

Isolation and purification of recombinant protein

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(GROUP 1)

Flow of procedure

- Calculations for Broth preparation
- Preparation of LB broth and agar
- Competent Cell Preparation
- Bacterial Transformation
- Induction of protein
- Protein isolation and degeneration using SDS-PAGE and sonicator
- Protein Purification

Preparation of LB Broth and Media

MATERIALS REQUIRED:-

- Conical Flasks
- Distilled water
- LB Media
- Agar
- Weighing Balance
- Distilled water
- Measuring flasks
- Cotton plug

Preparation of LB Broth and Media

PROCEDURE:-

- LB (Luria-Bertani) Broth- (25g/1000ml) was prepared for 60ml and 100ml
- Calculations were done as required
- The required amount of LB powder was weighed using a weighing machine
- The volume was made up to 60ml and 100ml by adding distilled water in conical flasks
- Agar (15g/1000ml) was also prepared in the same way
- Gentle mixing was done for even composition of the solution
- The flasks were sealed using cotton plug and paper using a rubber band
- The flasks were then autoclaved for around 2 hrs at 121°C at 15psi

Autoclave



Fig 1.a



Fig 1.b



Fig 1.c

Inoculation of Bacterial cells

MATERIALS REQUIRED:-

- Kanamycin50
- Shaker
- BL21(D3) Bacterial strain
- Pipette and micro-tips

PROCEDURE:-

- BL21(D3) Bacterial strain was used for producing competent cells
- A single colony of the bacterial strain was inoculated in 5ml of prepared LB Broth containing suitable antibiotics
- It was then grown overnight in shaker at 37 °C at 150 rpm

Competent Cell Preparation

MATERIALS REQUIRED:-

□ Prepared LB Media

□ Shaker

□ Pipette and micro-tips

□ Centrifuge

□ Falcon tubes

□ Ice cubes

□ Prepared CaCl₂

□ Autoclave

□ Parafilm

Competent Cell Preparation

- 1% of overnight grown culture was transferred to 10ml of LB Broth containing suitable antibiotics (1% = 100 μ L in 10ml)
- It was then grown in shaker till the OD at 600nm reached 0.4
- The culture was transferred to 50ml centrifuge tube
- Centrifuged at 5000rpm for 10mins at 4°C
- Dissolved the pellet in 10ml of 0.1M CaCl₂ (ice-cold)
- Incubated in ice for 30 mins
- Centrifuged at 5000rpm for 10 mins at 4°C
- Dissolved pellet in 500 μ L of 0.1M of CaCl₂
- Aliquot 100 μ L in 1.5ml autoclaved falcon tube
- Parafilmed the tube and stored at 4°C

Bacterial Transformation

MATERIALS REQUIRED:-

- Plasmid PS107
- Prepared Competent cells
- Pipette and micro-tips
- Ice cubes
- Prepared LB medium
- Centrifuge tubes
- Water bath

Bacterial Transformation

PROCEDURE:-

- 1 μ L of plasmid PS107 was transformed into BL21(DE3) strain by chemical method
- Thaw the competent cells on ice
- 5ng (2 μ L) of plasmid construct mixture in 1.5ml centrifuge tube was taken
- 100 μ L of competent cells to plasmid DNA was added
- Mixed gently by pipette mixing
- The mixture was placed on ice for 30mins

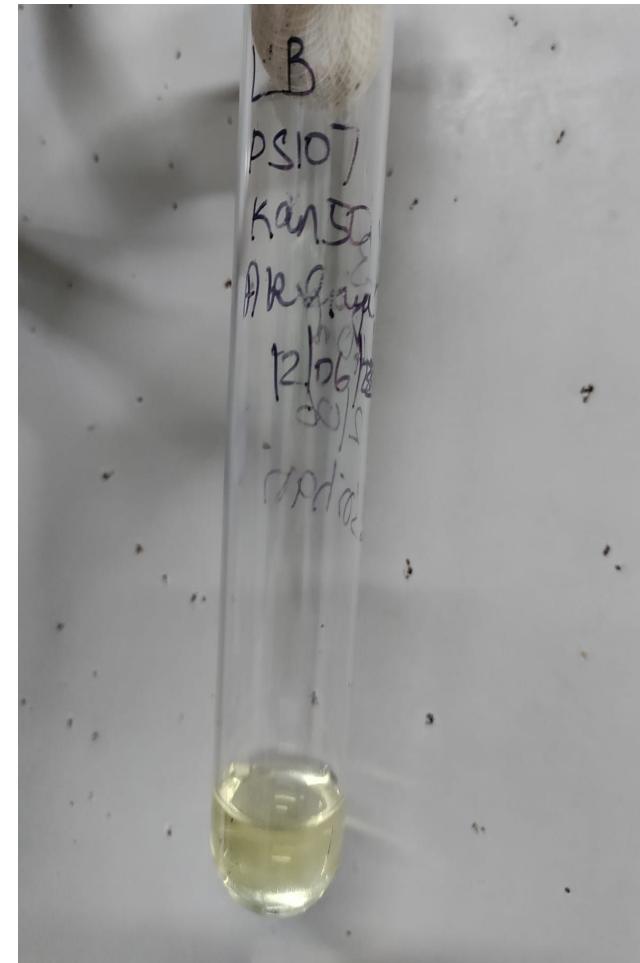


Fig 2.

Bacterial Transformation

- Heat shocked at 42°C for 90s
- The mixture was suddenly placed on ice for 5mins
- Added 1000µL of fresh LB medium into the tube
- Incubated the tubes on shaker at 150rpm for 1hr at 37°C
- After 1hr incubation, 100µL of mixture was taken and spread on the plates that contain LB and kanamycin50
- Incubated the plates overnight at 37°C for 16 hours

