EFFECT OF IRON ON BACTERIAL GROWTH & FBP PRODUCTION

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Abstract

FBP is an iron binding protein in gram negative bacteria that has the molecular weight of 37 kDa. It sequesters iron from transferrin which is an iron binding protein in humans. If the iron-binding of FBP is prevented, the bacteria would die as it cannot take iron from transferrin. Since an alternative drug to antibiotics can be found, further investigations of FBP structure are needed. We focused on the optimization of iron concentration to synthesize more recombinant FBP in bacteria to be used in structural studies. After the optimization of iron concentration, we have made the purification of our protein of interest.

Keywords: Iron-binding, FBP, protein purification, bacterial growth, iron.

1 Introduction

Bacteria need some conditions to maintain their life and grow. They require nutrients to provide energy and water to use in their metabolic activities to grow. Some environmental conditions are necessary in bacterial growth such as the pH, temperature range, amount of light, concentration of gasses and amount of pressure present[1]. When optimum conditions are provided, bacteria can grow very quickly and they are easy to be grown in the lab. Since it has been determined to grow bacteria in a closed system, they will grow in a predictable pattern. This pattern refers to growth curve composed of four different phases of growth: the lag phase, the exponential or log phase, the stationary phase, and the death or decline phase. In lag phase, bacteria try to adjust to their new conditions. Once bacteria adapt to the environment, they start multiplication exponentially. And the phase in which very rapid bacterial division occurs is called exponential or log phase. In this phase bacterial growth rate can be calculated by the formula: $P_{(t)} = P_{(0)} \times e^{rt}$. $P_{(t)}$ is the population at a specific time, $P_{(0)}$ is the initial population and r is the

[1] Sciencing. (2018). *What Three Conditions Are Ideal for Bacteria to Grow?*. [online] Available at: https://sciencing.com/three-conditions-ideal-bacteria-grow-9122.html [Accessed 10 Jul. 2018]

growth rate of bacteria. The last phase is the death or decline phase in which the number of bacterial population decreases[2].

Iron is essential for all living organisms. In mammalians it is responsible for carrying oxygen and in all organisms it is used in metabolic processes[3]. Iron is found in the nature as its Fe^{2+} or Fe^{3+} ions. And these ions damage to biological macromolecules. That is why organisms need a protein to carry iron ions. Transferrin is an iron carrying protein in human cells. However, FBP (ferric binding protein) is an iron binding protein in gram negative bacteria. When iron concentration lacks in the environment, bacteria that have FBP can overcome with this problem by hijacking iron from iron carrying proteins like transferrin. Receptors of outer membrane of the bacteria catches transferrin and separates iron from it and iron is shuttled to the periplasm. In periplasm, FBP gets iron and carries it to the cytoplasm[4]. Because of the iron stealing mechanism of the bacteria, it is needed to be understood the structure of FBP for further studies.

H. Influenzae is a gram negative bacterium that has FBP coding gene. Since it is hard to purify FBP from other proteins in *H. Influenzae*, recombinant technology can be used to produce FBP from BL21 strain of *E.Coli* which is efficient for protein expression. Normally, *E.Coli* cannot produce FBP and thus FBP coding gene can be inserted in a plasmid called pET28a (+) with recombinant technology. In this technology it is possible to insert the gene of interest between the restriction sites which can be digest by restriction enzymes. In this project pET28a (+) was used as plasmid and it includes two restriction sites, Kanamycin resistance gene, TEV and His-Tag. His-Tag consists six Histidine. The importance of this amino acid is it has an aromatic carbon in its side chain and aromatic carbons bind to metal ions. So this quality can be used to separate the protein of interest from others if FBP coding gene inserted near to the His-Tag. TEV is a cleavage site discovered in Tobacco Etch Virus which provides to separate FBP from His-Tag. Even the gene has been inserted in the plasmid, it cannot be expressed. Since repressors suppress the production of FBP, IPTG, an inducer, (Isopropyl-ß-D-thiogalactopyranoside) is needed to induce FBP gene expression.

After inducing the ferric binding gene expression in constructed BL21 strain of *E. coli*, in order to obtain the expressed proteins, cells were lysed with the BugBuster protocol by Novagen to burst the cell membrane and destroy the DNA. So, by the EDTA free protease inhibitor addition, only remaining is the cytoplasm of the bacteria with all proteins in it since the protease are inhibited. Additionally, being EDTA free is crucial to avoid EDTA from stealing iron from the environment.

The purification step requires Metal Affinity Chromatography that includes a metal column with metal ions inside. The recombinant protein with its 6xHistidine residues provide the binding to immobilized metal ions such as nickel, copper and cobalt. Since the metal used in this experiment is Nickel, His-tagged proteins can be separated easily by binding to nickel ions in column with proper buffers[5]. The buffers used for purifications are mainly purification, washing and elution buffers. The elution buffer plays a significant role as the imidazole in it has higher affinity than His-tag in FBP to nickel-resin. Thus, the protein of interest is replaced with the imidazole and it can be obtained from the flow through.

For protein dialysis the mainly used materials are the dialysis buffer and membrane with specific cut-off. That cut-off is the pores in the membrane which determines a specific molecular

[2] Bruslind, L. (n.d.). Microbiology. Retrieved from http://library.open.oregonstate.edu/microbiology/chapter/microbial-growth/

[3] Liu, G. (2015, December). *Design and development of a microfluidic device to monitor iron binding dynamics in iron transport proteins*.

