45 sec and then immediately place on ice for 2 - 3 min.
4. Add 900 µl of SOC or LB liquid medium (without antibiotics). Then, shake at 37℃ for 1 h at 200 - 250 rpm.
5. Preheat the corresponding resistant LB solid medium plates in a 37℃ incubator.
6. Centrifuge the culture at 5,000 rpm (2,400 × g) for 5 min, discard 900 µl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile bent glass rod to gently spread on the plate which contains the appropriate selection antibiotic.
7. Incubate at 37℃ for 12 - 16 h.

Colony PCR

✧ Pick up the colony

1. Take out the agar plate and observe the colony growth.

It can be observed that E. coli transformed with YEP and EGFP fluorescent protein has grown six lines, so each line corresponds to one PCR verification tube. Whereas, RFP has only one line, indicating a possible issue, so only one tube will be prepared. Further evaluation will be based on the results of gel electrophoresis and sequencing.



2. Prepare 13 new 1.5ml EP tubes and label them (G1, G2, G3, G4, G5, G6, Y1, Y2, Y3, Y4, Y5, Y6, R). Add 20μl of ddH2O to each tube.
3. After preparing the agar plates, pipettes, tips, forceps, EP tubes, and other equipment, open the laminar flow hood (set the first button to a wind speed of "003" and turn on the light with the second button), and disinfect hands by spraying them with alcohol before reaching inside.
4. Use forceps to pick up the yellow gun head, gently stick a small amount of bacteria, and then insert it into the EP tube filled with water and rotate it back and forth. If you observe that the liquid becomes turbid, it indicates that the bacteria has entered the water. At this point, discard the gun head, and proceed with the remaining lines in the same manner.

✧ Lyse the bacterial cells

Place 13 EP tubes in a 98°C metal bath for 10 minutes, then centrifuge and collect the supernatant.

✧ Prepare the PCR system [20μl]

1. Prepare 13 PCR tubes and label them as G1, G2, G3, G4, G5, G6, Y1, Y2, Y3, Y4, Y5, Y6, and R. Prepare the system as follows.

2×Taq	10 μl
Primer-F	1 μl
Primer-R	1 μl
Template	2μl
ddH_2O	6 μl
total	20 μl

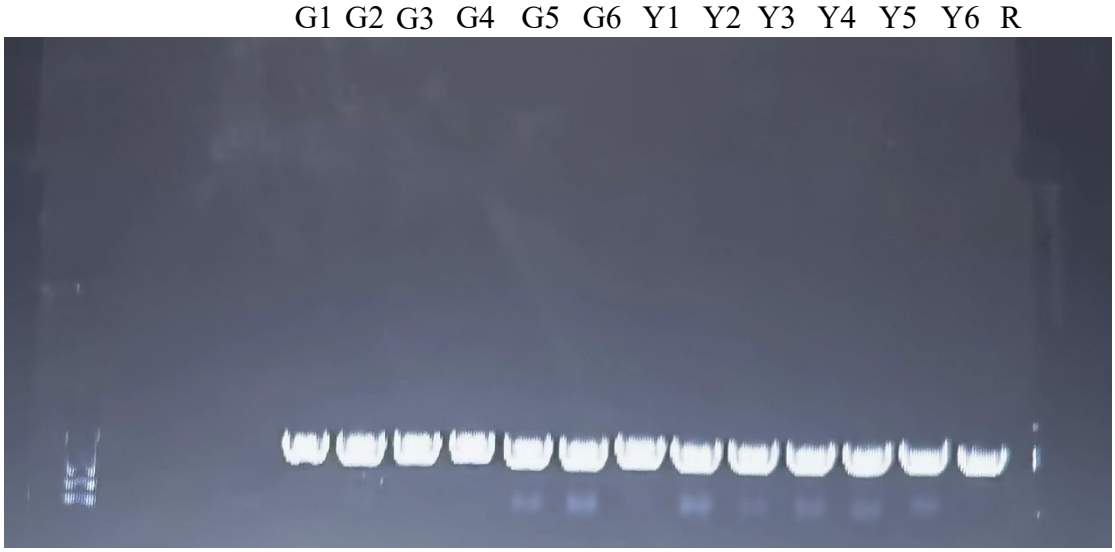
Note:
1. Combine G (Y) first and then distribute it into PCR tubes. For example, in a new EP tube, add 10 × 7 (the volume may decrease after mixing, to ensure there's enough for distribution, add an extra portion) = 70 μl Taq, 7 μl primers F and R, and 6 × 7 = 42 μl ddH2O. Then, distribute 18 μl into each of the 6 PCR tubes.
2. Add Taq enzyme last.

