EXPERIMENTAL EVOLUTION: ADAPTATION OF MICROORGANISMS TO EXTREME ENVIRONMENTS

QR code of interview with the group

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Abstract

To observe evolution in a short time period, microorganisms are best candidates because they grow very fast and have large populations. Experimental evolution is a technique used to evolve microorganisms in a laboratory.

Organisms may adapt to dynamic conditions of their environment through genetic mutations. The aim of this study is to conduct directed evolution experiments. Therefore, microorganisms are grown under a particular stressful condition and their adaptive capacity to the condition is observed.

Chemostats and morbidostats are widely used set-ups in directed evolution experiments. Two chemostats and two morbidostats systems were set up for this study. In addition, parameters of these systems were controlled by Arduino board, which provides flexibility in designing distinct systems.

Keywords: Experimental Evolution, *Escherichia Coli(E. coli)*, Mutation, Adaptation, Fitness

1 Introduction

Mutations are errors which may occur during several processes such as DNA replication of a cell. If these mutations increase the fitness of an organism to its environment it is passed from generation to generation over time. This process is called as adaptation in evolution. Experimental evolution is a method to investigate evolutionary processes in a short period of time in controlled laboratory conditions. Microorganisms are commonly used in such experiments due to their certain properties. They have fast reproduction rate, large populations in a small volume and are more error prone than eukaryotes. In addition, the high amount of data and studies

concerning microorganisms is another reason behind the usage of microorganisms (Lenski and Elena, 2003).

Evolution of microorganisms in a laboratory environment can be studied in different ways. Evolution is a slow and long term process under standard conditions however with certain changes and adjustments on the environment, it is possible to speed up the process. By arranging a medium in which microorganisms will be under evolutionary stress, directed mutation can be achieved. Directed evolution is the system of selecting the random mutants occuring in the culture that became more fit to the changes and limitations of the environment. This system of limiting environment does not speed up or cause mutations, yet, expedites the selection of mutants and enables them to survive while others struggle. The case of different experimental evolution techniques can easily be discussed when Lenski's long-term evolution and Van Hofwegen's rapid evolution experiments are considered. Lenski, even though no pressure and restriction was present for bacteria to struggle, observed citrate-utilizing *E. coli* mutants in a period of 30 years (Lenski et al., 2012). On the other hand, other studies show that when bacteria were subjected to evolutionary pressure that cause the direction of population to shift to the wanted mutants (citrate-utilizing, in this case), these results can be gained in a shorter period of time. Van Hofwegen, by lowering the amount of usable carbon sources in the media, created a directed pressure that eventually resulted in the isolation of *E. coli* strain using citrate as a carbon source. While Lenski isolated these mutants in 33.000 generations, in Van Hofwegen's experiment this was achieved within 100 generations (Van Hofwegen et al., 2016).

There are two types of systems to study experimental evolution: chemostat and morbidostat. Chemostat provides chemically static environment for microorganisms in which growth rate is controlled by a limiting factor (Moore, Robson, & Trinci, 2013). Morbidostat provides chemically dynamic environments where the amount of the limiting factor is arranged regularly (Toprak et al., 2013).

Arduino board is a hardware which is useful for creating interactive projects. Coding in Arduino IDE software and then uploading the code into the Arduino hardware the electronic components of a bioreactor system can be controlled via electronic circuits and connections.

1.1 Projects

1.1.a Increasing the Flow Rate

Since some of the bacteria –along with waste medium- are removed from the bioreactor, bacteria must reproduce proportional to the flow rate to sustain their lives in the bioreactor. At the beginning of the experiment, flow rate is arranged such that bacteria reach their physiological reproduction capacity.

Secondly, the system is started as a morbidostat and the flow rate is increased gradually. So, bacterial population is expected to genetically adapt to the increasing flow rates and reproduce faster under the evolutionary pressure of more bacteria removal.

$$\mu = \frac{lnN_2 - lnN_1}{t_2 - t_1}$$

Figure 1: Calculation of specific growth rate from bacteria amount in different times (1)

$$\mu = \frac{F}{D}$$

Figure 2: F: Flow Rate D: volume of the bioreactor(2)

It has been shown that specific growth rate of *E. coli* can increase up to 2.13 after physiological adaptation to its environment (Marr 1991). The main aim of this experiment is to create mutants whose specific growth rate is more than this value in the rich medium where the only limitation is their genetic composition.

The maximum value of specific growth rate of *E. coli* in the literature and a study of bacteria colonization against physical stress, where biofilm formation, bacteria colonization against fluid flow were mentioned and verified (Marr, 1991; Otto, 2014).

1.1.b Nitrogen Fixation

DNA is the hereditary material in living organisms. Information about the organism is stored inside the codons which are composed of bases A, T, G and C (U.S National Library of Medicine). They are the nitrogenous bases found in the DNA (Figure 3). By using the code in DNA; proteins, which are the main components of a living organism, are produced. The monomer of proteins are amino acids, they have four different parts; hydrogen, carboxyl, radical and amino groups (Figure 4). Since nitrogen is found in the structure of DNA and proteins it is an essential element for living organisms.

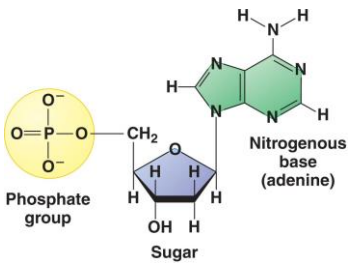

Figure 3: Structure of DNA (3)

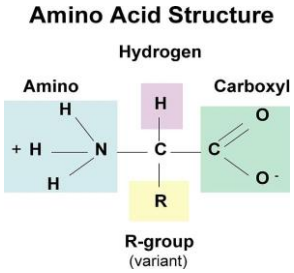

Figure 4: Structure of amino acid (4).

Normally, *E. coli* uses ammonia from its environment as a nitrogen source under aerobic conditions (Reuse, 1970). However; in a specific case, Nielson and Sparell showed that a strain of it can fix gaseous nitrogen under anaerobic conditions by synthesizing nitrogenase. In that experiment they also observed this nitrogen fixation activity is inactivated by oxygen (Neilson & Sparell, 1976). This implies that there is a background for nitrogen fixation in *E.coli.* In this experiment the aim is to see whether they are able to fix nitrogen under aerobic conditions or not. In this study, optimal nitrogen deficient medium in which *E. coli* can survive is found. Additionally, it has been shown that system can be setup and sustained by troubleshooting the problems and preventing the contamination problem.

1.1.c Cellulose Utilization

Carbon is an essential component for living organisms since it is needed for energy production. For *E. coli*, the preferred carbon source is generally glucose under standard conditions. (Chen and Strevett, 2003). As glucose sources in the medium start getting low, organisms move on to other possible carbon sources. (Görke and Stülke, 2008). Alternatives of glucose are found in nature and can be used by organisms including *E. coli* however, there are some alternatives similar to glucose but cannot be hydrolyzed by *E. coli.* Cellulose, a polysaccharide composed of linked D-glucose molecules and cellobiose, a disaccharide of two glucose molecules are examples of sugars that *E. coli* cannot hydrolyze due to lack of certain enzymes. In a directed evolution study, however, it was shown that cellobiose can be the carbon source for *E. coli* under evolutionary stress caused by the lack of glucose (Kricker and Hall, 1984). β-glucosidase enzyme is needed to obtain glucose from cellobiose and the gene

encoding it exists in *E. coli* genome as a cryptic gene which is not expressed in the wild type (Betts and Hall, 1986). With two classes of mutations, it is possible for *E. coli* to express β-glucosidase and in a directed evolution setup, the pressure to use another carbon source would help to select the strain that achieved this ability. (Joseph et al., 2017)(Kricker and Hall, 1984). Similarly, the purpose of this experiment is to see whether *E. coli* can use cellulose instead of glucose in an environment where the glucose level is restricting the growth rate of bacteria in a chemostat.

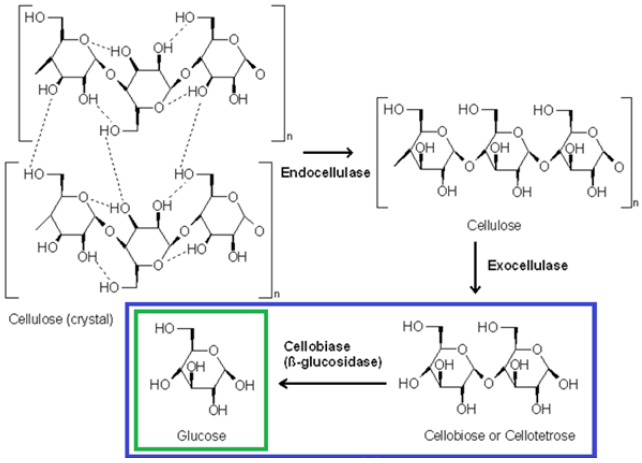

Figure 5: Hydrolysis of cellulose (5)

1.1.d Increasing Metal Binding Capability

Iron is one of the essential elements for living organisms. Iron molecules required for crucial essential metabolic activities such as transportation of oxygen, DNA repair and replication (Lasocki, Gaillard, & Rineau, 2014). However, ferric ions are poorly soluble under aerobic conditions at neutral pH, thus organisms have developed to catch iron as it is in the *E. coli* (Krewulak & Vogel, 2008). In this study, it is aimed to increase the ability of E.coli to catch iron by using a morbidostat designed as shown in the figure 4. Although, the system could not observed for a long period to observe significant changes on iron concentration ,was provided alternative method to produce bacterial iron binding protein, which is not commercially available, for iron binding dynamics researches(Liu, 2015). Also, it provided controlled medium preparation for iron.