[4] Krewulak, K. D., & Vogel, H. J. (2007, August 19). Structural biology of bacterial iron uptake. Retrieved from https://www.sciencedirect.com/science/article/pii/S0005273607002738

[5] His-tagged Proteins–Production and Purification | Thermo Fisher Scientific - US. (n.d.). Retrieved from https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/his-tagged-proteins-production-purification.html

weight to enable the free passage of molecules smaller than that specific molecular weight by the help of dialysis buffer. Hence, the molecules above that cut-off range stay inside the membrane to ensure its purification, in this case membrane with 12 kDa cut-off ensures with purification of the protein of interest with 37 kDa.

Another method called SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is for the separation of proteins based on their molecular weight[6]. SDS is an amphipathic detergent that causes the protein denaturation and disassociation with each other. It is negatively charged and masks the charge of a protein. During SDS-PAGE, all proteins migrate from cathode to anode. Proteins which have less molecular weight move further than others[7].

2 Methods & Results

2.1 Preparing the Plates and Seeding the Bacteria

For LB agar preparation, 20 grams of LB Agar mixture was weighed carefully to prevent its spreading and added into a labeled 1 L glass bottle that has the compound name, date and the owner written on it. Then 500 ml ddH_2O was put on it. In order to obtain a homogenous fluid, magnetic fish was used for mixing.

For LB broth, 25 gr of LB medium mixture was weighed and put into another labeled 1 L glass bottle. 1 L ddH_2O was added slowly onto our chemical compound.

Both of these steps above were performed on the bench since the bottles were autoclaved to sterilize after preparation. It is important to pay attention to put the bottles with loose lids into autoclave machine to prevent any explosion inside the machine due to the difference in pressure.

LB Agar stock was immediately mixed via magnetic fish before it solidifies. LB Agar was poured slowly into plates by avoiding formation of bubbles. 16 agar plates were obtained from 500 ml of LB Agar mixture. The plates were left open to cool, after some time the lids were half closed. The hood was closed and the plates were left to cool down. When plates were cold and solidified, the lids were closed and the sides were covered with parafilm. The plates were placed upside down to avoid any drops from vaporization. Then they were put into cold room after labeling appropriately to use later.

From the cold room, 2 plates of LB Agar and 2 plates of Kanamycin containing LB Agar were taken. 10 uL of BL21 was spread on to one of the LB with KAN containing plate as a control and also on to one of the only LB containing agar plate by streaking via pipette tip. After that, 10 uL of BL21 with pET28a (+) was spread on to both plates of LB Agar with and without Kanamycin. Then streaking was performed carefully and softly via pipette tip in order to observe the colony formation clearly. After labeling, 4 plates were incubated at 37º C for 16 hours.

As it can be seen from the Figure 3, in the BL21 containing LB plate with Kanamycin, no colony was observed. On the other hand, colony formation was observed in the other three plates.

[6] Oswald, N. (2018, February 16). How SDS-PAGE Works. Retrieved from https://bitesizebio.com/580/how-sds-page-works/

[7] SDS-PAGE. (n.d.) Retrieved from https://ww2.chemistry.gatech.edu/~lw26/course_Information/4581/techniques/gel_elect/page_protein.html

2.2 Growth Culture with Four Different Iron Concentration

In order to prepare big culture, small culture was prepared with 5 ml of LB medium, 5 uL of Kanamycin and the chosen colony of Kanamycin containing LB Agar plate with plasmid containing BL21 into a culture tube and then it was incubated overnight by shaking at 37ºC.

Four different Erlenmeyer flasks were prepared with different $FeCl_2$ concentrations. At first, 100 ml of LB medium was added into each flask. Then, 100 uL of 50 mg/ml Kanamycin antibiotic was added. The labeled first, second, third and fourth flasks were put no $FeCl_2$, 0.2 mM, 0.5 mM and 1 mM $FeCl_2$, respectively. Lastly, 1 ml of overnight starter culture was added into each flask. After shaking briefly, 1 ml of sample was obtained from four flasks into labeled cuvettes to measure the initial OD value at 600 nm. Additionally, as a blank, 1 ml of sample was taken from the LB medium. After that, incubation was started in shaking incubator at 221 rpm and 37ºC for all four flasks.

The OD_{600} was measured twice per hour until the OD value reaches around 0.65. When the OD was measured as 0.70, measurements were performed more often as four times per hour. Meanwhile, centrifuge was performed with taken samples at OD value equals around to 0.3, 0.6 and once per hour after the 0.6 value. Then, the supernatant was discarded whereas the pellets in eppendorfs were kept at -20 ºC fridge.

2.3 FBP Expression via Induction Culture with Four Different Iron Concentration

As in the growth culture, a small culture with modified BL21 was prepared and incubated overnight at 37ºC before starting.

Each of four different flasks were added with 100 ml of LB medium, 100 uL of 50 mg/ml Kanamycin and four different concentrations of $FeCl_2$ that are 0, 0,2, 0,5 and 1 mM. It is crucial to shake $FeCl_2$ before adding as it easily precipitates. After that, 1 ml of overnight starter culture was added in each four. Incubation of flasks was started at 37 ºC and 221 rpm. Meanwhile, the OD_{600} measurements were performed for the samples taken twice per hour. Blank was again taken from the LB medium. As taking sample to measure as the initial OD value was forgotten, first OD value was considered as initial. Additionally, the samples taken per hour were centrifuged to keep their pellets at -20 ºC fridge.