2. Add the corresponding primers separately.

✧ Run PCR reaction

95℃	3min	
95℃	15s	30-35 cycles
60℃	15s	
72℃	15s/kb	
72℃	5min	

✧ Run Agarose Gel Electrophoresis
1.5% agarose. 80V, 1h.
The result is as follows.



All are successful.

✧ Pick up the correct colony

Because there were still many bacteria on the original agar plate, it is possible to directly pick colonies from it, amplify them, and extract plasmids, without the need to recover them from an agarose gel.

- **Extract the PSB plasmid from bacteria culture.**

✧ Use FastPure Plasmid Mini Kit (DC201) to extract the plasmid.

1. Take about 15ml of overnight cultured bacteria, centrifuge at approximately 8000rpm for 3 minutes, and pour off the supernatant.
2. Add 500µl of Buffer P1 to the centrifuge tube containing the bacterial pellet (the original protocol used 5ml of bacterial culture with 250 µl of P1, but since there is not much bacterial pellet from step 1, we will use twice the amount). Vortex gently to mix (first open all caps, use one tip to add to all tubes, then vortex them together).
3. Add 500µl of Buffer P2 and gently invert the tube 8-10 times, until the solution becomes viscous and clear, indicating complete bacterial lysis (again, add all the liquid first, then invert all together).
4. Add 350×2=700µl of Buffer P3, gently invert the tube 8-10 times to completely neutralize P2. A white flocculent precipitate will form at this point (add all the liquid first, then invert all together).
5. Centrifuge at approximately 9500rpm for 10 minutes.
6. Place the adsorption column in a 2ml collection tube. Pass the supernatant through the column in two equal portions, approximately 600µl each time. Centrifuge at 12000rpm for 30s, discard the waste liquid in the tube, and place the adsorption column back into the collection tube.
7. Add 600µl of Buffer PW2, centrifuge at 12000rpm for 30s, and discard the waste.
8. Repeat step 7.
9. Centrifuge at 12000rpm for 1 minute to remove any residual wash liquid from the adsorption column.
10. Dry the adsorption column in a 65°C metal bath for 10-15 minutes. Also, heat up the H2O that will be used for elution.
11. Prepare 7 new 1.5ml EP tubes and label them. Place the adsorption column into each tube and add 50µl of H2O to the center of the column membrane. Allow it to sit at room temperature for 2 minutes, then centrifuge at 12000rpm for 1 minute to elute the DNA.

✧ Check the concentration.

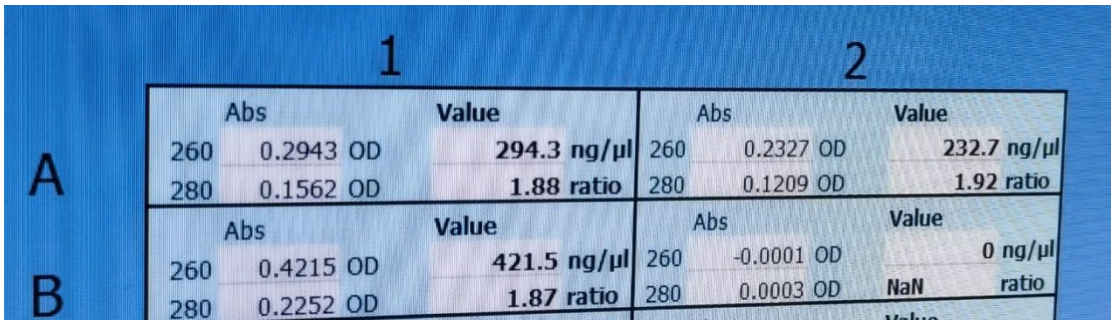
- **Extract the PSB plasmid from bacteria culture.**

✧ Use FastPure Plasmid Mini Kit (DC201) to extract the plasmid.