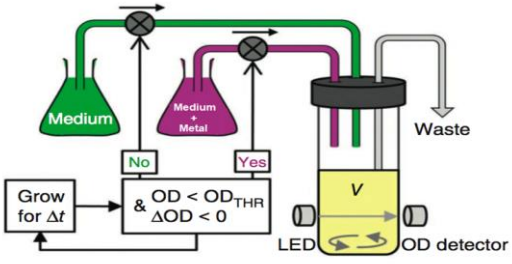

Figure 4: Designed morbidostat (6)

2 Materials and Methods

2.1 Software

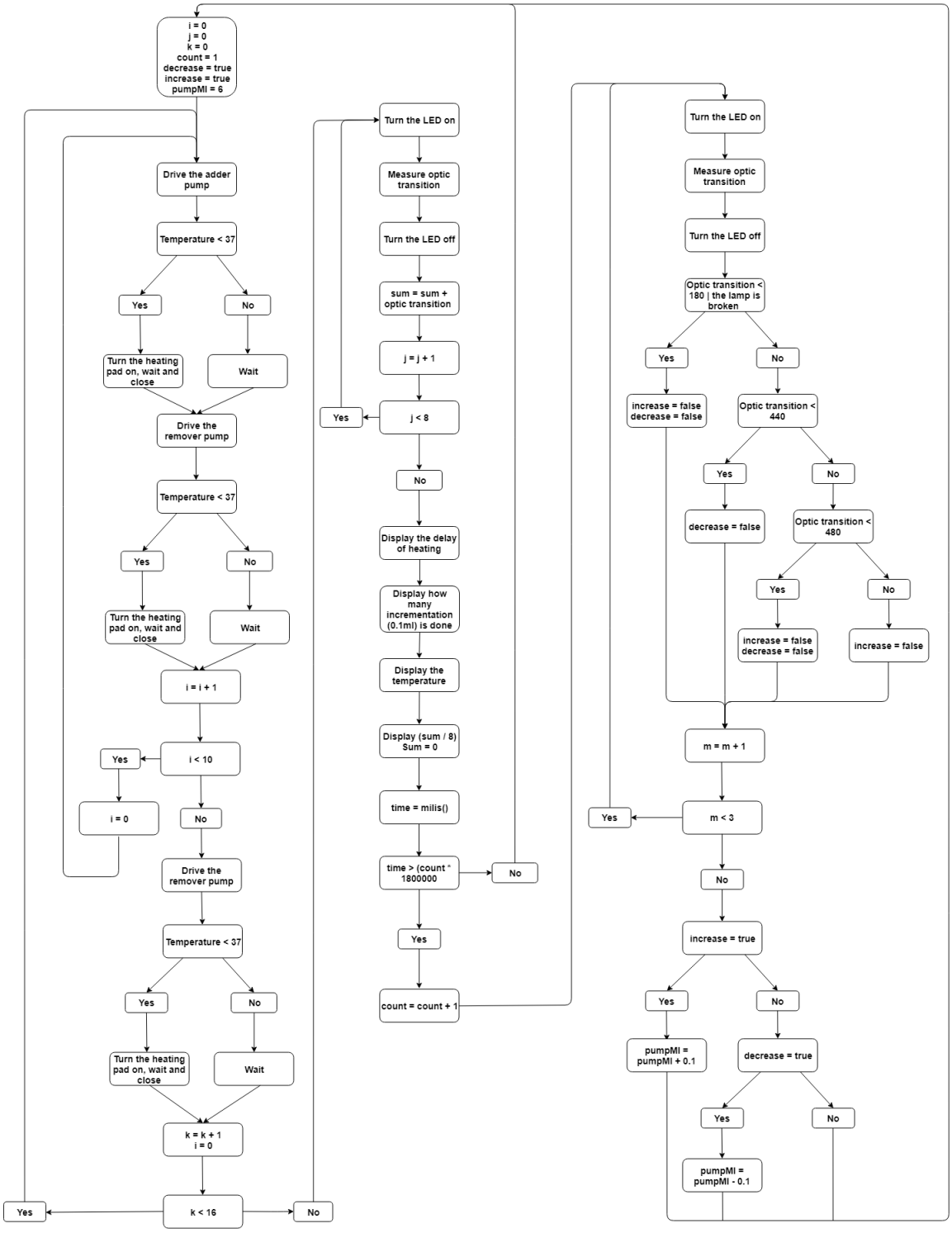

Figure 5: The Algorithm Diagram of Increasing the Flow Rate Experiment in Arduino Software

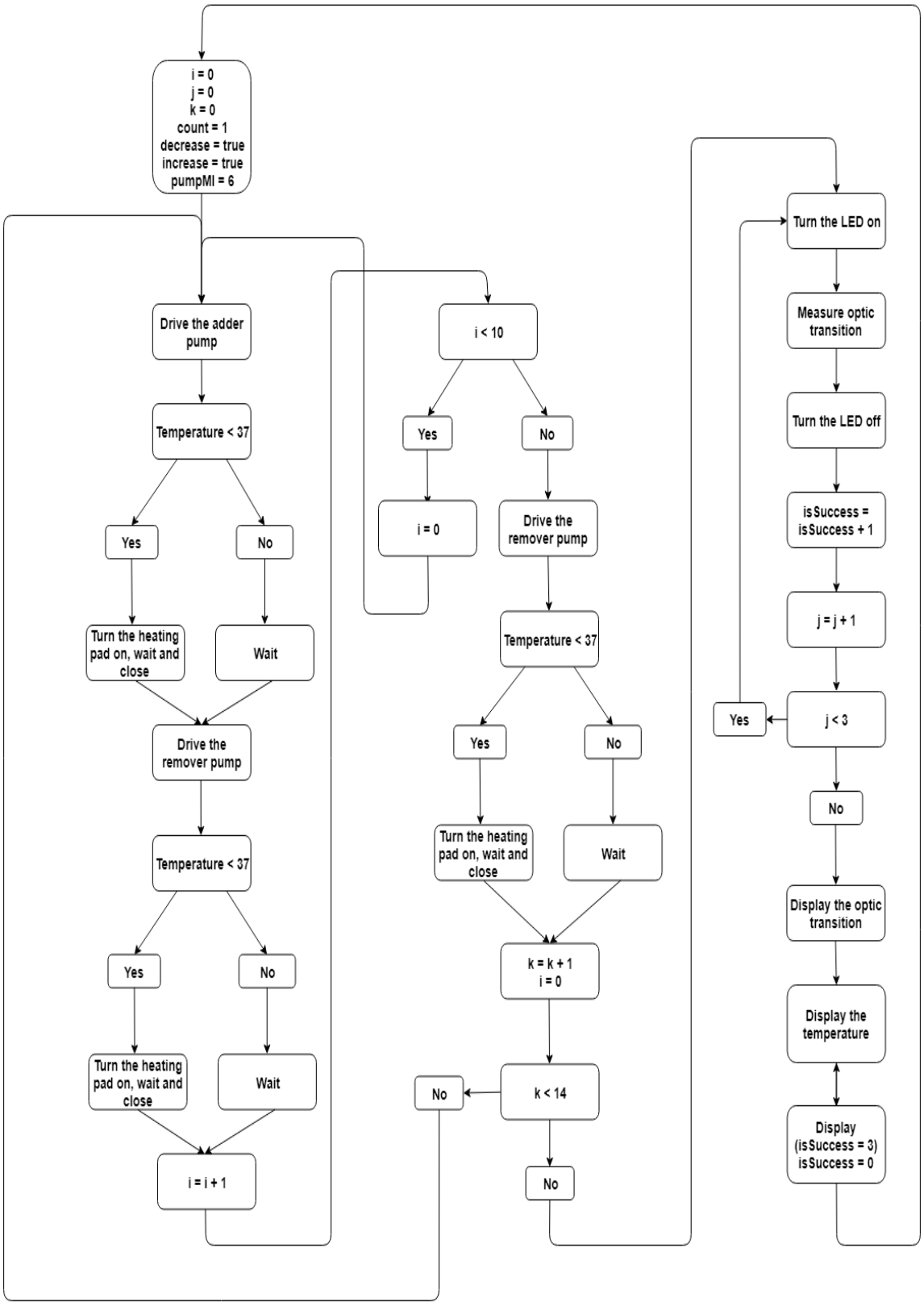

Figure 6: The Algorithm Diagram of Nitrogen Fixation and Cellulose Utilization Experiments Experiment in Arduino Software.