As the OD_{600} value had reached around 0.65, flasks were taken from the shaking incubator to stay at room temperature for 15 minutes. Then, 0.7 mM of IPTG was added into each flask to induce FBP gene expression. After that, incubation of flasks was continued at 26 ºC and 221 rpm for 5 hours under shaking. After 5 hours of incubation, the flasks were taken from the incubator and incubated on ice for 20 minutes to avoid heat shock. During that time, last samples were taken from each flask to measure OD_{600} value.

After ice incubation, each culture was transferred into labeled falcons to be centrifuged at 4750 rpm and 4 ºC for 30 minutes. At the end, supernatant was discarded whereas the pellets were stored at -80 ºC freezer.

2.4 12% SDS-PAGE Gel Running for Checking the Dilution Ratios

Lysis with Lysozyme

Weight of centrifuged cells in 2 mL eppendorf tubes were measured to calculate the amount of lysozyme that should be added which is shown in Table 3. Then cells in each tube were resuspended in 200 uL of lysis solution.

Lysis solution:

- 50 mM Tris-HCl, pH 8.0
- 0.5 M NaCl
- 1 mM PMSF
- 30 U/uL lysozyme for 4000 U/g cell paste
- 1mM EDTA, pH 8.0

Resuspended cells were sonicated for approximately 5 minutes and 5mM $MgCl_2$ and 100 ug/mL DNAse were added in each tube. Cells were incubated on ice for 30 minutes and then centrifuged in eppendorfs for 10 minutes at maximum speed. After centrifugation, supernatants were separated into clean tubes. According to their OD_{600} values, samples were diluted with dilution buffer which includes 10mM Tris, pH 8.0; 15 mM NaCl; 1mM $MgCl_2$. Dilution ratios were shown in the Table 4&5. Before loading the samples to SDS-PAGE gel, samples were dyed via mixing 5 uL of 6X loading dye (beta-mercaptoethanol) with 25 uL of sample to provide dilution. After heating them at 95°C for 8 minutes, samples were loaded into wells of the gel.

12% SDS-PAGE Gel Running

After loading the samples, running was started at 29 Ampere and increased to 40 Ampere after 10 minutes of running. Gels were run approximately 60 minutes and then stained with Coomassie Blue about an hour. After staining, gels were destained to remove Coomassie Blue for 2 hours and left to overnight.

Sample ID	OD_{600} value	Weight (mg)	Lysozyme amount
3A	0,305	0.013	2 uL
3a	0,335	0.012	2 uL
3C	1,528	0.007	1 uL
3c	1,240	0.043	6 uL

Table 3: Lysozyme that was used according to the weights of the samples.

Gel no	Sample no	Sample ID	OD_{600} value
1	1	3A	0,305
	2	3a	0,335
	3	3A-D1	0,1525
	4	3a-D1	0,1675
	5	3A-D2	0,07625
	6	3a-D2	0,08375
	7	3A-D3	0,03813
	8	3a-D3	0,04188

Gel no	Sample no	Sample ID	OD_{600} value
2	9	3C	1,528
	10	3c	1,24
	11	3C-D0	0,3056
	12	3c-D0	0,31
	13	3C-D1	0,1528
	14	3c-D1	0,155
	15	3C-D2	0,0764
	16	3c-D2	0,0775
	17	3C-D3	0,0382
	18	3c-D3	0,03875

Table 4&5: Samples were diluted to different OD_{600} values.

2.5 12% SDS-PAGE Analysis of 0.2 mM and 1 mM Iron Concentration for FBP Expression

Gel Preparation

12% SDS-PAGE gels were prepared by making 10 ml of 12% separating gel and 5 ml of 5% stacking gel for 2 gels .Ingredients were mixed with the order in the protocol of Table 6.

12% Separating	**10 ml for 2 gels**
1. ddH_2O	4.62 ml
2. 3M Tris, pH 8.9	1.25 ml
3. 20% SDS	50 uL
4. 30% Polyacrylamide	4 ml
5. 20% APS	75 uL
6. TEMED	5 uL
5% Stacking	**10 ml for 2 gels**
1. ddH_2O	3.85 ml
2. 1M Tris, pH 6.8	250 uL
3. 20% SDS	10 uL
4. 30% Polyacrylamide	850 uL
5. 20% APS	37.5 uL
6. TEMED	2.5 uL

Table 6: 12% SDS-PAGE gel ingredients.

First, the separating part was made and placed into glass plates and then the stacking part was added on it for both gels. When the solutions were solidified, they were removed from the instrument and placed in wet towels rolled with aluminum folio at 4 ºC fridge to avoid drying as they were not been used immediately.