1. Take out approximately 10ml of bacterial culture containing PSB plasmid, centrifuge at around 8000rpm for 3 minutes, and pour off the supernatant.
2. Add 500µl of Buffer P1 to the centrifuge tube containing the bacterial pellet (250×2=500µl for each tube). Vortex to mix.

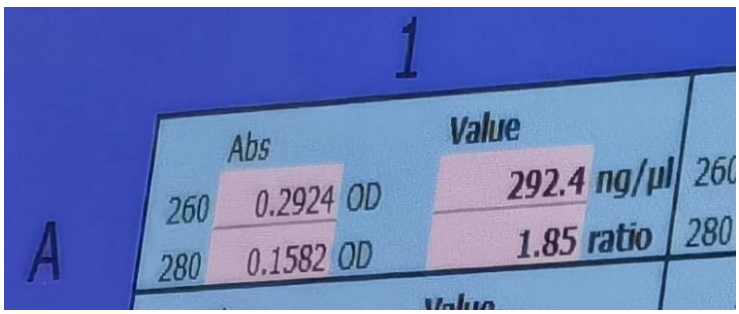
3. Add 500μl of Buffer P2 to each tube (250×2=500μl for each tube) and gently invert the tubes 8-10 times until the solution becomes viscous and clear, indicating complete bacterial lysis.
4. Add 700μl of Buffer P3 to each tube (350×2=700μl for each tube) and gently invert the tubes 8-10 times to thoroughly neutralize P2. A white flocculent precipitate will form.
5. Centrifuge at approximately 9500rpm for 10 minutes.
6. Place the adsorption column in a 2ml collection tube. Pass the supernatant through the column in three equal portions, approximately 600μl each time. Centrifuge at 12000rpm for 30s, discard the waste liquid in the tube, and place the adsorption column back into the collection tube.
7. Add 600μl of Buffer PW2 to the column, centrifuge at 12000rpm for 30s, and discard the waste.
8. Repeat step 7.
9. Centrifuge at 12000rpm for 1 minute to remove any residual wash liquid from the adsorption column.
10. Dry the adsorption column in a 65°C metal bath for 10-15 minutes. Heat up the H_2O that will be used for elution at the same time.
11. Prepare 7 new 1.5ml EP tubes and label them. Place the adsorption column into each tube and add 50μl of H2O to the center of the column membrane. Allow it to sit at room temperature for 2 minutes, then centrifuge at 12000rpm for 1 minute to elute the DNA.

✧ Check the concentration.



	1			2		
	Abs		Value	Abs		Value
A	260	0.2943 OD	294.3 ng/μl	260	0.2327 OD	232.7 ng/μl
	280	0.1562 OD	1.88 ratio	280	0.1209 OD	1.92 ratio
	Abs		Value	Abs		Value
B	260	0.4215 OD	421.5 ng/μl	260	-0.0001 OD	0 ng/μl
	280	0.2252 OD	1.87 ratio	280	0.0003 OD	NaN ratio

After mixing the two tubes of PSB provided this time with the one extracted earlier, the concentration was measured as follows.



	1			
	Abs		Value	
A	260	0.2924 OD	292.4 ng/μl	260
	280	0.1582 OD	1.85 ratio	280

13rd~20th September

We conducted a literature review and consulted with technical experts from Synmetabio company. Eventually, we developed a method for preparing competent cells of K. xylinus and an electroporation process.
After the plasmid construction was completed, we transferred it into K. xylinus via electroporation and then measured the fluorescence expression intensity and the indigoidine expression.

● Preparation of Competent Cells

1. Inoculate the bacteria into 5ml of culture medium and cultivate on a shaker at 30°C for 2 days.

The following steps must be carried out entirely on ice and within a clean bench.