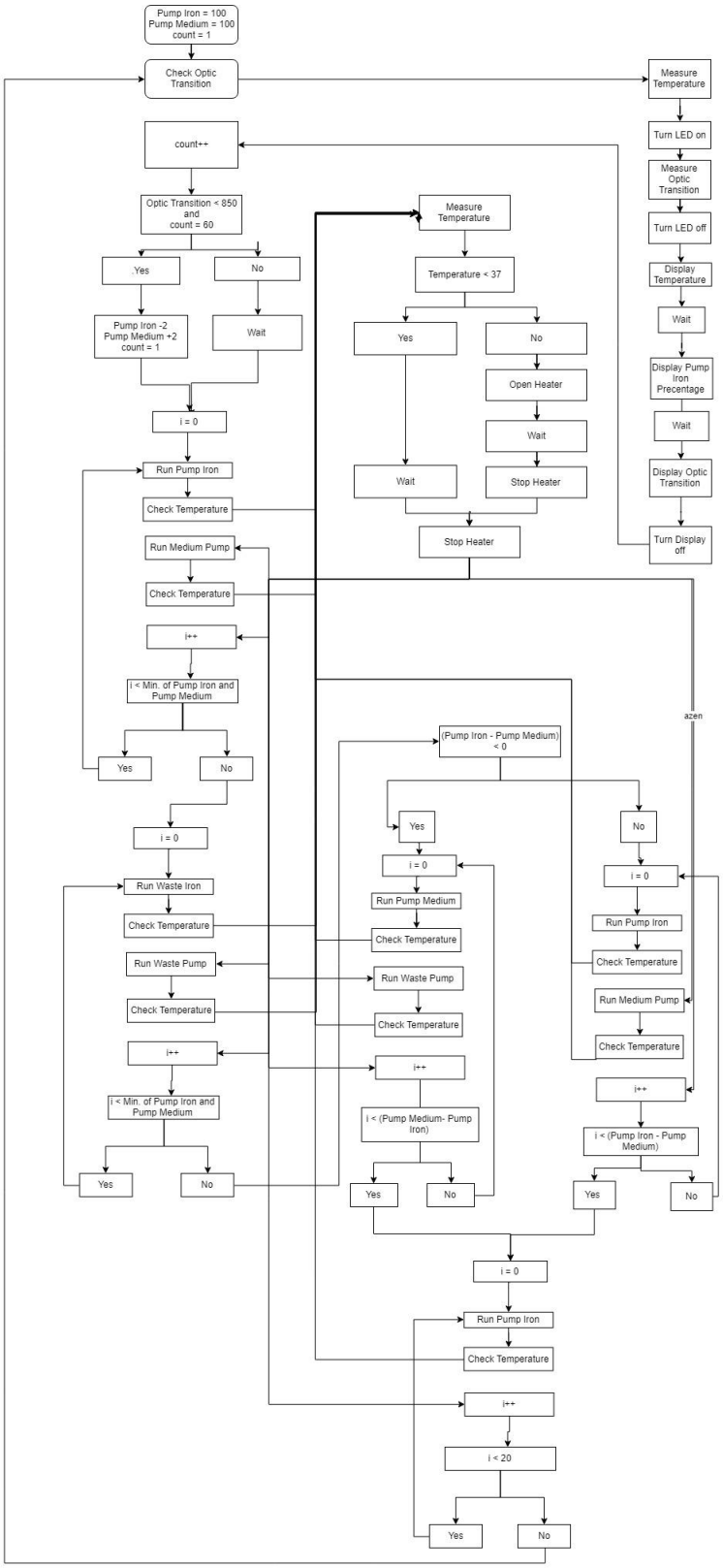

Figure 7: The Algorithm Diagram of Increasing the Iron Binding Protein Production

2.2 Technical Details of Arduino Based Bioreactor System

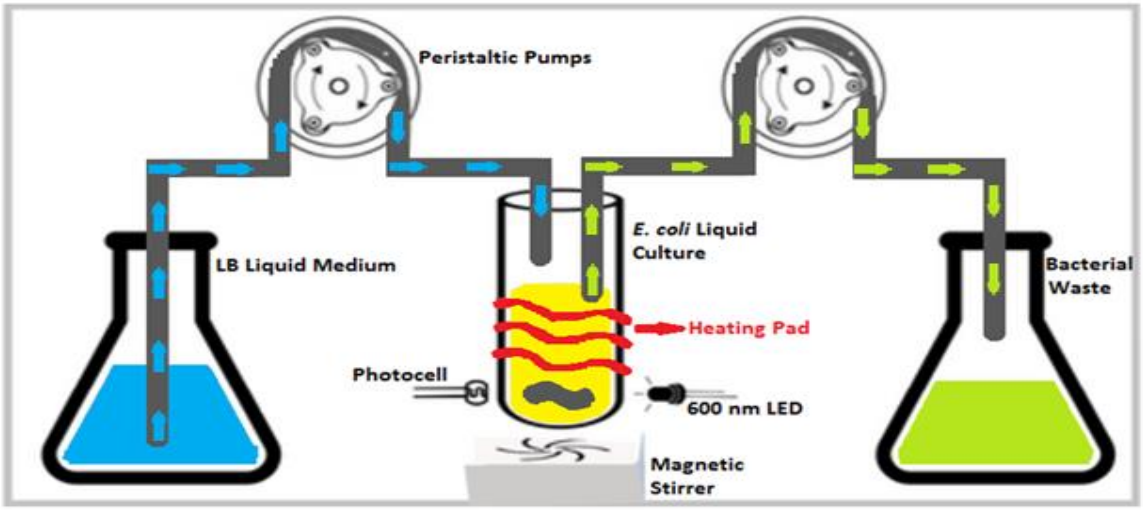

Figure 8: Overview of chemostat system

How microorganisms adapt to the extreme environments is the main question of this study. To answer this question two chemostats and two morbidostat systems were conducted. In each experiment, *Escherichia coli(E. coli)* were put into 4 distinct environments with 4 different media. Each media was arranged such that a mutant which could survive better than the others adapted to system better and survive. In this study, Arduino board is used since creating flexible systems is possible with it. Two peristaltic pumps and two TB6560 motor drivers for peristaltic pumps, TM1637 seven segment display, two heating pads, MLX90614 infrared temperature sensor, a photocell, a LED, a magnetic stirrer, a tube, silicon pipe, two containers, L298N motor driver for the heater, were used for each project. Coding in Arduino IDE software and then uploading the code into the Arduino hardware the electronic components of the bioreactor system were driven and the parameters of the systems were controlled via these electronic components. via electronic circuits and connections. Optic densities of the media were measured regularly to observe whether the target mutation was occured or not in genome of any of the bacteria in two chemostat systems. If optic density implied the mutation the genetic composition of the mutant and the genetic composition of an original one would be sent to analysis and the results would be compared.

There are two separate medium containers for fresh medium and waste medium. Peristaltic pumps create temporary pressure difference in the hose and moves the medium in the desired direction consequently. One of them adds fresh medium from the fresh medium container into the bioreactor while other one removes the waste medium from the bioreactor into the waste medium container. A temperature sensor and a heating pad are required to keep the temperature of the bioreactor constant, which is 37°C. A magnetic stirrer is very useful for homogeneous spread of the compounds in the medium.

There is a LED at one side of the bioreactor at the bottom of it and there is a photocell at the other side of the bioreactor. Photocell's resistance changes according to amount of light coming onto it. And, we measure the voltage changes depending on the photocell's resistance. It means that, when the bacteria concentration is high enough, light emitted from the LED cannot reach to the photocell effectively and the system increases the evolutionary pressure. Additionally, in the nitrogen fixation experiment, an air pump was used to allow air circulation in the medium. In addition, an air pump is used for pouring of the medium in the bioreactor during overflowing by creating pressure difference and additional evacuation pipe is used for this purpose.

The morbidostat designed in the Increasing the Iron Binding Capability project has different structure than the other two chemostat and the morbidostat in the Increasing the Flow Rate project. Instead of one fresh medium container, it has two medium containers with only difference is the presence of ferric ions. Other components of the system are same as other projects. It is designed to control the iron concentration in the bioreactor by controlling the pump rate of both mediums. Initially, the morbidostat programmed to pump both mediums equally (50/50). Then, the morbidostat's iron containing medium was ½ diluted, morbidostat reprogrammed as it would start at %100 iron containing medium pump rate and continued from the previous set-up's concentration.

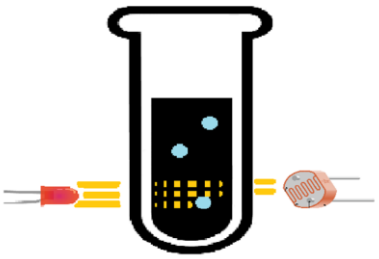

Figure 9: Photocell and bioreactor

2.3 Medium Optimization

2.3.a Measurement of Bacterial Growth in Distinct Media

To observe growth of bacteria in a specific medium, spectrophotometer is used. 1000 µL samples are taken from the media before inoculation of bacteria into the media. The samples are put into cuvettes, their tops are covered with parafilm and they are stored at +4 °C to prevent any bacterial growth till the OD measurement. Before OD measurement, samples are taken from refrigerator. The sample of the medium, where bacterial growth is wanted to be measured, is set into the spectrophotometer. Also, the cuvette including the medium in which bacteria inoculated is set into the spectrophotometer. The sample is used to determine the tare optic density of the medium to measure net optic density. The processes is repeated for each pair of the samples and the cultures.

2.3.b Increasing the Flow Rate

Media\Ingrediens	LB (g)	Glucose (g)	$CaCl_2$ (mL)	$FeCl_2$ (mL)	$MgSO_4$ (g)
S1	1.25	-	-	-	-
S2	1.25	-	0.09	0.158	0.01
S3	1.25	0.05	-	-	-
S4	1.25	0.05	0.09	0.158	0.01

Table 1: Media prepared for the medium optimization experiment

4 x 250 mL Erlenmeyer flasks were prepared for each of media. Lysogeny broth (LB) was added into each flask to be used as control. And, the effects of presence of glucose or minerals (Calcium chloride, Iron chloride and Magnesium sulfate) both separately and together on bacterial growth are observed with OD measurements. LB was added into all media since it was selected as the main nutrient source. Effect of glucose on bacterial growth was observed since carbon necessity of bacteria was assumed to increase proportionally to specific growth rate as long as the flow rate increased. Additionally, effect of minerals on bacterial growth was

observed since they are essential for the bacteria and reorganization of the intracellular activities after the mutation happened.