Lysis with Lysozyme

The centrifuged cells' weights were measured to calculate amounts of lysis buffer to be added. Then, cells were resuspended in lysis solution and sonicated for 5 minutes. After that, 5mM $MgCl_2$ and 100 ug/mL DNAse were added in each tube. Since cells in falcon tubes were not resuspended well, they were resuspended and sonicated again before adding DNAse.
Cells were incubated on ice for 30 minutes, then centrifuged in eppendorfs for 10 minutes at maximum speed and supernatants were taken into clean tubes. All of samples were diluted as their OD_{600} values equal to 0.155.This time samples were diluted with water mistakenly. However, it does not affect the SDS-PAGE running and only causes the protein dysfunction. Samples were dyed by 1X loading dye via mixing 5 uL of 6X loading dye with 25 uL of samples for each tube. Samples were heated at 95°C for 5 minutes.

time	sample ID	lysozyme (uL)	lysis buffer (uL)
0 h	2b	2,5	197,5
	4b	4,3	195,7
1 h	2c	0,9	199,1
	4c	4,3	195,7
3 h	2e	1,7	198,3
	4e	0,7	199,3
5 h	2z	3,1	3496,9
	4z	2,3	3497,7

Table 7: Sample descriptions and the lysozyme amount to be added.

Staining and Destaining Buffer Preparation

Staining Buffer (1L) includes 50% Methanol, 10% Acetic Acid and 2.5 grams of Coomassie Blue whereas Destaining Buffer has the same contents except Coomassie Blue. They both filled with distilled water until solution reaches 1 liter.

12% SDS-PAGE Gel Running

Samples were loaded into the gel as in the Figure 9. As a start, gel was run at 20 Ampere and currency was increased after 10 minutes to 30 Ampere. The gel was run for 40 minutes. Then stained and destained with buffers which were previously prepared. Since buffers have the higher concentration of methanol, the gel shrank. However, when it was rehydrated with water, it turned back to its original size without any damage on samples.

2.6 Lysis with BugBuster Protocol

Preparation of 1X BugBuster Solution

10X BugBuster was diluted with HEPES buffer (pH 7.5) and EDTA free protease inhibitor tablet (1tablet/400 mL) was dissolved in the solution.

Lysis of Pellets

Pellets were resuspended in 1X BugBuster solution. 5 mL solution was used for 1 gram of pellet. Then, 1uL benzonase was put per 5 mL resuspended solution and it transfered into falcon tube to incubate it in incubating rocker for 20 minutes at room temperature. Since resuspended solution includes proteins, further steps were performed at 4°C, on ice and solution was transfered into a centrifuge tube to centrifuge for 20 minutes at 16000xg and 4°C. After centrifugation, supernatant was kept in a falcon tube and 50 uL of it was taken as a sample to load the SDS-PAGE gel later.

2.7 Purification of FBP

All steps in purification were performed in cold room at 4°C.

Since nickel resin might be dried, it was needed to wash before using Ni-affinity column. So, column was washed with EtOH, ddH_2O respectively and equilibrated with washing buffer. After column was equilibrated, the liquid was discarded. Supernatant from lysed cells was mixed with Ni resin and incubated for 1 hour at 4°C shaking incubator. Since FBP was bound to Ni resin, other proteins flowed down and flow through was taken to load SDS-PAGE gel later. Then column was washed with washing buffer. First, 8 mL of washing buffer added and incubated for 5 minutes in shacking incubator and 50 uL of sample was taken from it. Then column was washed with 4 mL washing buffer two times. After that, 8 mL of elution buffer was added in the column. Since imidazole in elution buffer binds to Ni and separates FBP from Ni beads, FBP was flowed down. And flow through was obtained to load the gel later. 4 mL of elution buffer was needed to be added into the column three times but mistakenly first of the 4 mL additions was performed with washing buffer. Because of that, elution buffer was added one more time. In each addition, samples were taken from flow through to load the gel later. And FBP including solution was kept in 4°C to use it in dialysis. Lastly, Ni-affinity column was washed with washing buffer, ddH_2O and EtOH respectively.

<u>Buffers Used in Purification:</u>

Washing buffer (150 mL):

- 1X Purification Buffer
- 10 mM BME (beta mercaptoethanol)
- 0.2 mM PMSF
- Filled with water

Elution buffer (50 mL):

- 280 mM Imidazole
- Filled with water

2.8 Dialysis

Dialysis was performed in cold room at 4°C.

Dialysis membrane was cut and waited in PBS buffer for 10 minutes before using it. It is the best to cut 1 cm for 2 mL of FBP solution. Therefore, 10 cm membrane was used for 20 mL solution. Membrane was tied from one end and then, FBP solution was transferred into the membrane. After that membrane was tied from the other end. And membrane that was including FBP solution was put in 2 L PBS buffer to incubate for 7 hours. After 7 hours of incubation, the buffer was renewed and incubation continued for overnight. After that 50 uL sample was kept to load the gel. Then, remaining FBP was frozen by liquid nitrogen and kept in -80°C.

PBS Buffer (2 L):

- 137 mM NaCl
- 2.7 mM KCl
- 38 mM NaH_2PO_4
- 1.8 mM KH_2PO_4
- pH was adjusted to 7.4

2.9 Concentration Measurement with NanoDrop

Concentration of samples taken from lysed supernatant, flow throughs and FBP after dialysis was measured with NanoDrop as in the Table 8. Elution buffer was used as blank for lysed supernatant and different flow throughs, dialysis buffer was used as blank for FBP taken after dialysis.