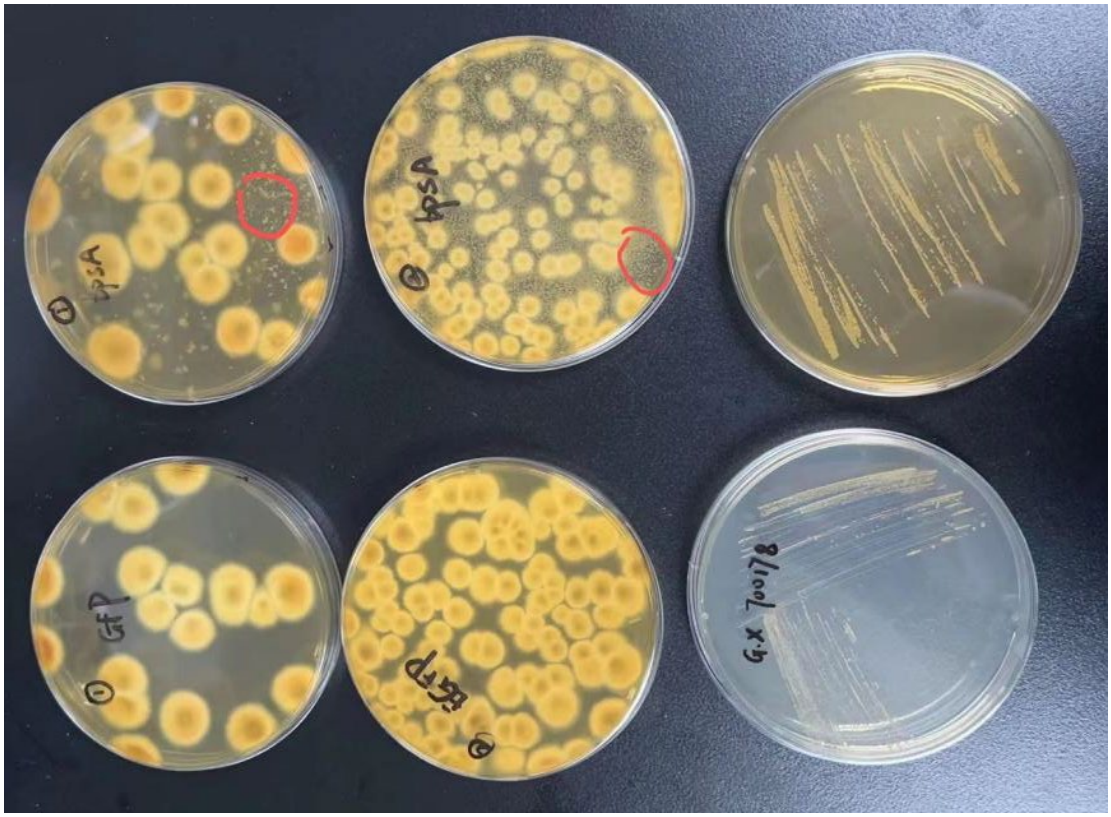
2. Inoculate the cultured bacteria into 100ml of culture medium at a ratio of 1:100. Cultivate at 30°C with agitation at 180rpm until the optical density (OD) reaches 0.4-0.6.
3. Place the culture on ice for 30 minutes, then transfer it into 50ml EP tubes within the clean bench.
4. Centrifuge at 6000rpm at 4°C for 10 minutes and discard the supernatant.
5. Add 35ml of sterile pre-chilled water to resuspend the bacterial pellet.
6. Centrifuge again at 6000rpm at 4°C for 10 minutes and discard the supernatant.
7. Resuspend the bacterial pellet in 20ml of sterile pre-chilled water.
8. Repeat the previous step.
9.Suspend the bacterial pellet in 2.5ml of sterile pre-chilled 10% glycerol, and aliquot 150μL into 1.5ml EP tubes. Store at -80°C for later use.

Calcium ions can be added for optimization during the preparation of competent cells.

● Electroporation

1. Clean the 0.2 cm electroporation cuvette with sterile water, followed by 75% ethanol, and then absolute ethanol.
2. Thaw a certain amount of competent cells (approximately 40μL) on ice, add 6μL of recombinant plasmid (specific volume to be determined). After a 30-minute ice bath, transfer the mixture into the electroporation cuvette, taking care to avoid bubble formation.
3. Perform electroporation at 2.5kV for 5ms. In the clean bench, add 1ml of culture medium, then transfer the mixture into a 1.5ml EP tube and incubate at 30°C with shaking at 180rpm for recovery.
4. After 7 hours, centrifuge at 8000rpm for 1 minute, discard 900μL of the supernatant, resuspend the pellet, and streak onto a selective solid culture medium. Incubate at 37°C.