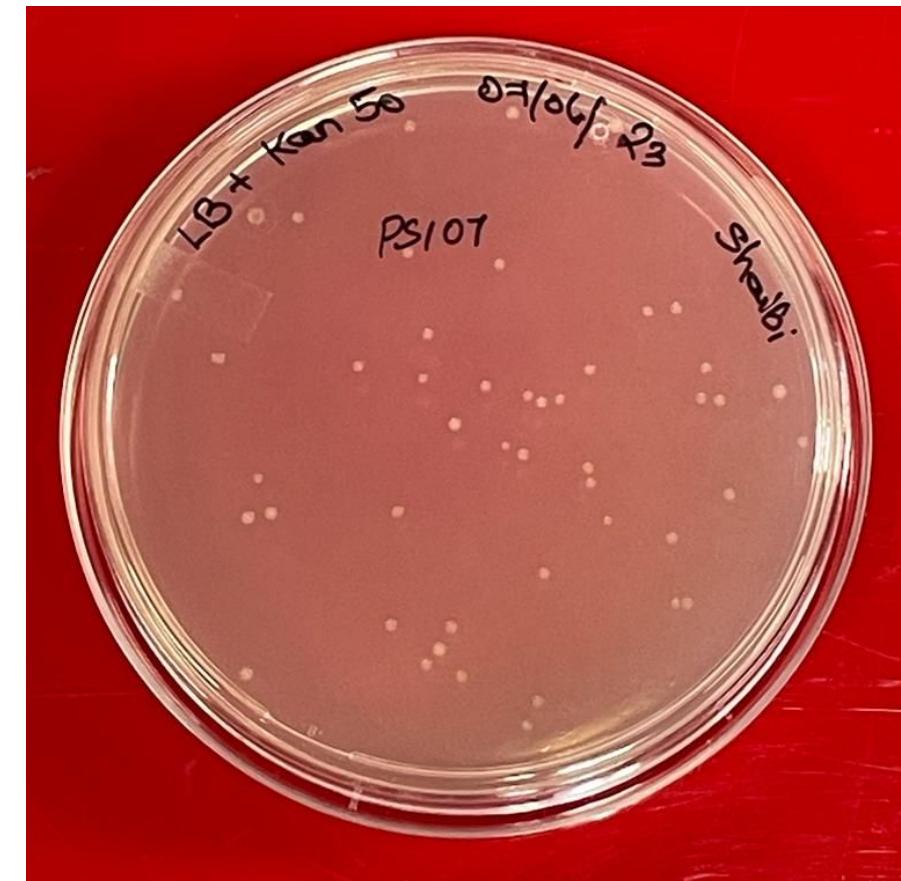
Visible transformed bacterial colonies

Fig. 3.a



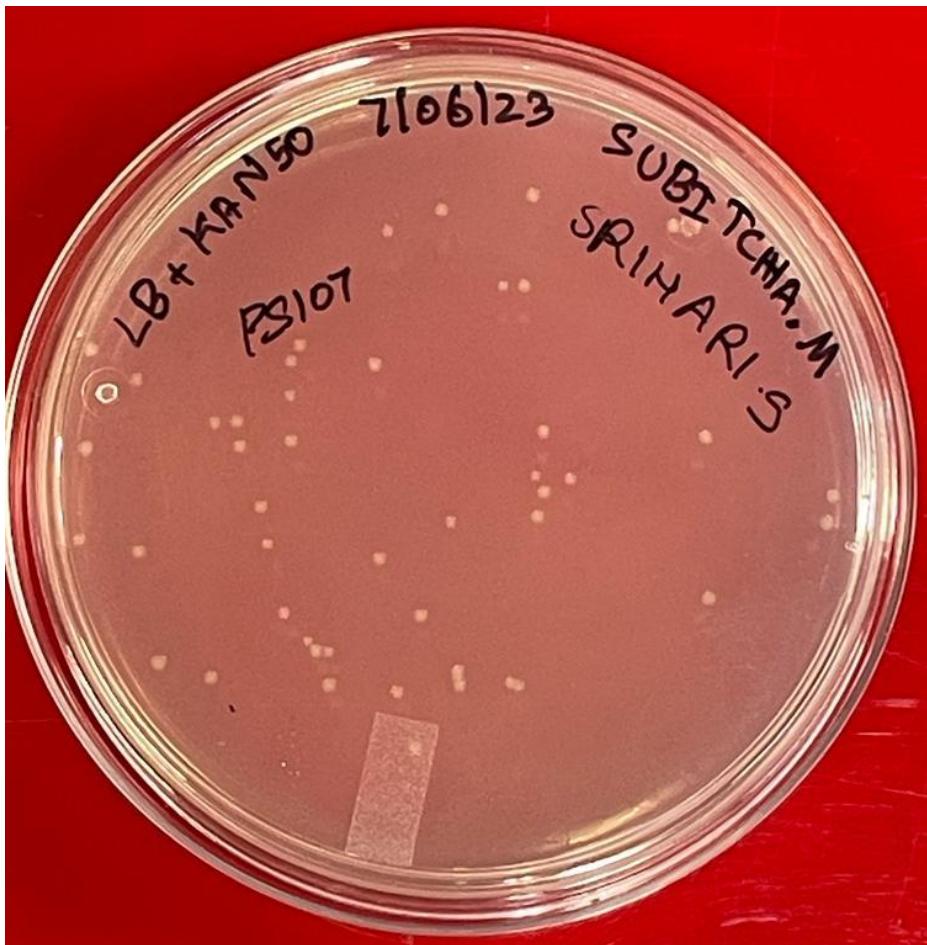
Subitcha got 112 transformed colonies

Fig. 3.b



Sabareesh got 46 transformed colonies

Fig. 3.c



Srihari got 33 transformed colonies

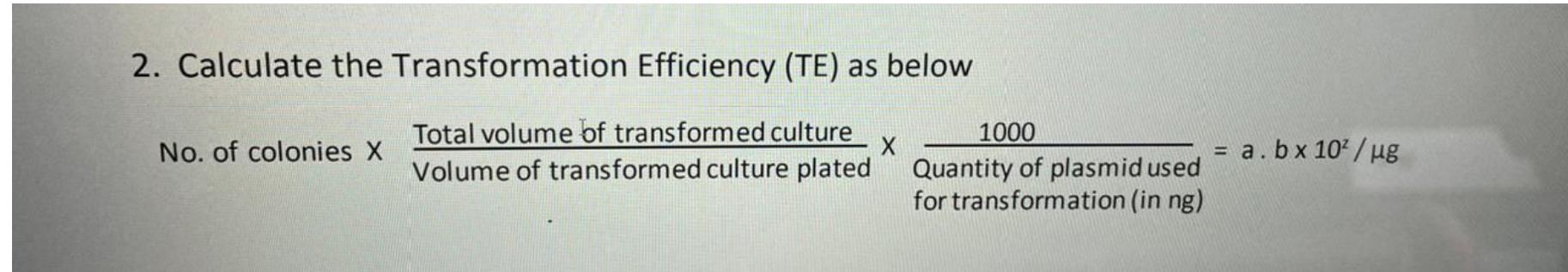
Fig. 3.d



Sunandini got 23 transformed colonies

Visualize colonies and Transformation efficiency

- Pure colonies were then visualized by naked eye
- Transformation efficiency was calculated by the following formula:



$$\begin{aligned}&=> 46 * (1100/100) * (1000/100) \\&= 5060 \text{ CFU}/\mu\text{g} \\&= 5.06 * 10^3\end{aligned}$$

Streaking of colonies



Fig 4.a



Fig 4.b

Visualizing the streaking plates with purified colonies

Fig 5.a



Fig 5.b



Fig 5.c

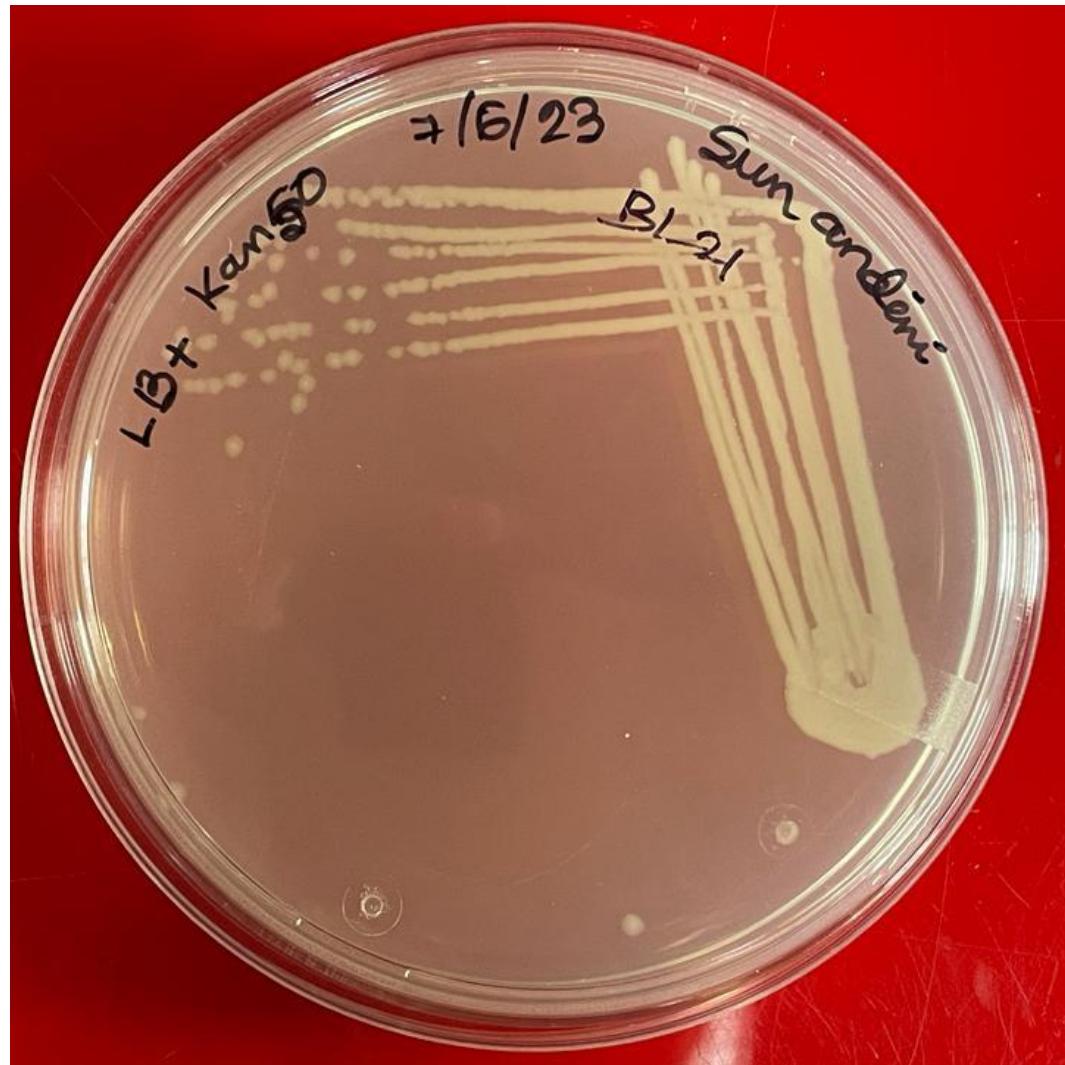
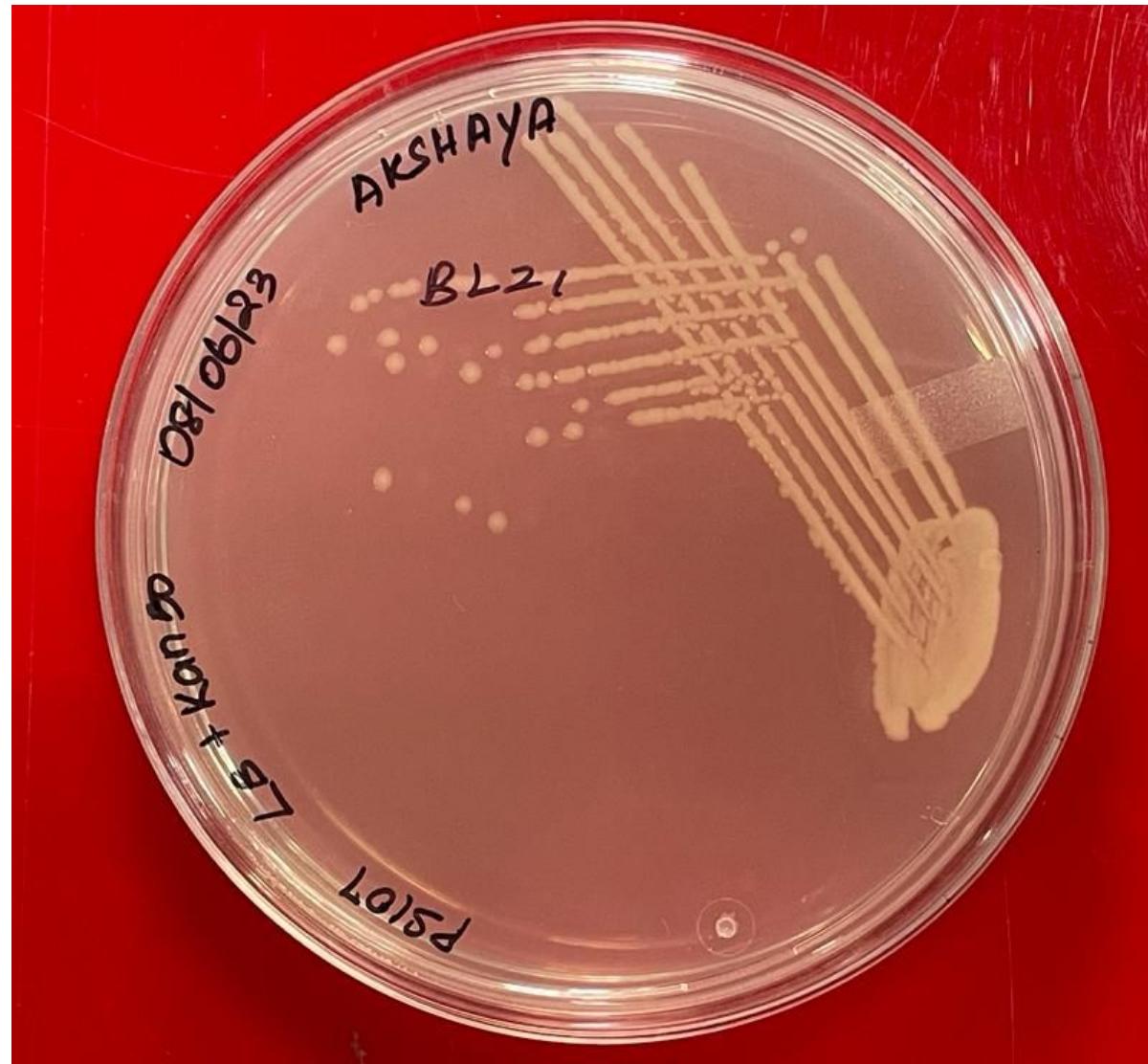