2.10 Protein Characterization via SDS-PAGE Analysis

Samples were obtained from lysed supernatant, flow throughs and FBP after dialysis. Flow through after 5th elution buffer addition and FBP after dialysis were diluted as in the Table 8. Then all samples were dyed with 1X of 6X loaded dye and heated at 95°C for 5 minutes and dyed samples were loaded to the gel. Running currency was started at 19 Ampere. After 10 minutes it was increased to 28 Ampere. 45 minutes later, running of the gel was stopped and the gel was stained with Coomassie Blue dye that was prepared before. Since dye includes high concentration of methanol, the gel was waited only for 10 minutes in the solution and then, the gel was waited for 1 hour in the staining buffer diluted with water. After that, the gel was destained with diluted destain solution. Since SDS-PAGE gel was not observed clear due to overloading, SDS-PAGE gel electrophoresis was repeated which can be seen in the Figure 12. But this time, all samples were diluted as in the Table 8. After dilution, the same procedure was applied and the clear gel screening was obtained.

Sample name	Label	Concentration (ug/uL)	Diluted concentration (ug/uL)
Lysed supernatant	LS	4.31	0.69
1st Flow through(FT)	FT	2.48	0.60
FT after washing buffer	WS	4.74	0.76
FT after 1st elution buffer(EB)	ES1	1.53	0.49
FT after 2nd EB*	E2	9.48	0.76
FT after 3th EB	E3	0.70	0.56
FT after 4th EB	E4	3.17	0.51
FT after 5th EB	E5	2.44	0.59**
FBP from dialysis	FBP	2.54	0.61**

Table 8: Initial and diluted concentrations of samples. * Washing buffer was added instead of elution buffer mistakenly. **Samples were diluted to given concentration for both first and second SDS-PAGE analysis.

3 Results

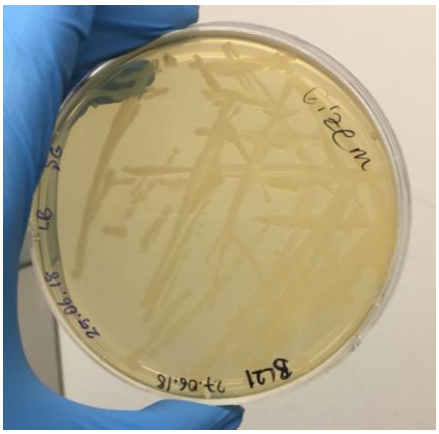


Figure 1: LB plate with 10 Ul BL21

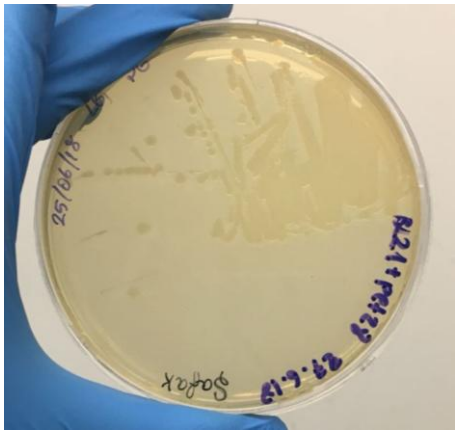


Figure 2: LB plate with 10 uL BL21+ pET28a(+)

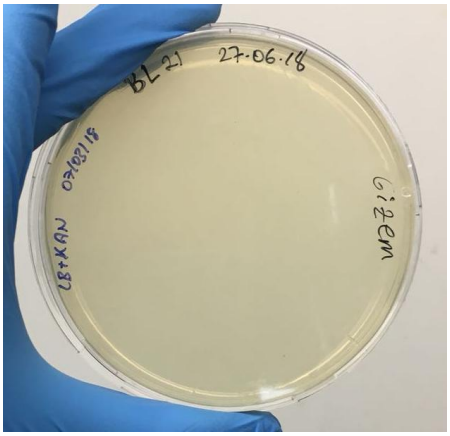


Figure 3: LB + KAN plate with 10 uL BL21

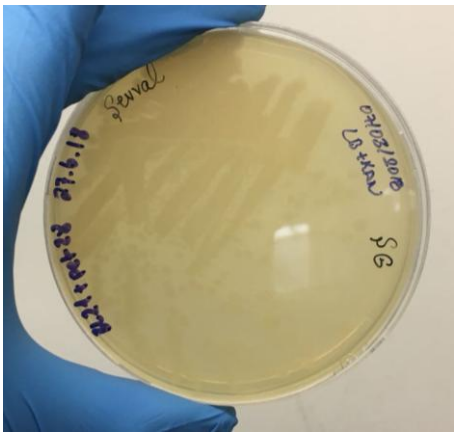


Figure 4: LB + KAN plate with 10 uL BL21+ pET28a(+)