2.3.c Nitrogen Fixation

	5X M9 (g)	$MgSO_4$ (g)	Glucose (g)	LB (g)	K_2HPO_4 (g)	KH_2PO_4 (g)	NaCl (g)	$FeCl_2$ (50Mm) (mL)	$CaCl_2$ (80 mM) (µL)	MilliQ water (mL)
C1	0.01	0.01	0.5	0.005	0.34	0.15	0.025	0.79	1125	50
C2	0.05	0.01	0.5	0.005	0.34	0.15	0.025	0.79	1125	50
C3	0.1	0.01	0.5	0.005	0.34	0.15	0.025	0.79	1125	50

Table 2: Mediums that prepared at 10.07.2018

To prepare a 50 mL medium, all ingredients were added to the erlenmeyer flasks and 50 mL distilled water was added on top of it. Top of the flask was covered with aluminum and flasks were autoclaved. After taking 1000 µL sample as blank, 500 µL overnight grown *E. coli* was inoculated to each medium. They were incubated at 37°C and 200 RPM and OD was measured at different periods of time.

The results appeared so close (Table 14) and bacteria were not suffering from the low amount of nitrogen so, a new medium set was prepared. Since both LB and M9 contains nitrogen source inside, different amounts of LB and M9 were tried to optimize the nitrogen amount.

	5X M9 (g)	$MgSO_4$ (g)	Glucose (g)	LB (g)	Na_2HPO_4 (g)	KH_2PO_4 (g)	NaCl (g)	$FeCl_2$ (50Mm) (µL)	$CaCl_2$ (0.1M) (µL)	MilliQ water (mL)
C1	-	0.001	0.05	0.005	0.039	0.015	0.0025	15.8	9	45
C2	0.006	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	45
C3	0.004	0.001	0.05	0.005	0.039	0.015	0.0025	15.8	9	45
C4	0.0009	0.001	0.05	0.005	0.039	0.015	0.0025	15.8	9	45

Table 3: Medium that prepared at 13.07.2018

To prepare a 50 mL medium, all ingredients except glucose were added to Erlenmeyer flasks. 45 mL distilled water was added and medium was autoclaved. Later, 5 mL of 100 g/L glucose stock was taken and added to each medium through filter. 1000 µL sample was taken from each medium to be used as a blank. 100 µL overnight grown *E.coli* was added. They were incubated at 37°C and 200 RPM. OD was measured at different periods of time.

OD results also appeared so close (Table 15) and there no control tubes that can be used to compare. So, a new medium set is prepared.

	5X M9 (g)	$MgSO_4$ (g)	Glucose (g)	Yeast (g)	Na_2HPO_4 (g)	KH_2PO_4 (g)	NaCl (g)	$FeCl_2$ (50Mm) (µL)	$CaCl_2$ (0.1M) (µL)	NH_4Cl (g)	MilliQ water (mL)
C1	0.0564	-	-	-	-	-	-	-	-	-	4
C2	0.0564	-	0.05	-	-	-	-	-	-	-	3.5
C3	0.0564	0.001	0.05	-	-	-	-	15.8	9	-	3.5
C4	0.0564	0.001	0.05	0.0005	-	-	-	15.8	9	-	3.5
C5	-	-	-	-	0.039	0.015	0.0025	-	-	0.0002	4.5
C6	-	-	0.05	-	0.039	0.015	0.0025	-	-	0.0002	4
C7	-	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	0.0002	4
C8	-	0.001	0.05	0.0005	0.039	0.015	0.0025	15.8	9	0.0002	4
C9	-	-	-	-	0.039	0.015	0.0025	-	-	0.0001	4.5
C10	-	-	0.05	-	0.039	0.015	0.0025	-	-	0.0001	4
C11	-	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	0.0001	4
C12	-	0.001	0.05	0.0005	0.039	0.015	0.0025	15.8	9	0.0001	4
C13	-	-	-	-	0.039	0.015	0.0025	-	-	0.0005	4.5
C14	-	-	0.05	-	0.039	0.015	0.0025	-	-	0.0005	4
C15	-	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	0.0005	4
C16	-	0.001	0.05	0.0005	0.039	0.015	0.0025	15.8	9	0.0005	4

Table 4: Medium that prepared at 17.07.2018

25 mL 56.4 g/L M9, 10 mL 100 g/L glucose, 5 mL 50 g/L yeast extract, 20 mL 5 g/L NH_4Cl, 20 mL 20 g/L $MgSO_4$, 600 mL Milli-Q water stocks were prepared. M9 salts, except nitrogen source were prepared as a 50 mL salt mixture. In the mixture there were 78 g/L Na_2HPO_4, 30 g/L KH_2PO_4 and 5 g/L NaCl. For $FeCl_2$ and $CaCl_2$ previously prepared stocks were used. Since disposable sterile tubes cannot be autoclaved; stocks were prepared, autoclaved and added inside the laminar flow. Total volume was completed to 5 mL in each tube by using sterile water. 1000 µL sample was taken from each tube to be used as a blank. 10 µL overnight grown *E.coli* was added to each medium and they were incubated at 37°C, 200 RPM. OD was measured at different periods of time (Table 16).

	5X M9 (g)	$MgSO_4$ (g)	Glucose (g)	Yeast (g)	Na_2HPO_4 (g)	KH_2PO_4 (g)	NaCl (g)	$FeCl_2$ (50Mm) (µL)	$CaCl_2$ (0.1M) (µL)	NH_4Cl (g)	MilliQ water (mL)
C3	0.0564	0.001	0.05	-	-	-	-	15.8	9	-	3.5
C4	0.0564	0.001	0.05	0.0005	-	-	-	15.8	9	-	3.5
C11	-	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	0.0001	4
C12	-	0.001	0.05	0.0005	0.039	0.015	0.0025	15.8	9	0.0001	4
CA-Y-	-	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	-	4
CA-Y+	-	0.001	0.05	0.0005	0.039	0.015	0.0025	15.8	9	-	4
C17	-	0.001	0.05	-	0.039	0.015	0.0025	15.8	9	0.00005	4
C18	-	0.001	0.05	0.0005	0.039	0.015	0.0025	15.8	9	0.00005	4

Table 5: Medium that prepared at 20.07.2018

To see the effect of yeast and nitrogen, a new medium set was prepared. M9 and glucose were not autoclaved so they needed to be added through filter but it was forgotten and added directly to the sterile disposable tubes. All ingredients were added and the whole medium was filtered. Since yeast cells are big and they are not able to pass through the filter it could have caused a problem but previously stock was autoclaved and cells were disrupted so it didn't cause a problem. Each tube was prepared twice to be used as a control. 1000 µL sample was taken to be used as a blank and 8 µL overnight grown *E.coli* was added to each medium and incubated at 37°C, 200 RPM. OD was measured at different periods of time (Table 17).

2.3.d Cellulose Utilization

	5X M9 (g)	LB (g)	$MgSO_4$ (g)	$FeCl_2$ (50 mM) (mL)	$CaCl_2$ (80 mM) (mL)	Glucose (g)	MilliQ Water (mL)
D1	0.564	0.005	0.01	0.79	0.1125	0.005	50
D2	0.564	0.005	0.01	0.79	0.1125	0.025	50
D3	0.564	0.005	0.01	0.79	0.1125	0.05	50

Table 6: Components of media prepared at 10.07.18

First, a 150 mL medium was prepared with the sum of all three media's ingredients except glucose. Then, 150 mL was distributed into three 50mL media in 150 mL Erlenmeyer flasks. Lastly, glucose was added to the amounts given in Table 6. Three Erlenmeyer flasks were autoclaved and after autoclave, 1000 µL from each media was put into cuvettes and stored at 4˚C in the refrigerator. These samples are used in OD measurement to tare accordingly. 500 µL of culture grown in LB overnight was taken and put into each medium. New culture flasks were all placed in the shaking incubator at 37˚C and 120 rpm. OD was measured in 3 hour periods.

	5X M9 (g)	LB (g)	$MgSO_4$(g)	$FeCl_2$ (50 mM) (mL)	$CaCl_2$ (100 mM) (mL)	Glucose (g)	Cellulose (cellulose filter paper pieces)	MilliQ Water (mL)
D1	0.564	0.005	0.01	0.79	0.09	0.005 g	+	50
D2	0.564	0.005	0.01	0.79	0.09	0.0025 g	-	50

Table 7: Components of media prepared at 12.07.18

Two of 50 mL media were prepared with the composition stated in Table 7. With a hole puncher, small pieces were pierced from cellulose filter papers and used as the cellulose source in the D1 medium. Flasks were autoclaved. After autoclave, blank cuvettes for OD measurements were taken again and stored in 4˚C. To the rest of the medium, 500 µL of overnight *E. coli* culture was added and the flasks were incubated at 37˚C, 200 rpm. OD was measured at different hours.