Fig 5.d



Fig 5.e



Media and Mother culture Preparation

- Media was prepared for 20ml, 50ml and 60ml
- Mother culture was also prepared
- Streaked a grown colony from spread plate into another spread plate
- Overnight incubation was done for 16hrs at 37°C

IPTG Induction for plasmid

- To a fresh 10ml LB media, 10 μ L of Kan50 and 1% of LB media with PS107 was added (1% = 100 μ L) to form the mother culture
- It was then grown in a shaking incubator at 37°C for about 3 hours until the OD reached 0.4
- To the 100ml and 20ml flasks, 100 μ L and 20 μ L of Kan50 were added each along with 1ml and 200 μ L of mother culture respectively (1% of mother culture)
- Added 1mM IPTG (0.119g)
- After induction, the flask was returned into the shaking incubator at 37 °C for 4 hours.
- After 4 hours, 1 ml of induced sample was transferred to 1.5ml centrifuge tube and centrifuged at 10,000rpm for 5 mins (only did for 2 mins but the pellet wasn't intact and compact, hence do it for 5 mins)
- Discarded the supernatant and the pellet remained
- The pellet was then frozen at -80°C until needed

IPTG Calculation

$$\text{M.W} = 238 \times \frac{100\text{MM}}{1000} \times \frac{5\text{mL}}{1000}$$

$$\approx 238 \times 0.1 \times 0.005$$

$$= 0.119 \text{ g}$$

$$= 119 \text{ mg}$$

IPTG Induction for GFP

- The same processes were repeated for GFP except:-
 - The incubation time was standardized as 2hrs, 3hrs, 4hrs and 20hrs.
 - Instead of kanamycin as antibiotic, ampicillin was used

Viewing mother culture cells

- The mother culture was serially diluted for 4 times and observed under the microscope at 400x and 1000x magnification
- Rod-like transparent cells were visible