We observed the plate delineation inoculation of already electrotransformed K. xylinus. We cut off a small piece of each and put them into YA medium to re-incubate at 30°C and observe the results the next day; The red boxed out should be the successful plasmid transformation of K. xylinus colonies, we re-picked a part of the inoculation to 50ml centrifuge tube, 30 ℃ static culture.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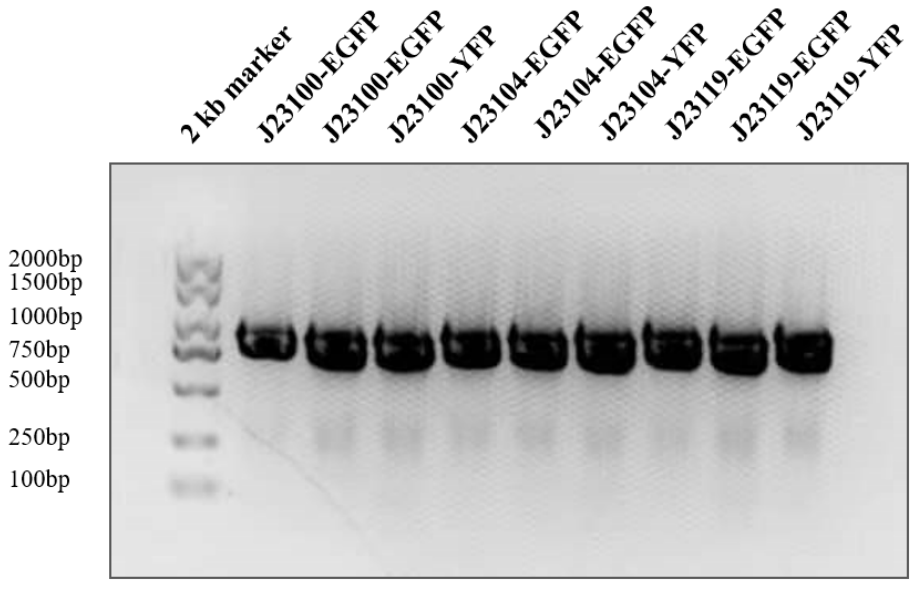
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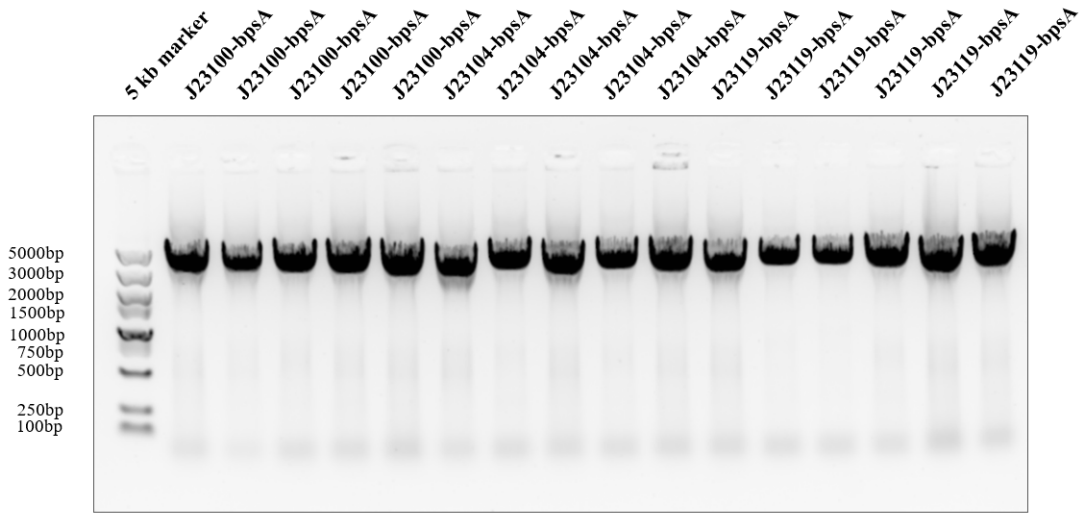

21st~31st September

We searched the literature and previous iGEM projects to optimize our plasmids. We reconstructed a plasmid with modifications to different composite promoters compared to the original plasmid, aiming to adjust the gene expression intensity.