	5X M9 (g)	$MgSO_4$(g)	$FeCl_2$ (50 mM) (µL)	$CaCl_2$ (100 mM) (µL)	LB(g)	Glucose (g)	Cellulose (cellulose filter paper pieces)	MilliQ Water (mL)
D1	0.0564	-	-	-	-	-	-	5
D2	0.0564	-	-	-	-	0.05	-	5
D3	0.0564	0.001	15.8	9	-	0.05	-	5
D4	0.0564	0.001	15.8	9	0.00005	0.05	-	5
D5	0.0564	-	-	-	-	0.0005	-	5
D6	0.0564	0.001	15.8	9	-	0.0005	-	5
D7	0.0564	0.001	15.8	9	0.00005	0.0005	-	5
D8	0.0564	0.001	15.8	9	0.00005	0.0005	+	5
D9	0.0564	-	-	-	-	0.00025	-	5
D10	0.0564	0.001	15.8	9	-	0.00025	-	5
D11	0.0564	0.001	15.8	9	0.00005	0.00025	-	5
D12	0.0564	0.001	15.8	9	0.00005	0.00025	+	5

Table 8: Media prepared 18.07.18 with controls

In sterilized disposable tubes, 5 mL of media was prepared with the compositions stated in Table 8. Since these tubes cannot be autoclaved, each component was first prepared as a stock, autoclaved and then added to the tubes. 25 mL of 5X M9 (56.4 g/L) and 20 mL of 20 g/L $MgSO_4$ stocks were prepared and autoclaved. 50 mM $FeCl_2$ and 100 mM $CaCl_2$ solutions were already autoclaved. On the other hand, glucose cannot be autoclaved as a stock since it caramelizes because of high temperature. Therefore, 10 mL of 100g/L glucose stock was prepared and filter sterilized when adding to the media. In addition, as the cellulose source, sterile nitrocellulose was used. After media preparation, 1000 µL for blank cuvettes were taken and cuvettes were stored at 4˚C. 50 µL of overnight culture was added to each tube and all were incubated at 200 rpm, 37˚C. OD was measured at 7 and 21 hours after inoculation.

2.3.e Increasing Metal Binding Capability

	M9 Medium (g)	Glucose (g)	MilliQ Water (mL)	50 mM FeCl2 (mL)
Fe+	0.2276	0.2	20	0.395
Fe-	0.2276	0.2	20	0.158
Fe+-	0.2276	0.2	20	0

Table 9: 12.07.2018's Medium experiment's components.

Three 50 mL Erlenmeyer flasks were labeled as Fe+, Fe-, Fe+- (+: highest, -: lowest). The mediums were prepared in the labeled flasks as listed materials in the table 9 were added to them. The Milli-Q water measured with volumetric cylinder and $FeCl_2$ was transferred with micropipette. When preparation of flasks were done, they were autoclaved. 1mL sample transferred to cuvette for blank OD measurements. After flasks were prepared, they were inoculated with 0.2mL overnight bacteria that was prepared previous day from stock *E.coli*. Flasks were incubated at 37°C and 200rpm. OD measurements were done when 3, 6 and 9 hours passed. 1mL samples from each time slots were transferred to cuvettes, tared with blanks and measured with spectrophotometer. At 6 and 9 hours blank measurements were recorded due to suspicion of contamination.

	M9 Medium (g)	Glucose (g)	MilliQ Water (mL)	50 mM FeCl2 (mL)
Fe+	0.2276	0.2	20	0.158
Fe-	0.2276	0.2	20	0
Fe+-	0.2276	0.2	20	0.063
Fe+--	0.2276	0.2	20	0.010

Table 10: 18.07.2018's Medium experiment's components.

Four 50 mL Erlenmeyer flasks were labeled as Fe+, Fe-, Fe+-, Fe+-- which represents their iron concentration (+: highest, -: lowest). Ingredients were added to labeled flasks according to table 10. Flasks were capped with aluminum foil and autoclaved. New iron stock was prepared for this experiment, which has different, reddish, color than previous stock. 1 mL transferred to cuvettes for blank OD measurements. When the blanks were completed, flasks were inoculated with 0.2 mL previously prepared overnight bacteria via micropipette. Inoculated flasks were incubated at 37°C and 200rpm. OD measurements were done when 4 and 17 hours were elapsed. 1mL sample from each time slot transferred to cuvette. The cuvettes of each medium and each time slot were tared with their blanks and OD measured with spectrophotometer.

	M9 Medium (g)	Glucose (9)	MgSO4 (g)	0.1M CaCl2 (mL)	MilliQ Water (mL)
Fe 1	0.564	0.5	-	-	50
Fe 2	0.564	0.5	0.01	0.09	50

Table 11: 19.07.2018's filtered stock mediums

	Fe 1 (mL)	Fe 2 (mL)	50mM FeCl2 (mL)
Fe 1+	5	-	0.1
Fe 1-	5	-	-
2Fe 1-	5	-	-
Fe 1+-	5	-	0.02
2Fe 1+-	5	-	0.02
Fe 2+	-	5	0.1
Fe 2-	-	5	-
2Fe 2-	-	5	-
Fe 2+-	-	5	0.02
2Fe 2+-	-	5	0.02

Table 12: 19.07.2018's new medium sets.

At 19/07/2018, to prepare test mediums, disposable sterile tubes were used. Since the tubes cannot be autoclaved, firstly stock mediums (Fe 1, Fe 2 and 50 mM FeCl2) were prepared as adding listed materials in the table 11 to falcons, then filtered. Then, disposable tubes were labeled. "+" has 1mM,"+-" has 0.2mM and "-" has zero iron concentration. Also, the 2 before the Fe means the parallel experiment. Ingredients of labeled tubes were added according to the table 12. Tubes were shaked to homogenize them, then 1 ml from each tube transferred to the cuvettes for blank OD measurements. The tubes were then inoculated with 40µL overnight bacteria prepared previous day. After inoculation, the medium set incubated at 37 at 37°C and 200rpm. Next day, 20/07/2018, 1 ml sample from each tubes were transferred to cuvettes. Overnight samples were tared with their blank and OD measured by using spectrophotometer.

2.4 System Preparation

2.4.a Increasing the Flow Rate

The medium S4 is selected according to OD results however; iron chloride is omitted because it turbidites the medium and thus prevents the correct OD measurement. The method used in the morbidostat was that the flow rate is increased slightly by the Arduino Board and whether the bacteria could adapt to the higher level of the flow rate was seen as increase in OD. When the bacteria adapted to the higher level of the flow rate, the system increase the flow rate again depending on the comparison of the OD value with the OD threshold, upper of which means that the bacteria adapted to the increased flow rate. However, if measured OD value is less than the OD threshold, down of which implies pre-washout, the flow rate is decreased to the previous value. OD measurement process is repeated by Arduino in each half an hour and the flow rate is increased or decreased by 0.1 mL/hour depending on the comparison of measured OD value to upper and lower boundary. The volume of the culture in the bioreactor was decided as 8 mL to minimize the required fresh medium amount.

2.4.b Nitrogen Fixation

Different media were prepared to find the optimal amount of nitrogen that enables bacteria to live but at the time suffer from the limited amount. By considering the results of the medium set that was prepared on 20.07.2018, it is obvious that yeast extract compensated the nitrogen deficiency since OD results appeared relatively high (Table 17). The OD difference

between normal nitrogen, low and zero nitrogen mediums were compared, and it has seen that decrease in the nitrogen amount obviously affected the growth of bacteria. C17 medium was chosen for the system because growth was relatively lower than normal nitrogen level and relatively higher than zero nitrogen containing medium. For 2 liters medium; 0.4 g $MgSO_4$, 6320 µL of 50 mM $FeCl_2$, 3600 µL of 0.1M $CaCl_2$, 0.02 g NH_4Cl, 20 g glucose, 15.6 g Na_2HPO_4, 6 g KH_2PO_4 and 1 g NaCl was used. After performing the steps that described in the initiation of the system part, by the help of the filter which is located at the cap of the fresh medium tank, glucose was added. Finally, 1000 µL overnight grown *E. coli* was added to the bioreactor and system was started as 4 mL/h.

2.4.c Cellulose Utilization

OD results of all 12 cultures (with the components shown in Table 6) were compared and according to the criteria of restricted growth, the D8 medium was chosen for the actual system. The fresh medium for the system consisted of 22.56 g of M9, 0.4 g of $MgSO_4$, 6.32 mL of 50 mM $FeCl_2$, 3.6 mL of 100 mM $CaCl_2$, 0.02 g of LB, 0.2 g of glucose and pieces of cellulose filter paper in 2 L water. The medium was prepared in a 2 L glass bottle. The medium, with the rest of the system, was autoclaved. Cellulose papers were also added to the bioreactor since the pieces are too big to travel through the silicone hoses. After autoclave, 6.32 mL of $FeCl_2$ was added through the filter with a syringe since 50 mM stock was not ready before autoclave. Into the bioreactor, 500 µL of overnight culture was added.

Due to blurry fresh medium, new medium was prepared with certain changes. First, instead of cellulose filter papers, it was decided to use a piece of cotton cloth as the cellulose source. With a thin metal wire going through the air hole of the bioreactor cap, cloth was hanged. Amount of $FeCl_2$ was decreased to 1.264 mL (1/5 of the original concentration) and LB was increased up to 0.2 g (10 times of the original concentration. The preparation steps of the medium were changed, also. First, all ingredients except $MgSO_4$ and $CaCl_2$ were added to 2 L of distilled water. 0.4 g of $MgSO_4$ in 25 mL water and 3.6 mL of 100 mM $CaCl_2$ in 25 mL water were prepared. After autoclave, $MgSO_4$ and $CaCl_2$ stocks were separately filter sterilized. The rest of the system preparation protocol was repeated.