Cells before lysis viewed under microscope

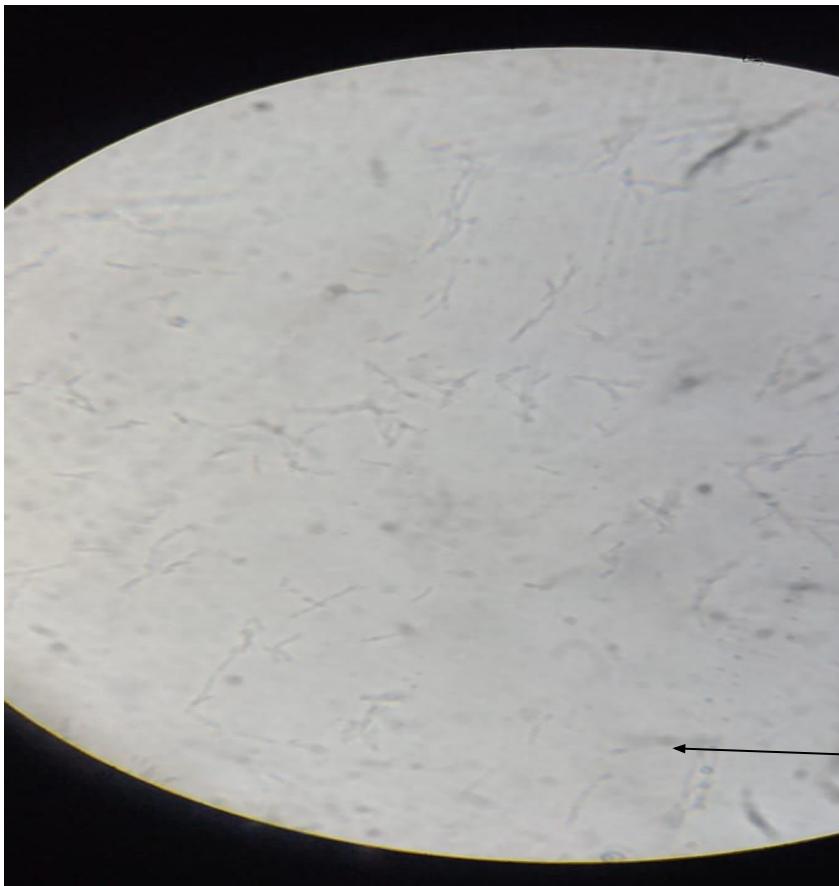


Fig. 6.a

Rod-shaped
bacterial cells

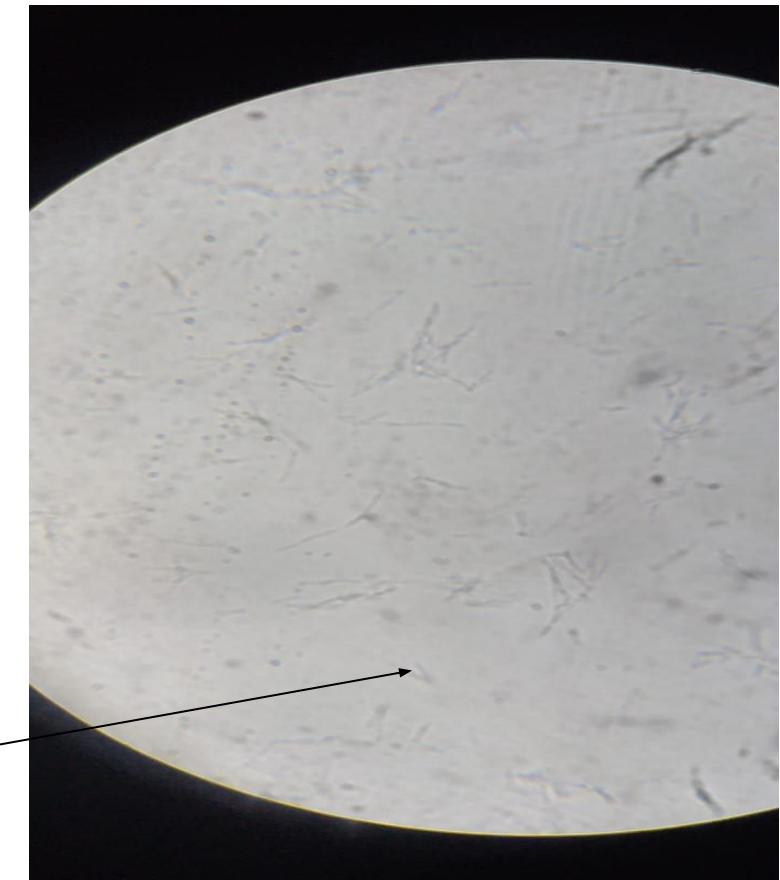


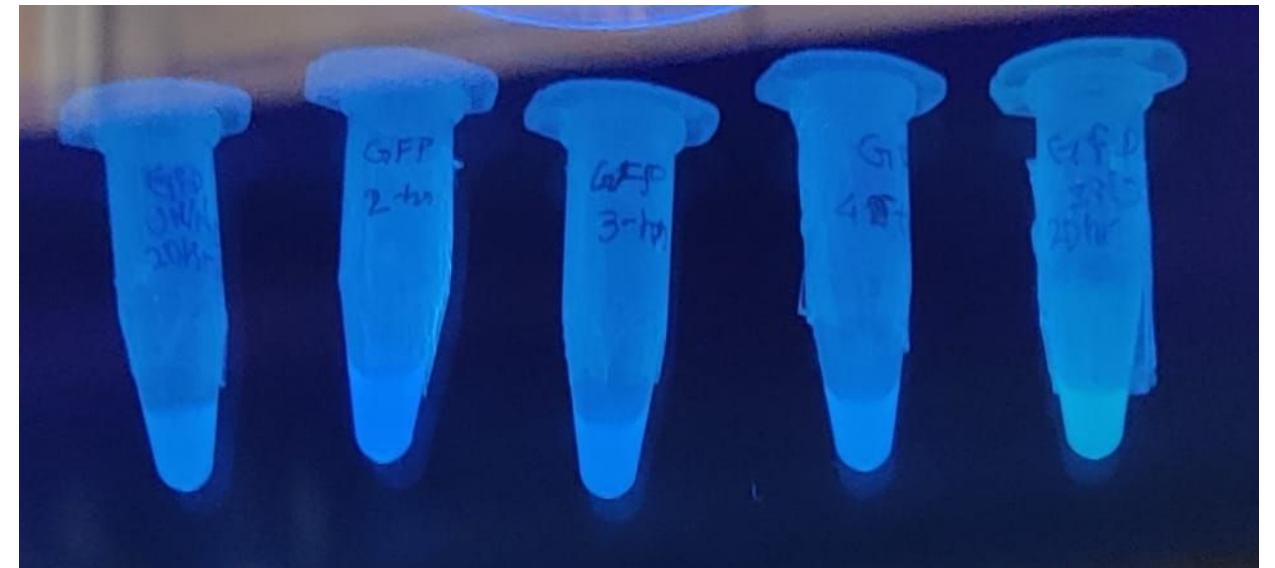
Fig. 6.b

Viewing GFP Protein

- The GFP proteins were viewed under the UV light
- The samples used where at a time interval of 2hrs, 3hrs, 4hrs and 20hrs
- It was observed that 20hrs showed a strong green color when viewed under UV

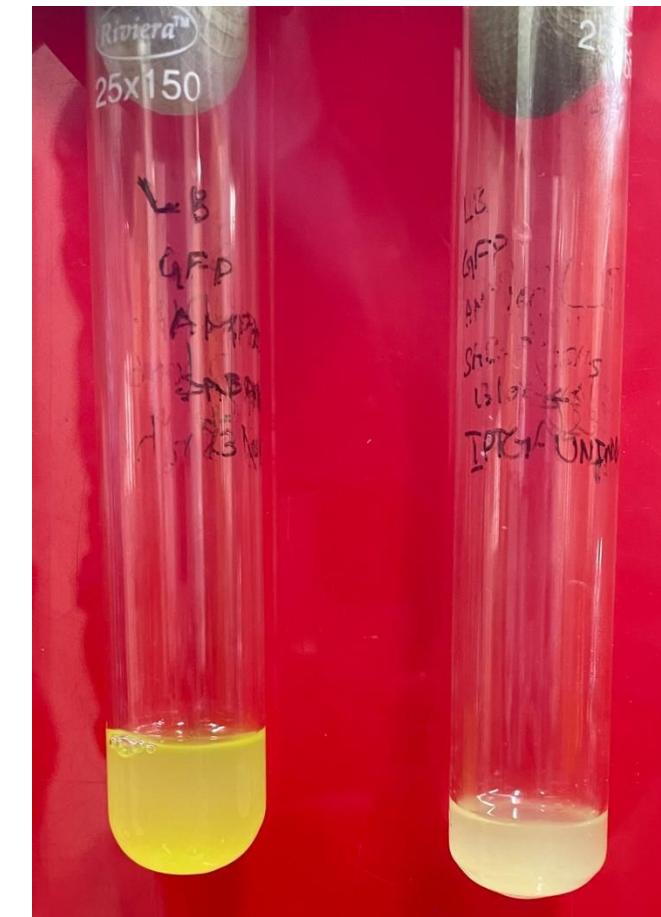


Before UV



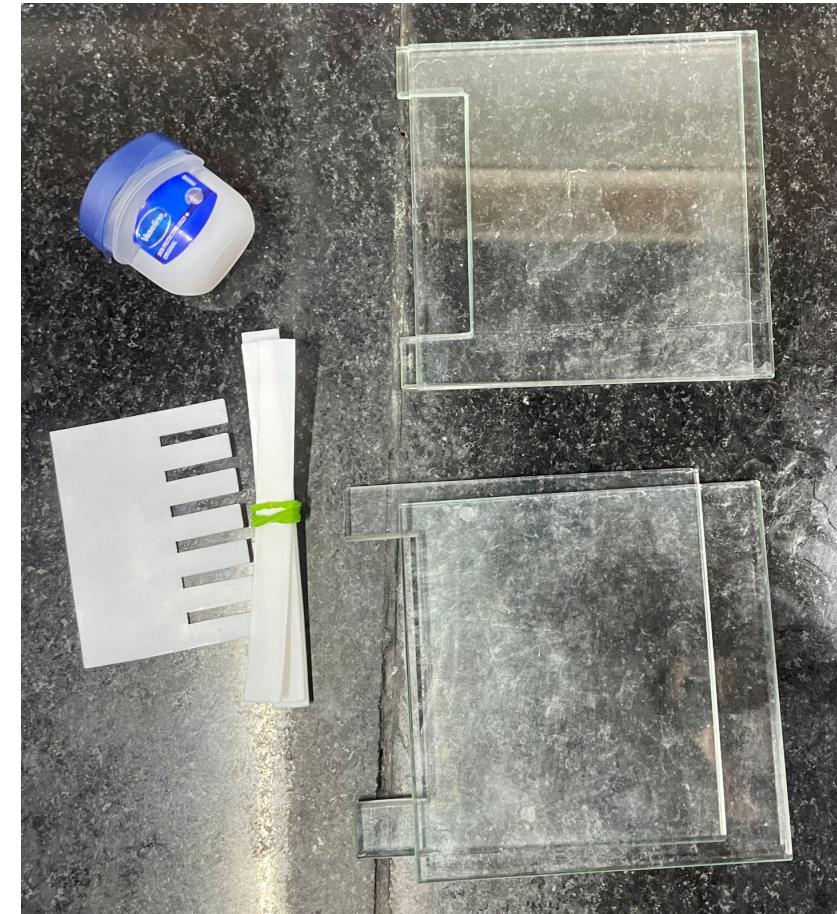
After UV

GFP Protein - Induced and Uninduced



SDS PAGE components

- Glass plates
- Spacers
- Vaseline
- Comb
- TGS buffer
- Paper clips
- Loading Apparatus



Calculations for SDS-PAGE

TABLE 18.3 Solutions for Preparing Resolving Gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

Solution components	Component volumes (ml) per gel mold volume of							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
6%								
H ₂ O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% acrylamide mix	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8%								
H ₂ O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% acrylamide mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10%								
H ₂ O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12%								
H ₂ O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% acrylamide mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15%								
H ₂ O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% acrylamide mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

TABLE 18.4 Solutions for Preparing 5% Stacking Gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

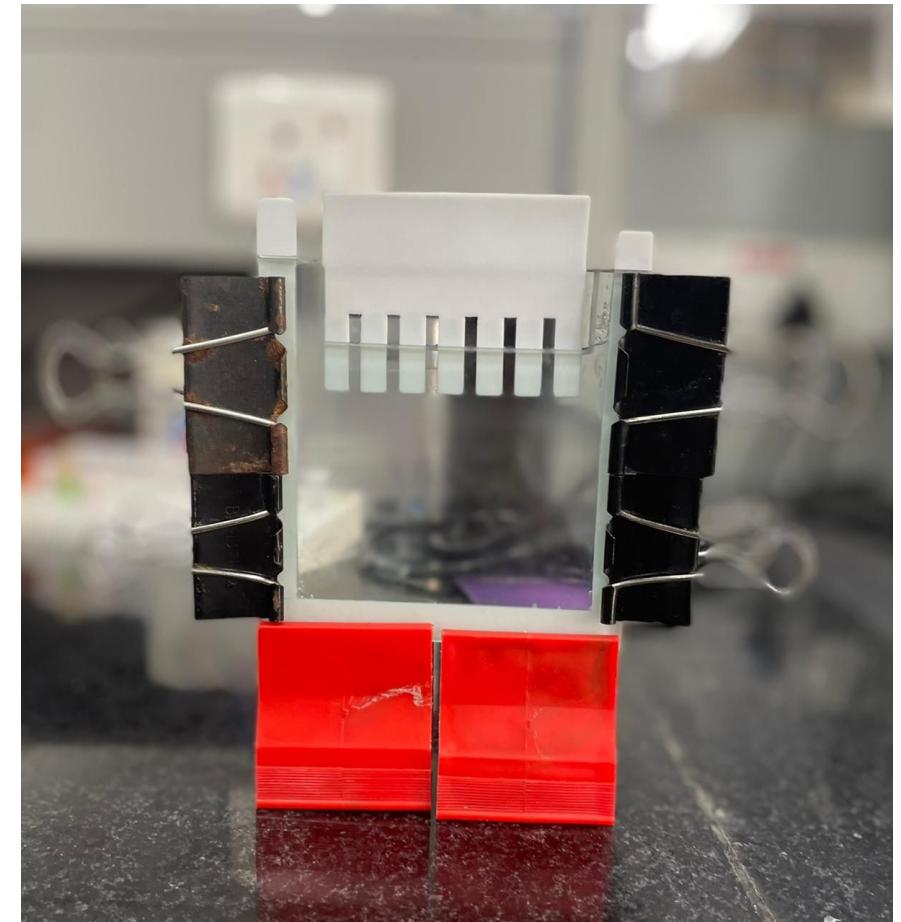
Solution components	Component volumes (ml) per gel mold volume of							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H ₂ O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.26	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Tables 18.3 and 18.4 are modified from Harlow and Lane (1985).