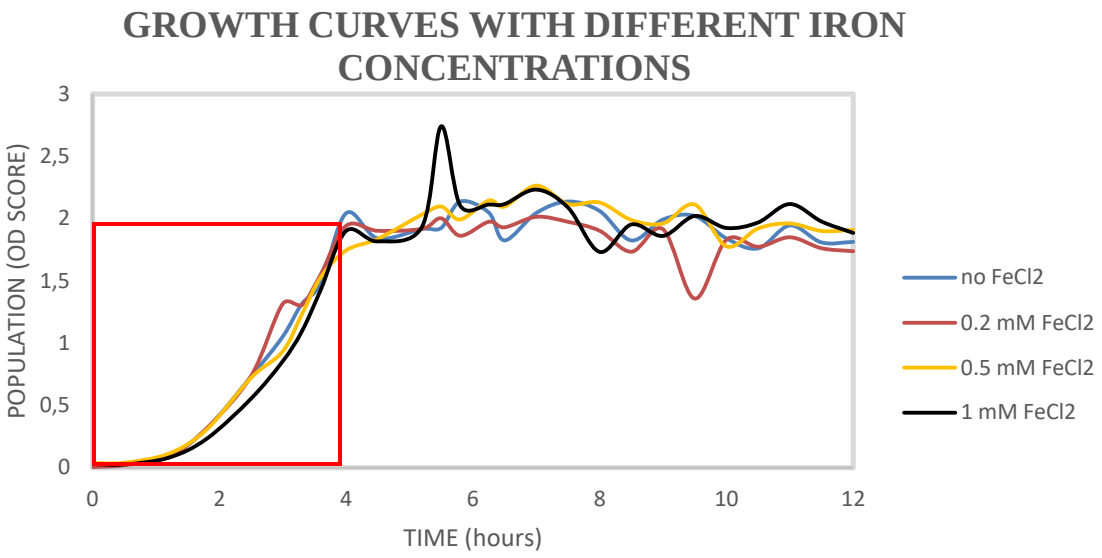


Figure 5: Growth curves with 0, 0.2, 0.5 and 1 mM iron concentrations

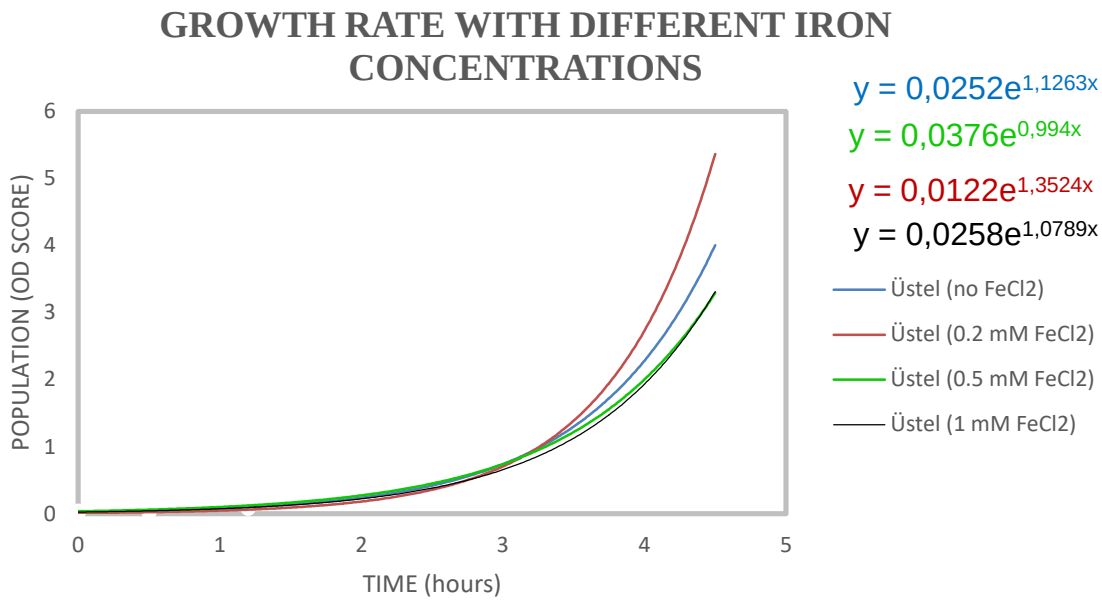


Figure 6: Graph that indicates the rates of exponential growth as shown in Figure 5 with red rectangle.

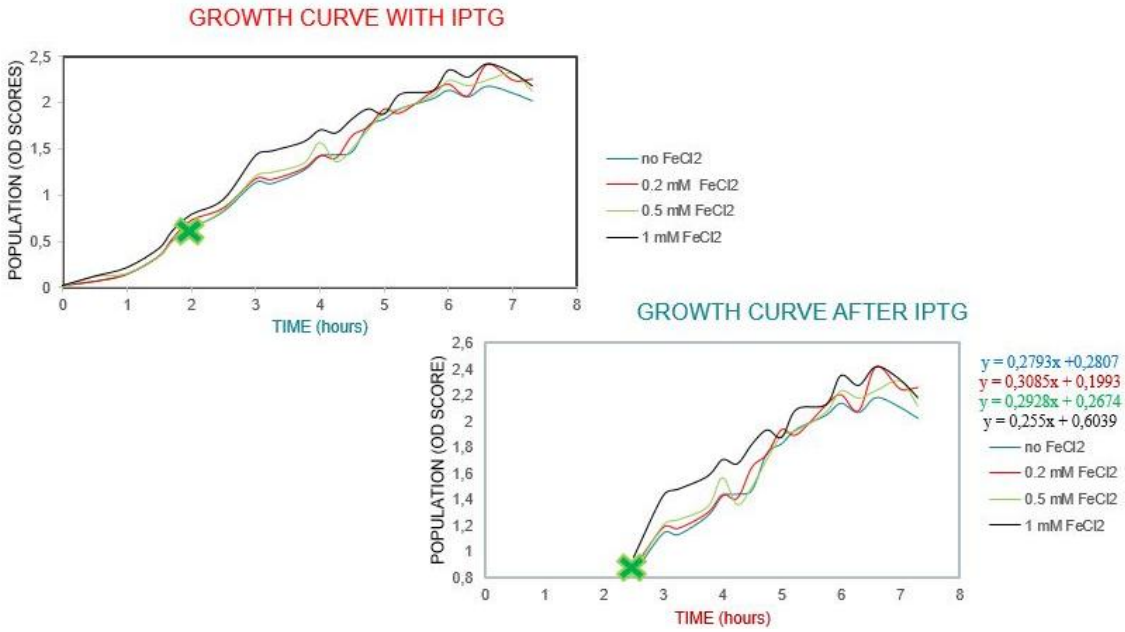


Figure 7: The graphs of the OD_{600} data of induction culture with different iron concentrations. Green cross symbolizes the time of IPTG addition.