2.4.d Increasing Iron Binding Capability

After deciding Fe 2+- was the optimum to start medium with 0.2mM iron concentration. To start with 0.2mM iron concentration in the bioreactor, medium with 0.4mM iron concentration was prepared for the system start 50% percent iron medium pump rate. To prepare mediums 22.56g M9 medium, 3.6mL 0.1M CaCl2 and 0.4g MgSO4 were measured and put into each medium bottle. 16mL from 50mM FeCl2 stock was added to the iron medium. The bottles filled with 2L of Milli-Q water by using graduated cylinder. Then, mediums were connected to the morbidostat system and all silicone pipes were covered with cotton and aluminum foil to prevent degradation of the pipes system during autoclave. Whole pipe system and bioreactor autoclaved after constricting the possible flow of mediums due to pressure with metal squeezers. After autoclave, 70g glucose added to the beaker with 350ml Milli-Q water(20g/100mL). In the laminar flow, 100 mL of glucose solution filtered to the both medium container from their filter. After glucose solution, very small amount of EtOH was injected through filter of medium containers. System connected with peristaltic pumps. Connection points of pipes to medium containers and waste pump to bioreactor were siliconed to prevent contamination. Finally, the morbidostat system was started and observed daily.

When it was decided to continue with the system, which would start at 100% iron medium pump rate, iron medium concentration changed as 0.2mM. 22.56g M9 medium was measured and added to each medium container. 8mL of 50mM FeCl2, half of previously used amount, put into the iron medium. Mediums were autoclaved. After autoclave process, pipes system, old mediums and bioreactor were transported to the laminar flow and new mediums were

replaced with old ones. 1.2g MgSO4 was measured and put into beaker containing 75mL Milli-Q water to prepare 0.4g/25 mL stock. 50g Glucose and 9 mL from 0.1M CaCl2 were added to another beaker with 241 mL Milli-Q water to inject to mediums through filter. 25mL from MgSO4 solution and 100 mL of glucose and CaCl2 solution added to each medium container. After glucose, CaCl2 and MgSO4, very small amount of EtOH was injected through filter of medium containers. The pipe system and bioreactor connected to the sensors and peristaltic pumps. The new Arduino code was uploaded and the system was started at previous systems concentration.

2.4.e Initiation of Experiments

After suitable media for each experiment were chosen and prepared accordingly, the system was prepared for autoclave. First, silicone pipes were connected to each other to complete the system and were squeezed with metal clips to prevent leakage from the medium container to bioreactor. A filter was inserted to the end of the silicone pipe for air sterilization and for possible addition of an ingredient. Another filter was inserted to the air pipe of the bioreactor. The hoses and filters were wrapped with cotton and aluminum foil for protection from the heat of the autoclave. The media were autoclaved with the rest of the parts of the system including the silicone hoses, waste container and the bioreactor.

When autoclave was finished, the whole system was taken to the laminar flow. As the first step, the necessary additional ingredients were added through the filter of the medium container with a syringe. After adding a component from the filter, a small amount of ethanol was also injected to sterilize the filter. The holes where pipes go through were all siliconed to prevent contamination and making sure they are all sealed. Only the hole for the medium supply pipe was not siliconed since the pipe will be heated and silicone would melt later. Finally, bioreactor was inoculated with the overnight culture.

Silicon hoses were placed into the appropriate peristaltic pumps. Heating jacket was wrapped around the bioreactor. Another one was wrapped around the metal part of the medium supply pipe tightly to prevent contamination in the fresh medium tank. Pumps started working until the medium in the bioreactor reached the previously determined level. Then, pumps were closed for some time while the rest of the system worked to let bacteria settle and grow. Later, the complete system was started.

3 Results

3.1 Increasing the Flow Rate

Hour(s)\Media	S1	S2	S3	**S4**
0	0.106	0.126	0.120	**0.125**
3	0.468	0.479	0.425	**0.497**
6	1.455	1.502	1.586	**1.608**
24	1.907	1.940	2.010	**2.008**

Table 13: OD results of medium optimization experiment

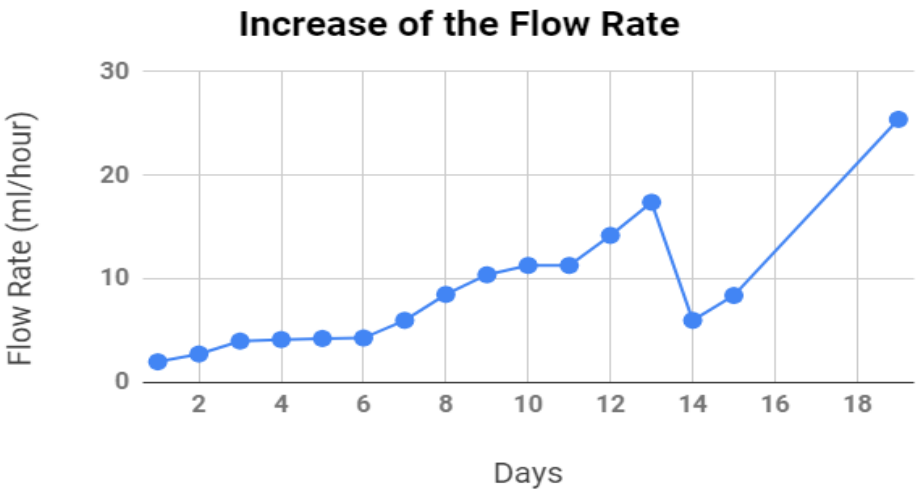

Figure 10: Increase of the flow rate

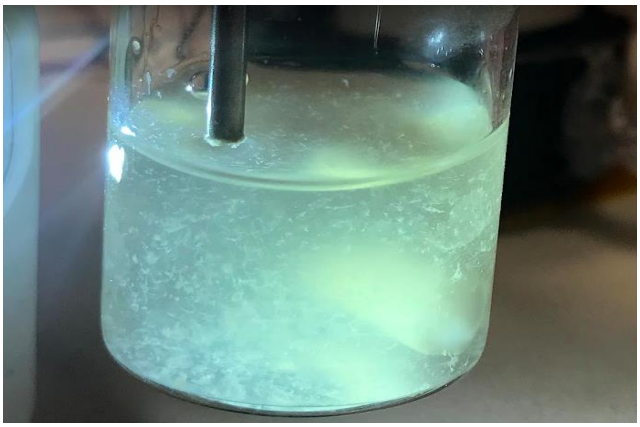

Figure 11: Agglomeration in the bioreactor

3.2 Nitrogen Fixation

	3 hours	9 hours	27 hours
C1	0.476	0.724	0.827
C2	0.491	0.827	0.832
C3	0.496	1.093	1.133

Table 14: OD results at different time points 10.07.2018

	3 hours	6 hours	22 hours
C1	0.113	0.142	0.173
C2	0.064	0.155	0.176
C3	0.100	0.214	0.211
C4	0.100	0.140	0.180

Table 15: OD results at different time points 13.07.2018

	7 hours	21 hours
C1	0.021	0.029
C2	0.151	0.166
C3	0.509	2.620
C4	0.473	1.980
C5	0.001	0.028
C6	0.092	0.105
C7	0.566	1.708
C8	0.547	1.956
C9	0.008	0.001
C10	0.110	0.113
C11	0.438	1.168
C12	0.524	1.764
C13	0.013	0.011
C14	0.119	0.103
C15	0.431	2.860
C16	0.446	2.600

Table 16: OD results at different time points 17.07.2018

	17 hours	Average
C3-1	2.092	2.114
C3-2	2.136	
C4-1	2.196	2.182
C4-2	2.168	
C11-1	1.032	0.956
C11-2	0.880	
C12-1	1.404	1.140
C12-2	0.876	
CA-Y-1	0.044	0.037
CA-Y-2	0.031	
CA-Y+1	0.432	0.482
CA-Y+2	0.532	
C17-1	0.612	0.606
C17-2	0.600	
C18-1	0.989	0.988
C18-2	0.986	

Table 17: OD results at different time points 20.07.2018

Days	Optic Transition Value	Average Optic Transition Value
1	258	258
2	251	250
	250	
	249	
3	260	253
	246	
4	256	256
5	275	275
6	277	277
7	295	320
	304	
	324	
	328	
	332	
	335	
8	344	344

Table 18: OD results of the system

Figure 12: Contaminated medium (left)