$$\begin{aligned}
 100 \text{ mL} &\rightarrow 10 \text{ g SDS} \\
 5 \text{ mL} &\rightarrow \frac{10 \times 5}{100} \text{ g SDS} \\
 &= 0.5 \text{ g SDS} \\
 \\
 +1000 \quad 100 \text{ mL} &\rightarrow 10 \text{ g APS} \\
 0.2 \text{ mL} &\rightarrow \frac{0.2 \times 10}{1000} \text{ g APS} \\
 &= 0.02 \text{ g APS} \\
 &= 20 \mu\text{g of APS}
 \end{aligned}$$

SDS-PAGE

- The SDS PAGE apparatus was set-up using the components
- Leaking was prevented by properly sealing the components using Vaseline
- It was then tested using water and set for 10mins
- The resolving gel for 12% and stacking gel for 5% was prepared for 10ml and 5ml respectively



Loading into SDS-PAGE

- To 1ml of pellet, 100 μ L of water was added and re-suspended
- To this, 100 μ L of 2x dye was added
- To the 90 μ L of pellet 9 μ L of water and 9 μ L of 2x xylene cyanol dye was added
- Mixed it well by pipette mixing
- Heated the samples in boiling water bath for 10mins
- Let it cool down
- Centrifuged at 10,000rpm for 5mins
- Loaded 5 μ L of 1ml sample and 9 μ L of 90 μ L sample by taking the sample from supernatant into SDS-PAGE

Stacking gel
Sample loaded
Resolving gel

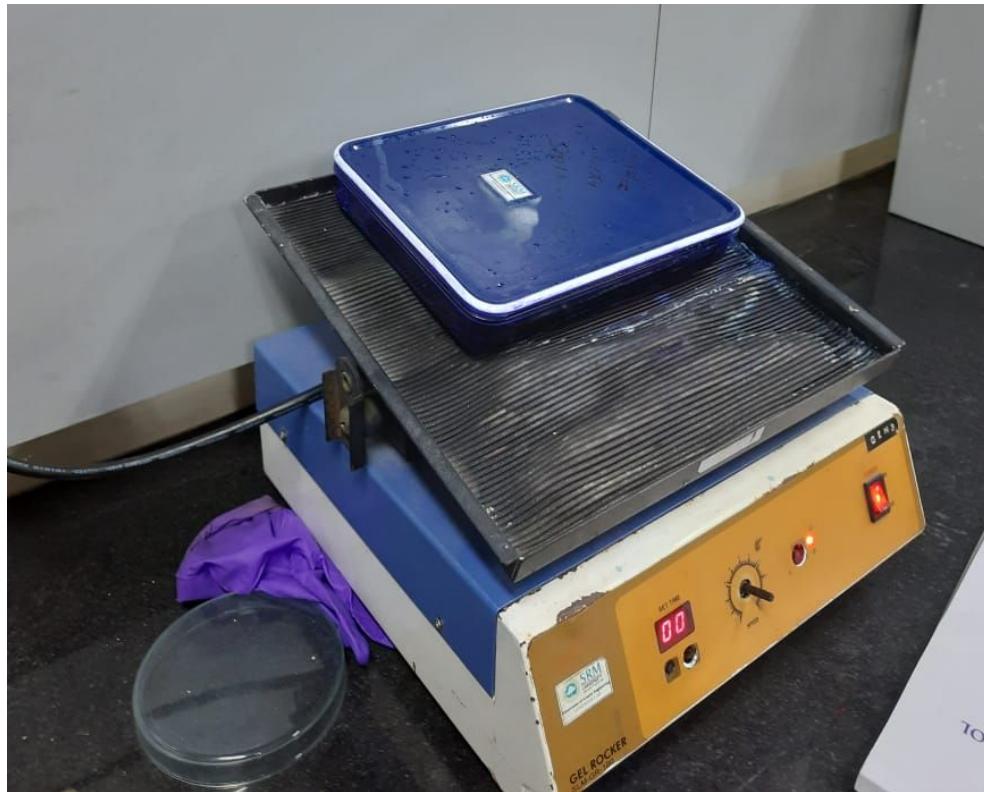


SDS-PAGE

- The samples were added into the wells in the following order:
 1. Ladder
 2. GFP Un-induced Sample
 3. 2hrs- GFP induced sample
 4. 4hrs- GFP induced sample
 5. 20hrs- GFP induced sample
 6. PS107 Un-induced sample
 7. PS107 Induced sample
- After 2hrs of running SDS-PAGE the gel was removed and stained by using the staining solution
- After 1hr the de-staining solution was added and left overnight

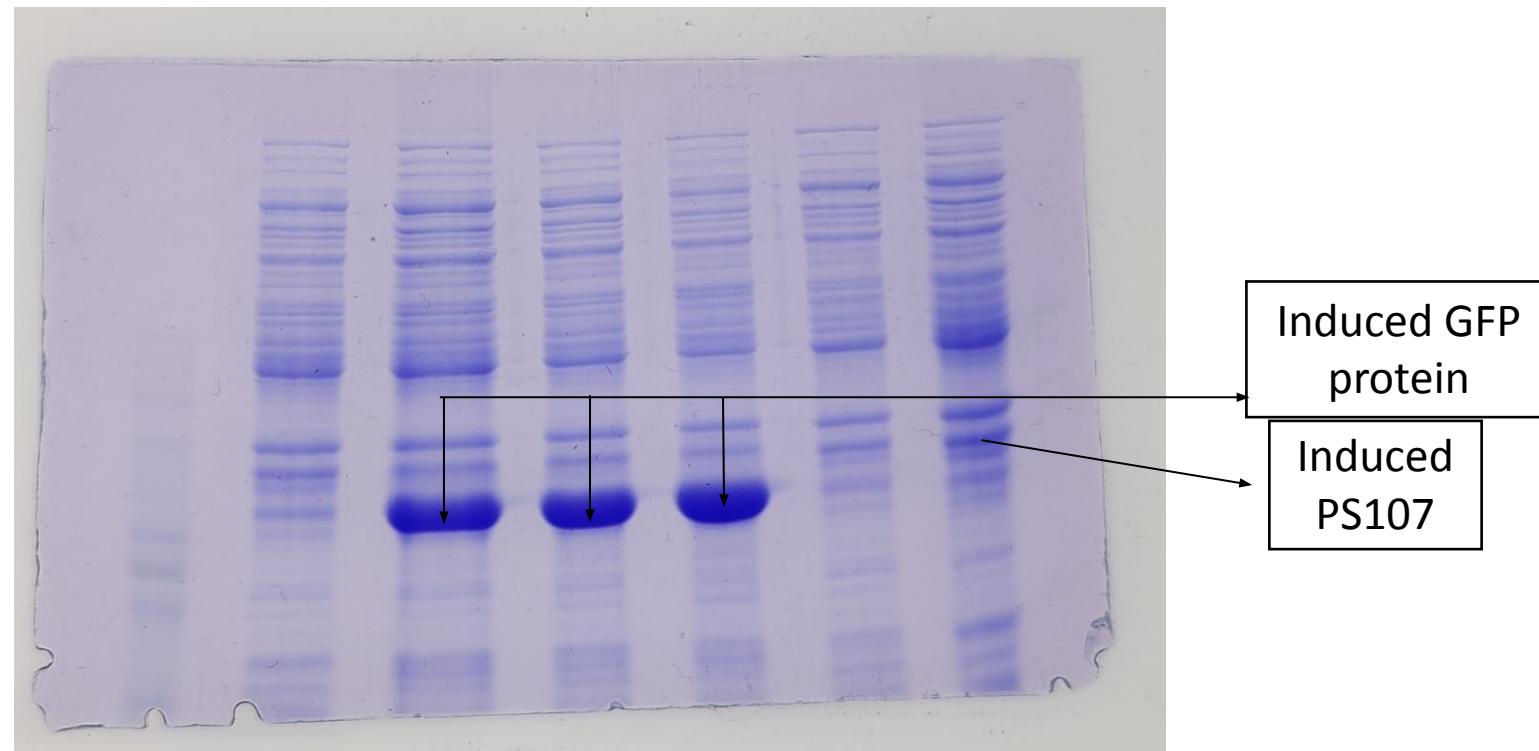
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SDS Gel Staining