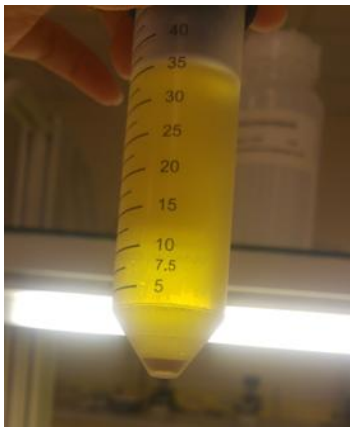


Figure 8: The precipitated pink yield is pellet whereas the supernatant is the LB medium.

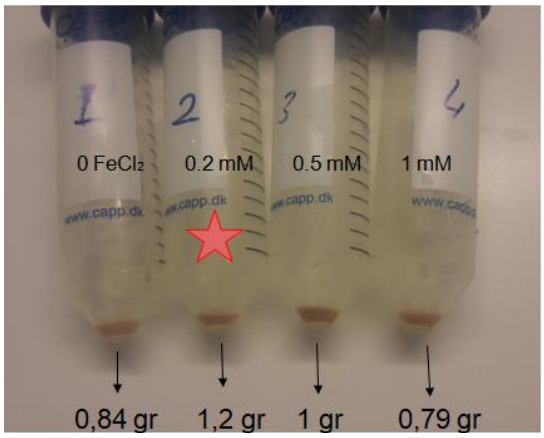


Figure 9: The precipitated pink yields of different iron concentrations. The higher yield is 1.2 grams from the 0.2 mM concentration as shown with a pink star.

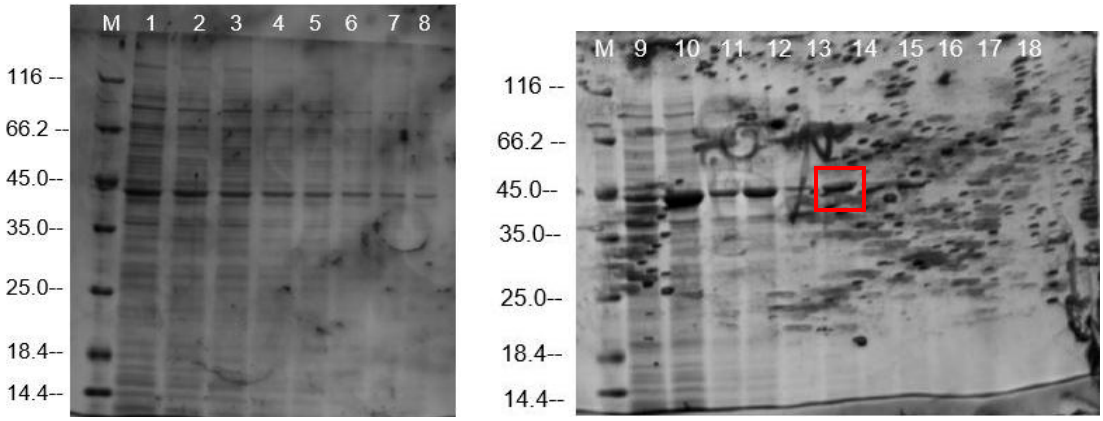


Figure 10: Diluted samples as shown in Table 4&5 were loaded to SDS-PAGE gel. Red rectangle indicates band with the optimum dilution ratio as 0.155.

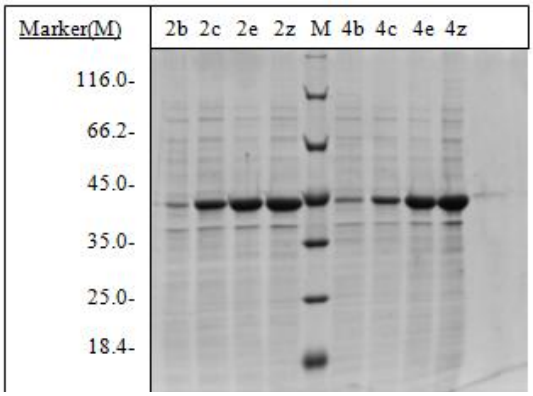
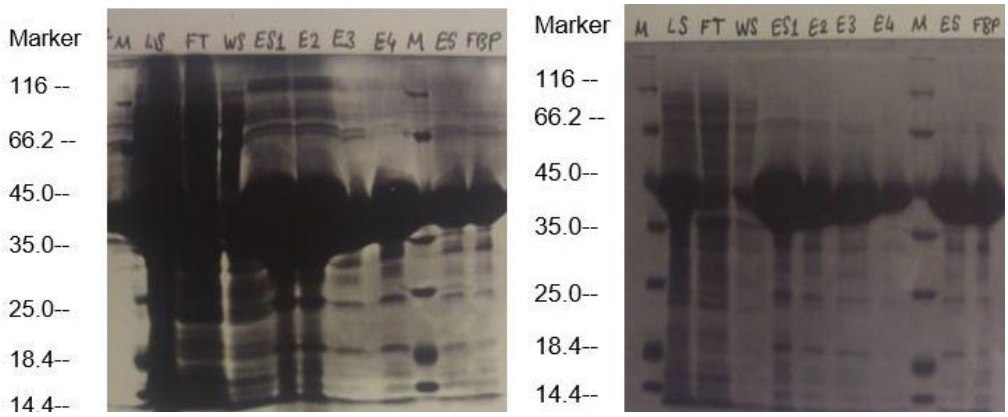


Figure 11: SDS-PAGE gel result. Samples named with 2 has the 0.2 mM and 4 has the 1 mM $FeCl_2$ whereas b: t=0, c: t=1 h, e: t=3 h, z: t=5 hours after IPTG addition.