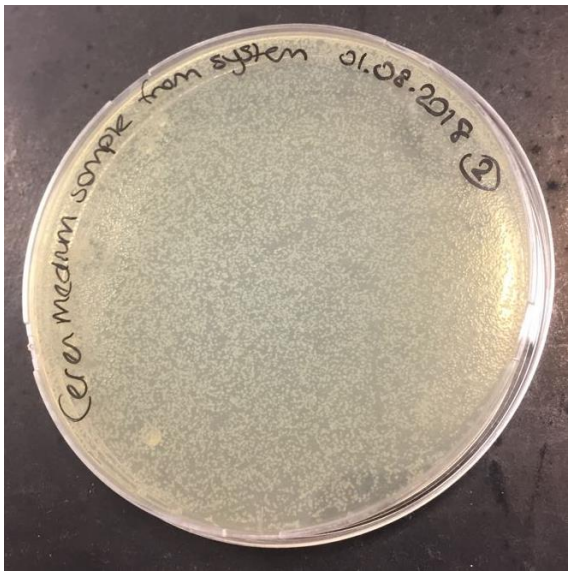

Figure 13: Plate results of contaminated medium

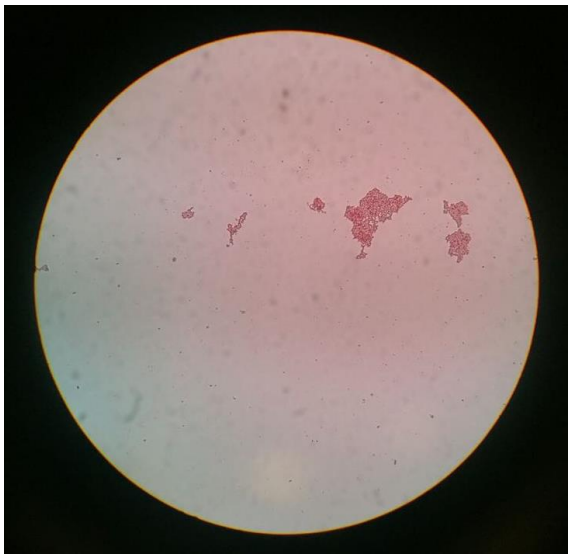

Figure 14: Gram staining result

3.3 Cellulose Utilization

	3 hours	9 hours	27 hours
D1	0.343	0.421	0.781
D2	0.657	0.803	1.029
D3	0.795	0.971	1.795

Table 19: OD results of cultures in media of table 4

	3 hours	6 hours	9 hours	22 hours
D1	0.248	0.315	0.280	0.263
D2	0.194	0.236	0.217	0.219

Table 20: OD results of cultures in media of table 5

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
7 hours	0.058	0.224	0.718	0.816	0.152	0.052	0.215	0.175	0.102	0.113	0.125	0.127
21 hours	0.070	0.245	1.936	1.664	0.131	0.111	0.213	0.188	0.128	0.139	0.211	0.172

Table 21: OD results of cultures of table 6

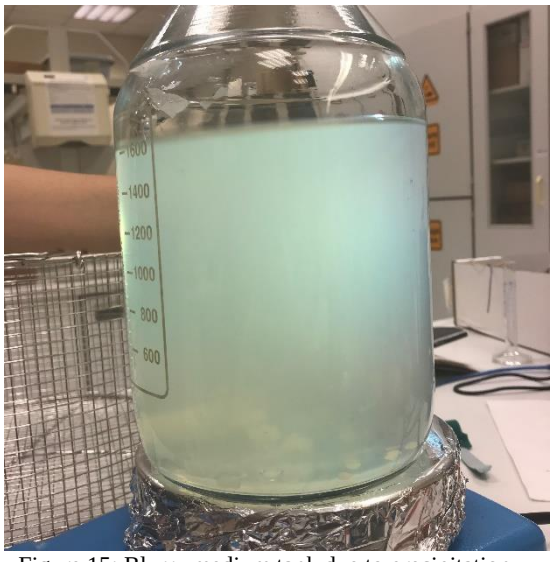

Figure 15: Blurry medium tank due to precipitation

Figure 16: Blurry bioreactor dure to disrupter cellulose papers

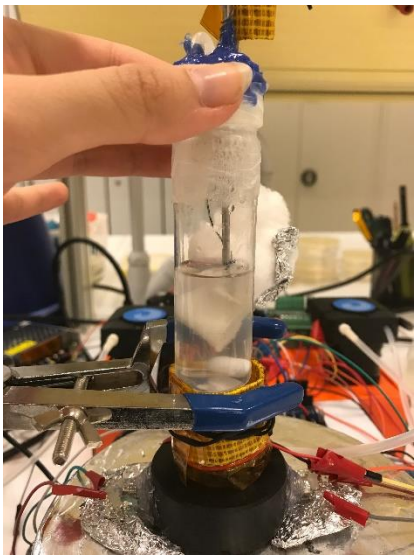

Figure 17: Final bioreactor with cotton cloth hanged inside.

3.4 Increasing Iron Binding Capability

	3 Hours	6 Hours Blank	6 Hours	9 Hours Blank	9 Hours
Fe+	0.379	-	0.380	0.460	0.154
Fe-	0.137	0.275	0.078	0.263	0.404
Fe+-	0.287	0.598	0.267	0.624	0.113

Table 22: 12.07.2018's Medium experiments OD results (Measured at 13.07.2018).

	4 Hours	17 Hours
Fe+	0.145	0.233
Fe-	0.072	0.246
Fe+-	0.150	0.101
Fe+--	0.165	0.222

Table 23: 18.07.2018's Medium experiment OD results.

	Fe 1+	Fe 1-	2Fe1-	Fe 1+-	2 Fe1 +-	Fe 2+	Fe 2-	2Fe 2-	Fe 2+-	2Fe 2+-
Overnight OD	0.233	0.077	0.057	0.068	0.065	2.130	0.840	0.837	2.020	2.040

Table 24: 19.07.2018's mediums' OD results (20.07.2018).

Percentage change 50% to 38% in the first set-up in one night and runned for 8 days. However, change in iron concentration did not occur since precipitation problems in the silicone pipes. Also, in the second set-up contamination, occurred after 1 day, caused system to not progress further.

Figure 18: Plating results of contaminated mediums.

4. Conclusion and Future Work

4.1 Increasing the Flow Rate

Bacterial growth was approximately the same in all media (S1, S2, S3, S4). Therefore, all possible components except Iron chloride (LB, glucose, Calcium chloride, Iron chloride and Magnesium sulfate) were decided to be added into the medium of the experiment since they have considerable advantages and do not have any drawback. LB includes all essential nutrients for bacteria to sustain their intracellular activities and organizations. Glucose is a carbon source, which was considered to be essential for fast reproduction during the further periods of the experiment. Minerals are significant for bacteria due to their usage as cofactor and roles in other basic intracellular reactions additionally; they support the processes after possible mutation and the expression of new genes. In other words, the rich medium is provided for bacteria to hinder any limitation different from their reproduction capacity originating from their current genomic composition.

The flow rate is set to 2 mL/hour at the beginning of the experiment to protect bacteria from wash out by allowing them to adapt to the medium physiologically. The flow rate was increased to 4 mL/hour gradually as long as they adapted. It stayed near 4 mL/hour for three days

by Arduino board since the optic density could not reach to the lower threshold rapidly after last increasing. However, it increased approximately 2 mL/day in following three days and reached 11.3 mL/hour in a day later. It stayed at this value for a day. During this interruption it might have been assumed that genetic adaptation occured however; it has been calculated that the flow rate could reach to 17.04 mL/hour without genetic adaptation discussing a study about growth of *E. coli* (2.13 = F/8 in Figure 2) (Marr, 1991). Thus, the interruption is considered to be caused by physiological adaptation processes. Additionally, the flow rate reached nearly to the calculated maximum value by thirteenth day after the physiological adaptation is completed nevertheless; agglomeration and biofilm formation is observed simultaneously. In addition, it has been observed that agglomerated bacteria could not been removed from the bioreactor. Therefore, formula in appendix 2 lost its validity for the experiment because the rate of bacteria amount in the removed medium to the amount of all bacteria is less than the rate of the removed medium volume to the whole medium volume. As a solution, mixture of medium was stopped, agglomerated bacteria particles were waited to sink and a sample was taken from the media to inoculate the bacteria which do not agglomerate to the new tube. Starting the experiment again with the flow rate of 6 ml/hour, agglomeration is started in hours again. It was assumed that chemicals triggering agglomeration were still in the sample and they were the cause of recurrence of the agglomeration that's why the speed of agglomeration is decelerated after 6-7 hours. Then, it is considered that the experiment had to be redesigned to prevent agglomeration and OD measurement was stopped since it was concluded that OD values were not reliable. Nonetheless, the flow rate continued to increased up to 25.4 ml/hour till the nineteenth day. During this period, it is considered that although the specific growth rate of the bacteria could not increase, the bacteria achieved to sustain their lives against the higher levels of the flow rate by agglomerating. Therefore, agglomeration was decided as the main focus, the literature was searched about colonization of bacteria and analyse of and discussion on the problem and possible solutions have been continuing in parallel with the literature.

4.2 Nitrogen Fixation

Since, OD results of the first (10.07.2018) and the second (13.07.2018) experiments (Table 14 - 15) appeared to be so close; new media with different nitrogen amounts were produced. This time, control media were also prepared to be able to compare the results with standard nitrogen levels.

If LB is to be used in the medium, it has yeast extract, which contains all sort of elements and nutrients for bacterial growth. This will allow bacteria to not be constricted to develop ways to fix nitrogen. For example, if it needs an enzyme that uses cobalt as a cofactor to fix nitrogen, it can find that element when yeast is used. On the other hand, yeast extract has every kind of nutrient including nitrogen-based ones'. Since the exact amount of nitrogen is unknown in LB, this limits the control over the medium. To solve this problem, third (17.07.2018) and fourth (20.07.2018) medium sets were prepared and effect of each component was observed by changing each factor at a time. In the last medium set of the experiment (20.07.2018), C17 medium was chosen, because when compared to media which has complete nitrogen and zero nitrogen, OD results of that medium were in the middle and this showed that there was an obvious influence of nitrogen in bacterial growth. It restricted the growth, so it seemed a reasonable medium for the system. C17 is preferred instead of C18, which has yeast, because it was observed that yeast compensated the lack of nitrogen, so it is not very useful for this experiment.