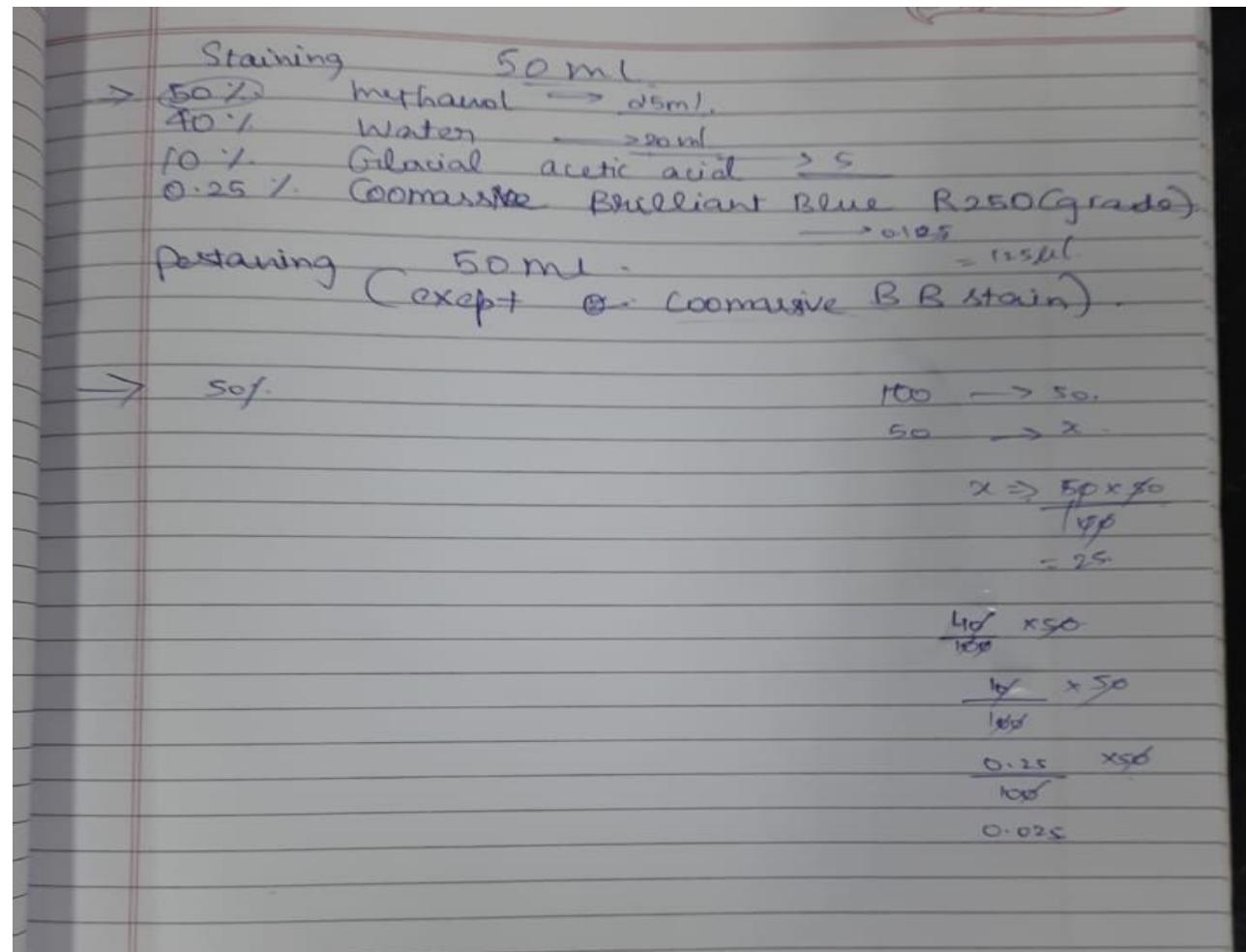


Viewing of gel

- The gel was viewed after de-staining the gel completely
- Thick bands were observed for GFP induced proteins in the recombinant region at 25-35kda which indicates that the proteins were induced using IPTG
- Thick bands were not observed at un-induced GFP and PS107
- A slightly thick band was observed at induced PS107 but was not that prominent which indicates that the proteins were only slightly induced at 4hrs



Staining and de-staining Solutions



Xylene-cyanol 2x dye

Xylene cyanol - 2x dye

5x (total 5ml)

β -mercaptoethanol - 100μL

1M tris (pH 6.8) - 2.5mL

xylene cyanol - pinch

sucrose - 2.5g

SDS - 0.25g

water - upto 5mL

Diagram:

$$5m \rightarrow 5x$$

$$x \leftarrow 2x$$

$$x = \frac{2}{5} \times 5$$

$$x = 2 \text{ mL}$$

$$(5x) \times x = (2x) \times 1 \text{ mL}$$

$$x = \frac{2}{5}$$

$$= 0.4 \text{ mL}$$

2.4 mL

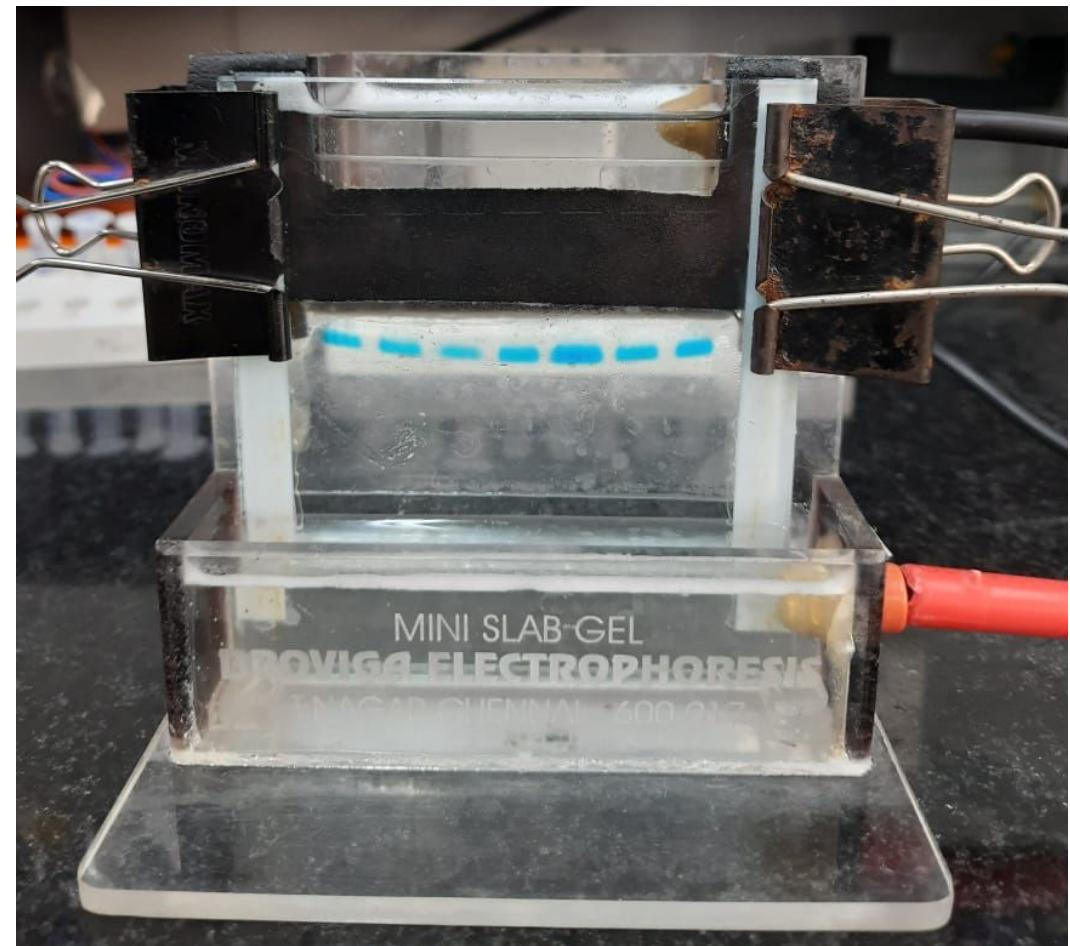
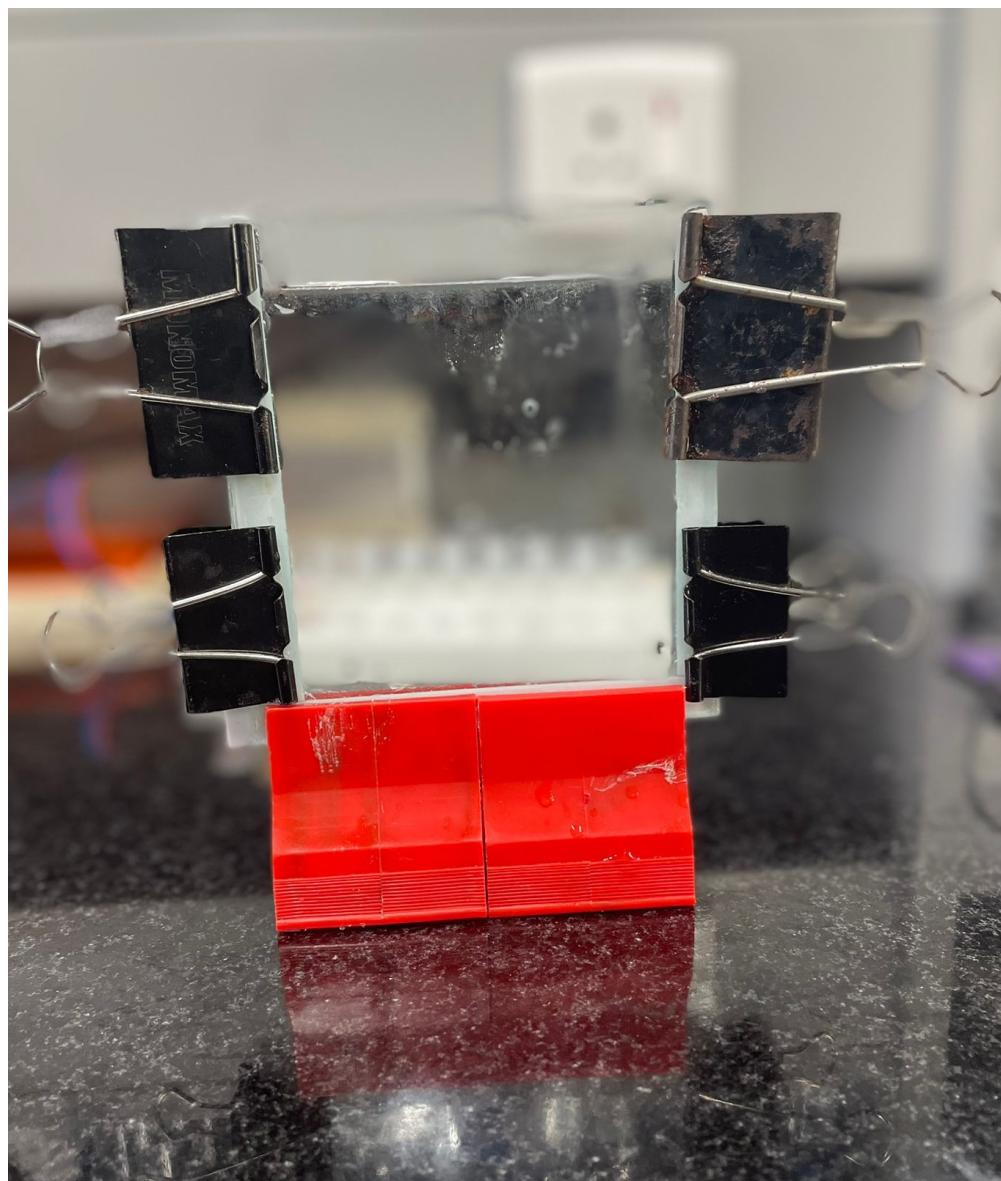
SDS-PAGE