(a) (b)

Figure 12: (a) is the first and (b) is the second SDS-PAGE gel. "M" refers to Marker and labels of the samples are indicated in the Table 8.

4 Conclusion and Future Work

Determined four different iron concentrations were examined by checking several modeling such as comparing yield weights, plotting the graph for modeling the growth rate by OD_{600} data and 12% SDS-PAGE gel running to compare the FBP production.

As in Figure 9, comparing yield weights suggest that the most efficient expression of FBP is from the 0.2 mM of $FeCl_2$ concentration with 1.2 grams of yield. The efficiency order of the rest from higher to lower is 0.5 mM, 0 mM, 1 Mm according to weights of the yields. Additionally, although no $FeCl_2$ was added to the first culture, it can be seen that first falcon has also pink pellet. Since the pink color occurs by the binding of FBP to iron source and there is no iron added into first culture, it may demonstrate the iron-binding from the LB medium. Thus, even from the light color of pink yield, one can conclude the LB medium has its own amounts of iron source. Therefore, the iron amount in the LB medium should be measured to be clear in the results.

The periodically taken data of OD_{600} refers to both population and turbidity of the sample. When the plots of the data of the growth culture were examined, the exponential growth can be detected clearly until the log phase around 0.65. As in the Figure 6, the highest growth rate from the formula is 1.352 that belongs to 0.2 mM concentration. After the log phase, the steady state can be seen as expected. To conclude, 0.2 mM iron concentration is the best for the bacterial growth.

In the induction culture graph, the bacterial growth slows down compare to growth culture since the induction of gene expression cause bacteria not only growing but also expressing the protein of interest. Additionally, after inducing by the IPTG addition, there is a linear increase observed which may refer to the continuous production of FBP. As in the Figure 7, the highest slope as 0.309 from 0.2 mM concentration refers to the greatest increase and so the FBP production.

The different SDS-PAGE gels was run for several reasons such as determining the best dilution ratio of the OD_{600} scores, comparing the FBP production of different $FeCl_2$ concentrations and checking the purification of FBP. As in the Figure 10, when the bands of the

gel are compared, the clearest band results from the OD_{600} diluted to 0.155. Therefore, dilution ratio of 0.155 was used for the further dilutions of the samples to load the gel. Moreover, the comparison of the thickness and the darkness of the bands indicate the proteins included in that sample with different molecular weights. Hence, according to the principle of the SDS-PAGE analysis, the protein of interest at 37 kDa can be seen easily with its greater thickness in Figure 11. So, one can conclude that the left-hand side of the marker has greater and increasing thickness by time with 0.2 mM of $FeCl_2$ than 1 mM.

During the staining of the former SDS-PAGE gel, it was realized that the gel size was decreased and then in the destaining step it was shrunk by half. The investigations on that issue were concluded as if the gel stays much in the access amounts of alcohol, this causes shrinking by its dehydration. On the other hand, if it is hydrated much, it gets larger. Therefore, the further shrinking was avoided by treating the gel with water.

The screenings of the last SDS-PAGE for the samples got from lysed supernatant, purification step and dialysis can be seen in the Figure 12. The reason of repeating the gel running is because the overload in the Figure 12(a) which prevents the clear seeing and further comments. Therefore, the second gel was run with increased dilutions as in the Figure 12(b) to be able to prevent the overload and to comment on the result. According to the samples called Elution Sample 5 (E5) and FBP that are expected to have the highest purified FBP in them, there are more bands observed rather than FBP at 37 kDa. Therefore, one can conclude that the FBP is not purified well and there are other proteins inside the solution. One reason behind this issue can be the old Nickel beads inside the affinity column. This issue can be based on the samples named Flow Through (FT) and Washing Sample (WS) in Figure 12(b). No band was expected at 37 kDa level of those samples. However, there are slightly thin band formations that say the solution has FBP inside. Thus, that old and unqualified Nickel resin may not ensure the purification by being unable to bind FBP. Furthermore, another cause of unpurified FBP may be the failure at the washing step due to not shaking well so that rest of the proteins did not leave the column. Later in the elution step, the proteins inside the column could flow through as well as the FBP. Hence, the last solution assumed to be the purified FBP contains the left proteins.

To conclude, the best iron concentration to be used in further experiments is 0.2 mM for both the optimum bacterial growth and FBP production. Although experiments on iron concentration was performed to optimize the FBP production and bacterial growth, not only iron concentration but also other conditions such as IPTG concentration, time and temperature affects the protein production and growth rate of bacteria. Hence, as future works those conditions should also be optimized so that FBP can be produced and investigated easily.

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