The bacteria that is used for inoculation was incubated at LB and they might store nitrogen that is present in the medium. This situation may result with uncontrolled nitrogen amount, although the prepared medium has controlled nitrogen levels. To be able to control the nitrogen source, inoculated bacteria amount is decreased to 100 µL instead of 500 µL.

When the system medium was prepared, a precipitation problem appeared. To solve this, a fish was added to the medium tank and system was stirred whole night. Next day, the medium was a bit blurry, but it was homogenous, so the system was started with that medium. It worked so well for 6 days. In the 7th day, wire of the heater which was placed on the fresh medium hose broke and it was noticed a bit late. Also, in the evening of 7th day, it was observed that because of the air pump, medium bubbled and resulted bubbles carried the medium which has bacteria in it to the top of the bioreactor. Air hole which was located on the cap of the bioreactor was filled with cotton to enable sterile gas exchange and when medium was carried to the cap, it soaked up with medium in the bioreactor. Since wet cotton is a good place for bacterial growth; to prevent contamination, a new piece of cotton was autoclaved and placed. At the 8th day of the system, the nutrient tank become yellowish (Figure 12) so we have suspected from a contamination problem. To understand this, we took a sample from the hose under laminar flow and plated the sample (Figure 13). To get a faster result, sample was also observed under microscope after performing Gram staining (Figure 14). When results of these two experiments were observed it was seen that there was a contamination problem. Since in Gram staining, contaminant appeared as a gram negative bacteria, we suspected from a back contamination. This might be occurred while carrying the system to the laminar flow to change the cotton, because we forgot to squeeze the hoses and medium started to flow uncontrollably. At that time, if medium from the bioreactor was carried to the fresh medium tank, this might be the reason for contamination. Also the optic transition values started to increase which shows that bacteria are dying. Since medium is contaminated, nutrients in the medium are consumed before reaching to the bioreactor and bacteria in the bioreactor cannot get fresh medium and starts to die.

The medium in the bioreactor started to become transparent however the color of the fresh medium was not transparent at the beginning of the experiment. When *E.coli* uses the medium, they might metabolite the ingredients that cause the blurry color and make the medium transparent. However, there is no clear evidence, so it needs to be further investigated.

Although a good stressed condition is obtained and system worked successfully, *E. coli* couldn't undergo mutations that cause nitrogen fixation under aerobic conditions in 8 days. However, if a solution is found to the contamination problem, different results might be obtained.

If they were to fix nitrogen, it could be understood by looking at the optic transition values. If it decreases, it means bacteria grew well and this shows that there might be a mutation that causes nitrogen fixation. If a situation like that happens, bacteria might be inoculated into a medium without nitrogen and observed. As they grow in that medium, they can be sent to genetic analysis for further investigation.

4.3 Cellulose Utilization

An important part of the whole experiment was to decide on the most applicable medium to achieve the goal of cellulose utilization in *E. coli*. The most useful environment for directed evolution is in which bacterial growth is restricted to a level but not prevented. In cellulose utilization experiment, such a medium should be lacking in glucose since the main objective is to observe if *E. coli* can use an alternative carbon sources, cellulose. Many experiments with different media compositions were performed in order to decide on the most suitable glucose concentration. First, three different glucose levels were tested, keeping everything else stable (Table 6). The OD results of this experiment (Table 19) shows that, as expected, decreasing glucose concentration decreases bacterial growth. However this experiment lacks the proper controls and does not give an idea on the effect of cellulose. Theoretically, *E. coli* cannot hydrolyze cellulose nevertheless it should be tested. In the other medium experiment, cellulose was added to a glucose concentration level that was tested previously (0.005 g) and an even lower amount of glucose was tested (Table 7). When OD results of this trial (Table 20) and the previous one were compared, addition of cellulose seemed to cause no increase and lower glucose kept decreasing growth. Although these comparisons gave an idea on the matter, none of these

experiments had the appropriate control mediums. For a controlled experiment, all parameters should be arranged carefully. An experiment consisting of 12 media was planned with this intention. Media with no glucose and normal glucose were prepared, all glucose levels were tested with and without LB separately, each were also tested with and without cellulose. From the bacterial growth in each media, as shown with the OD results in Table 21, it was concluded that the most fitting medium is the one with 0.1 g/L glucose concentration, 0.01 g/L LB and cellulose filter papers (rest of the ingredients were stated in Table 8). LB complicated the decision process since LB is not a completely known and standard medium. Composition of the yeast extract is not exactly known and can differ from batch to batch. Therefore, although it provides certain ions that might be needed for the growth and adaptation process, it also provides carbon. The carbon amount is however, not known. Nonetheless, it was ignored since LB does not contain glucose and when concentrations are compared, its carbon supply can be overlooked.

The system was prepared using the concentrations of D8 medium. After the autoclave, however, the medium had a large amount of precipitation. It was first thought that the cellulose papers were disrupted and looked as if salts did not dissolve, but after putting a magnetic fish in and stirring the medium, cellulose papers rose in the medium as whole pieces and the medium was still blurry (Figure 15). Later on, the cellulose papers in the bioreactor got disrupted, possibly due to stirring with the fish (Figure 16). Such medium and bioreactor prevent the detection of bacteria since the first detection needs be from the system getting blurry. It was suggested that the reason behind precipitation is the chemical reaction caused by $CaCl_2$ and $MgSO_4$. In order to solve the turbidity problem, $MgSO_4$ and $CaCl_2$ were separately prepared and after autoclaving the rest of the medium, was added through the filter with a syringe. This prevented the reaction that caused precipitation and the medium was clear. For the problem concerning cellulose paper pieces, another cellulose source was chosen. A small ball of cotton wrapped by a thin metal wire and a piece of cotton cloth hanged with a metal wire were the possible options. First, they were put into water to observe whether they break apart and blur the medium, neither of them did but cotton cloth seemed more intact and suitable for the system. In addition, concentrations of LB and $FeCl_2$ were also changed. Iron made the medium yellowish and to prevent this, it was decreased and compensated with increased LB. Also, bacteria seemed to suffer more than expected therefore LB was added to a concentration in which carbon amount can still be overlooked. After these changes, system was started again. The system was working without a problem until evaporation started in the bioreactor due to the cotton cloth. Cotton cloth caused the medium in the bioreactor to be evaporated and below the level of waste pipe. Therefore, waste could not be emptied for a while. By submerging the cloth fully into the medium, the problem was solved (Figure 17).

With the final form, system worked for a week and no other complications occurred. In a period of one week, bacteria were not able to gain the ability of cellulose utilization. Nevertheless, appropriate medium for a directed evolution experiment concerning cellulose utilization in addition to a working system was established. For future work, by taking the troubleshooting stated into consideration, further experiments could be constructed with a proper system.

4.4 Increasing Metal Binding Capability

In the Increasing the Metal Binding Capability project, it was aimed to increase this capability without genetic manipulation. Therefore, we did many experiments to construct a morbidostat. One of the most important is the medium experiments. 12/07/2018's experiment was controlled medium, which has controlled Fe levels, minimal salt medium and carbon source. It was expected to more bacterial growth in high iron concentration. However, results were unexpected at the 3rd hour. Therefore, we were suspected of contamination of blank mediums. To determine whether it was caused by contaminated blank mediums, the blank OD results were

recorded at the next time slots. The blanks were increased between 6th and 9th hours as we expected. This medium set was repeated again at 18/07/2018, however, results were unexpected as well. The reason behind the unexpected results was the newly prepared FeCl2 solution. This solution has red color, which is very different than the the stock we previously used. The stock could be oxidized due to bad storage conditions. On the final medium experiment, essential salts such as MgSO4 and CaCl2 were used as well as the their control groups. Also, FeCl2 solution was renewed and used in the experiment. It was aimed to compare how the essential salts affect the growth and determine that whether medium with these salts could provide iron as a limiting factor. This experiment was concluded as we expected, medium with additional salts provide selectivity. At 20/07/2018 OD measurements we decided to use medium with salts and 0.2mM FeCl2 concentration since the bacterial growth was decreased as the iron concentration was decreased. Therefore, this experiment contributed how to choose selective medium by using controlled medium. If LB medium was used in this experiment, due to yeast extract, many trace elements can be present and would not provide control on the selective medium. After the medium was decided, the mediums were prepared. The iron containing medium was started twice concentration as we decided in the medium experiments because system started 50% pump rate of mediums, which dilutes to half concentration. However, this set-up caused increasing waiting period of iron containing medium inside the silicone pipes goes to the bioreactor. Therefore, as a solution we proposed the ½ dilution of iron containing medium and continue the experiment with previous set-up's final iron concentration, which was 38% at that time, and reprogram the set-up to start from 76%. We shortened the waiting time of iron medium inside the pipes by increasing the running time of iron medium pump. Due to turbidity on newly prepared mediums of new set-up, we were suspected of contamination at that concentration. We plated the 100µL samples of mediums and incubated overnight. The plating results were as expected, there was bacterial growth. The bacterial growth was homogenic which we were suspected that was our strain. If contamination was caused by other organisms, the plate would not be homogenic. Although these problems, this system can be continued to decrease from %76 percent. When the system stuck at a percentage value, and start to decrease again, which may tell us possible adaptation mutation. Thus, genetic analysis can be done at that point or SDS-PAGE can be done to compare both starting strain and mutant strain. If any regulation of protein changed, this protein could be purified for further protein analysis.

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