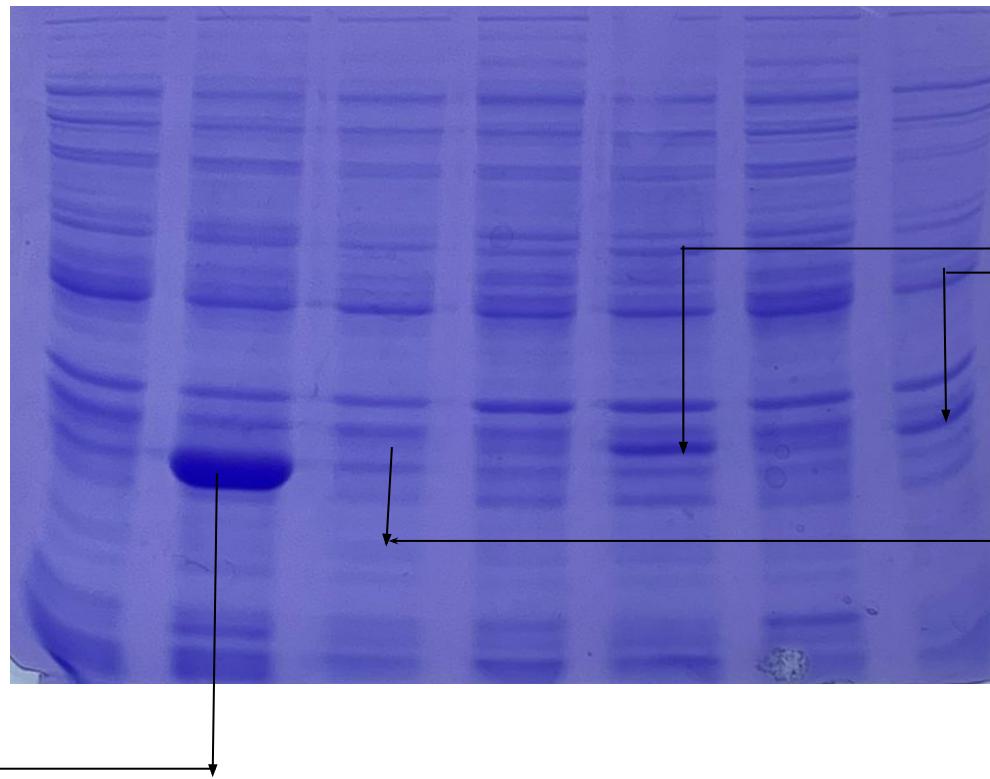
- The SDS-PAGE was repeated again for loading the other samples
- The 12% resolving gel was prepared for 8ml and 5% stacking gel was prepared for 3ml
- The samples were then loaded in the following order:
 1. Un-induced GFP
 2. Induced GFP at 20hrs
 3. Un-induced PS107
 4. Induced PS107
 5. Induced PS107
 6. Induced PS107
 7. Induced PS107
- The same procedure was then repeated again and the gel was viewed the next day

Calculations for SDS-PAGE

<u>15/06/23</u>		<u>12% SDS Resolving gel (8mL)</u>	<u>100 mL → 10g SDS</u>
①	$H_2O - 2.64 \text{ mL}$	$3.3 \leftarrow 16 \text{ mL}$	
②	$30\% \text{ Acry} - 3.2 \text{ mL}$	4.	
③	$1.5 \text{ M Tris (8.8)} = 2 \text{ mL}$	2.5.	
④	$10\% \text{ SDS} - 0.08 = 80 \mu\text{L}$	0.1	$100 \text{ mL} \rightarrow 10 \text{ g SDS}$
⑤	$10\% \text{ APS} - 0.08 = 80 \mu\text{L}$	0.1	8m
⑥	$\text{TEMED} - 0.0032 = 3.2 \mu\text{L}$	0.004.	
<u>5% SDS stacking gel (3mL)</u>		$10 - 3.2$	
①	$H_2O - 2.1 \text{ mL}$	$8 \rightarrow 3.3 \times 8$	
②	$30\% \text{ Acry} - 0.5 \text{ mL}$	10.	
③	$1 \text{ M Tris (6.8)} - 0.38 \text{ mL} = 380 \mu\text{L}$		
④	$10\% \text{ SDS} - 0.08 \text{ mL} = 30 \mu\text{L}$		$100 \text{ mL} \rightarrow 10 \text{ g SDS}$
⑤	$10\% \text{ APS} - 0.03 \text{ mL} = 30 \mu\text{L}$		$0.150 \text{ mL} \rightarrow \frac{15 \times 10}{100}$
⑥	$\text{TEMED} - 0.003 \text{ mL} = 3 \mu\text{L}$		$100 \text{ mL} \rightarrow 10 \text{ g SDS} \rightarrow 10 \cdot 015 \text{ g} \rightarrow 15 \text{ mg}$

15/06/2023





Thick band shows
recombinant
protein was
induced in GFP

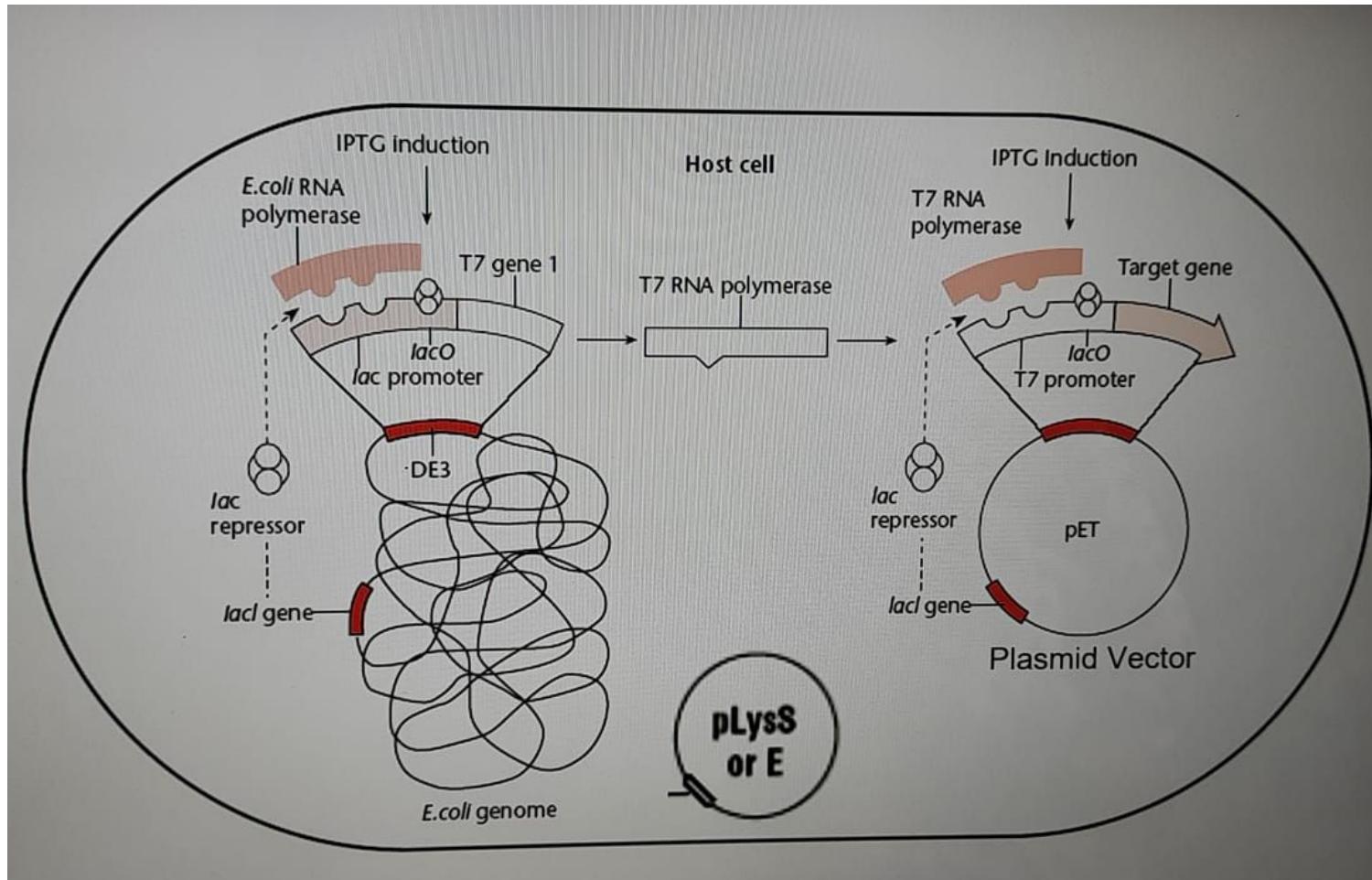
Thick band indicates that
protein was induced in
PS107

Band visible in
un-induced plasmid
indicates leaky
expression of proteins

Interpretations from gel

- The gel was observed and it was interpreted that all the proteins were induced in all the samples as bands were visible
- Some samples had thicker bands than the others
- Also a slight band was observed in un-induced PS107 also due to leaky basal expression of proteins

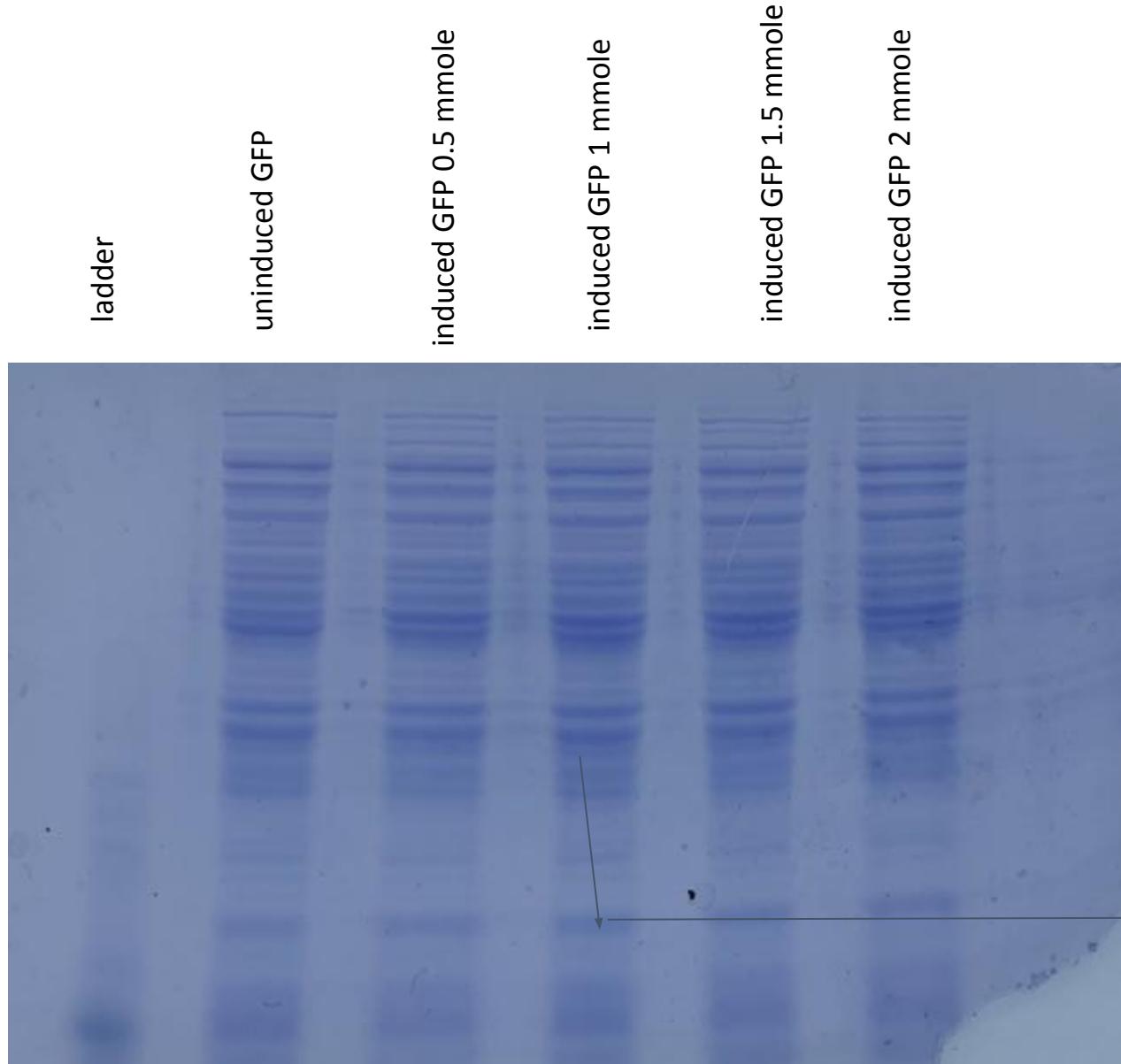
Principle of IPTG Induction



Function of basic T7 Lac Promoter System in *E. coli* (DE3)

Optimization of IPTG concentration

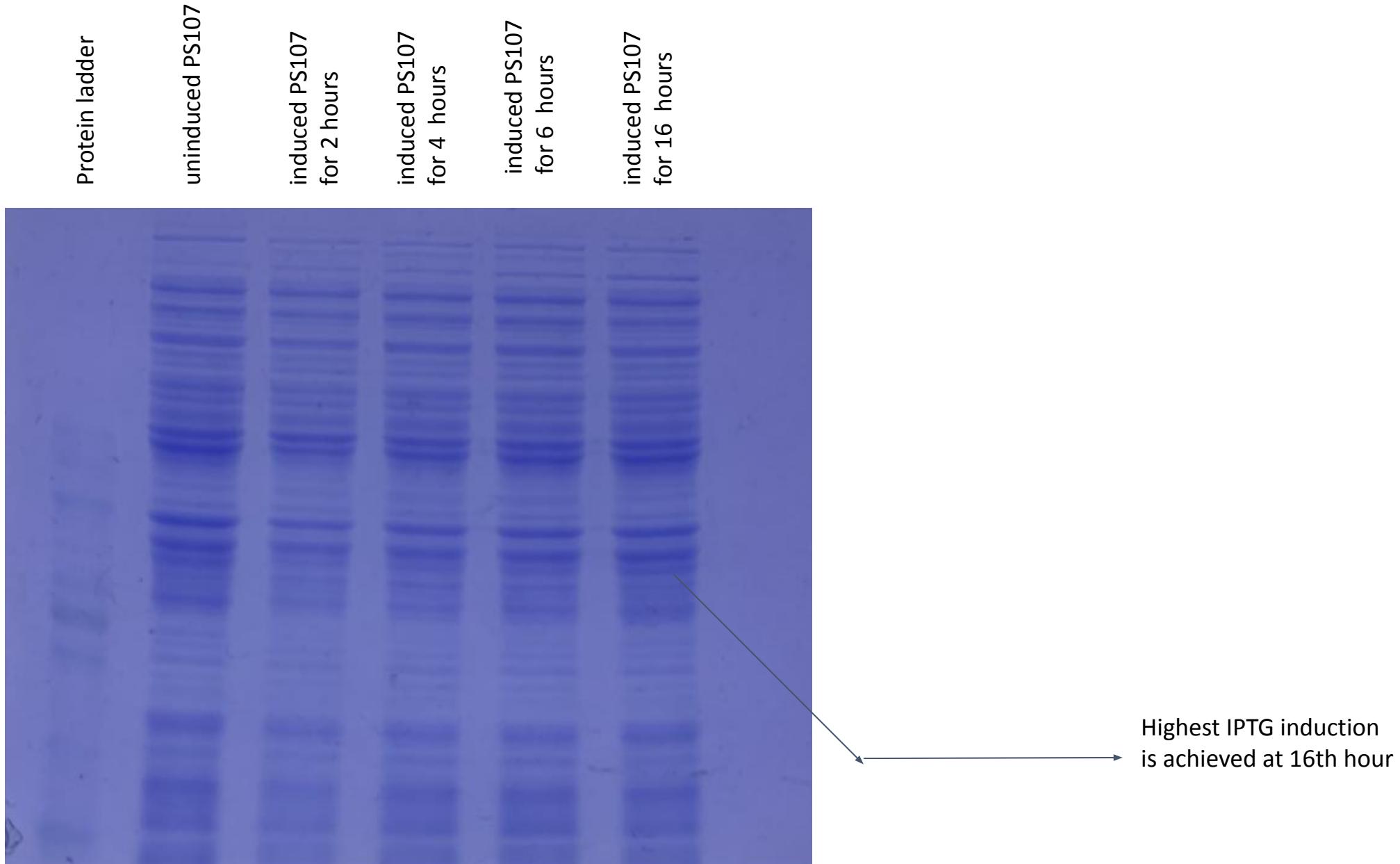
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we used 4 different concentration
of IPTG to induce i.e. 0.5 mmol, 1
mmol, 1.5 mmol, 2 mmol

Highest induced band is
at 1 mmol concentration of
IPTG

Optimization of duration of IPTG induction



Optimization of duration of IPTG induction

