

**SYNTHETIC CELLULAR SYSTEMS FOR WHOLE CELL
BIOCATALYSIS**

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SYNTHETIC CELLULAR SYSTEMS FOR WHOLE CELL BIOCATALYSIS

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

SYNTHETIC CELLULAR SYSTEMS FOR WHOLE CELL BIOCATALYSIS

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Synthetic biology is a field utilizing basic science and engineering approaches to create novel synthetic systems. Biocatalysis is one of those already existing processes which was reviewed intensely due to its advantages of using enzymes as catalysts. It is efficient, requires less additional reagents compared to chemical transformation methods, and it is environment friendly. Due to selectivity of enzymes it is easier to separate products. Enzymes are capable of carrying out many basic and complex reactions however some common problems occur in most strategies due to the nature of enzymes and mostly requirement of purification of the enzymes. Major issues are longevity-sustainability of the enzymes, modularity of the system, and yield of the enzymes. Thanks to the present advances in recombinant DNA technologies and discoveries in bacteria mechanisms like secretion, these pitfalls are addressable through Synthetic biology. We proposed a series of genetic circuits for the sustainability of

biocatalysis systems by employing engineered bacterial biofilms. The final, biofilm proteins made nanofibers are protecting both cells and enzymes thus providing an environment fit for replenishment of the enzymes along with modularity to the system. Here we present two synthetic cellular systems utilizing engineered biofilms to address the issues of biocatalysis and we propose an RNA based synthetic regulatory component to increase the robustness of our systems.

KEYWORDS: Functionalized curli protein, biofilm, biocatalysis, riboregulator

ÖZET

BAŞLIK

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Sentetik biyoloji disiplinler arası temel bilim ve mühendislik yaklaşımlarını kullanarak yeni sentetik sistemler yaratan veya halihazırda mevcut biyolojik sistemleri taklit etmeye çalışan bir bilim dalıdır. Biyokataliz bu halihazırda mevcut biyolojik sistemlerdendir ve kimyasal reaksiyonlar için enzim kullanılması gibi sıradışı bir yöntem içerdiği için yaygın olarak çalışılan bir konudur. Etkili, çevre dostu ve de kimyasal yöntemlere oranla çok daha az ekstra reaksiyona girecek madde gerektirdiği için ilgi çekici bir konudur. Enzimlerin seçici özelliklerinden dolayı, oluşan ürünleri ayırmak da daha kolaydır. Enzimler basitinden karmaşığın bir çok reaksiyonu katalizleyebilirler ancak onların da kendilerine özel, hassas yapılarından dolayı ve genellikle izole edilmeyi gerektirdikleri için oluşan sorunları vardır. Bunların en büyükleri, kurulan sistemlerin sürdürülebilirliği, enzimlerin çabuk bozulması ve genellikle sistemlerin modüler olmamalarıdır. Günümüzde oldukça gelişen rekombinant DNA teknolojiler ve bakterilerdeki salgılama gibi mekanizmalarla ilgili keşifler bu

sorunlara çözüm olacak sentetik biyoloji olanakların önünü açmıştır. Sürdürülebilirlik sorununa olan yaklaşımlardan biri biyofilm kullanmaktır. Biyofilmler sisteme modülerlik sağlarken hücreleri, dolayısıyla da enzimleri koruyup, enzimlerin yenilenmesi için uygun bir ortam hazırlamaya yardımcı olur. Bu çalışmada 2 tane sentetik hücre sistemi sunarak biyokatalizin sorunlarını çözmeye yöneliyoruz. Sistemlerimizi, biyofilmler üzerinde mühendislik çalışması yaparak oluşturuyoruz. Ayrıca sistemlerimizin etkinliğini artırmaya yönelik bir regülasyon parçası kullanımı da öneriyoruz ve test ediyoruz.

ANAHTAR SÖZCÜKLER: Fonksiyonel curli proteini, biyofilm, biyokataliz, riboregulator

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ABBREVIATIONS

aTc	Anhydrotetracycline
IPTG	Isopropyl B-D-1-Thiogalactopyranoside
ALP	Alkaline Phosphatase
<i>E. coli</i>	<i>Escherichia Coli</i>
PNPP	Para-Nitrophenylphosphate
CV	Crystal Violet
GFP	Green Fluorescent Protein
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
SEM	Scanning Electron Microscopy
TEM	Tunneling Electron Microscopy
CPD	Critical Point Dryer
ECM	Extra Cellular Matrix
RBS	Ribosome Binding Site
WT	Wild Type

CHAPTER 1 INTRODUCTION

1.1 SYNTHETIC BIOLOGY

Synthetic biology is an interdisciplinary field that utilizes principals of different engineering and basic science approaches like biology and computer sciences, to design and create novel synthetic systems or even rewrite the already existing ones in nature.[1]–[3] It primarily focuses on working with biological systems as primary playing field. Since there are a lot of natural products and with a lot of them already characterized, synthetic biology has various opportunities to focus on and manipulate to achieve synthetic molecules with pharmaceutical properties, with fuel properties or even novel samples for material science to improve already existing systems. All of these natural products are produced in several species and controlled and regulated by genes in their DNA. Advancing recombinant DNA technologies comes in to play in this context and let synthetic biologist use and design new synthetic gene clusters from known regulatory elements in these species.[1], [3] In this way Syntethic biology achieves its goal, creating synthetic systems. Normally in a bacterium for example, a product might be produced through several reactions that are regulated by a few transcriptional factors and enzymes.[1]–[3] In a way ,generally, there are more than minimally required factors in play when a natural product is being synthesized in cell due to complex environment of the cell. Synthetic biology tries to isolate key factors in a process and try to use minimum number of required factors and even modify them sometimes to achieve higher efficiency.[1]–[3] This approach results in the creation of before mentioned synthetic systems.

When the field was first emerging it was actually founded around *E. coli* and yeast mostly because at the time they were already well characterized and were good platforms for newly created synthetic systems.[1] So these two cells were the foundation of this field and the field mostly evolved around them. In time though, several species become available to be grown in laboratory environment and with these developments, new platforms that were impossible to implement in *E. coli* or yeast are now possible. Aforementioned recombinant DNA technologies also advanced with the field and in the end it was even easier to isolate parts from even an uncharacterized species' genome and this even further increased the possibility to create multi layered, more complex synthetic systems. In Figure 1.1 it can be seen a few of the first synthetic systems created using DNA technologies, very early in the field. [1] Synthetic biology as mentioned before, rely on engineering approaches. Created synthetic systems are usually designed and named after electronic engineering terms and systems.[1]–[3] One of the basic concepts among these systems is designing a genetic circuit for the syntethic production of a natural product. These designs can include multiple regulatory elements again taken from different species, multiple input methods, in other terms inducer in biological systems, and several genes of course to facilitate the production. [2] Now it was possible to create complex, multi layered and even systems responsive in a digital manner.[4] In some cases even minimal versions of eukaryotic genome is synthesized.[5]

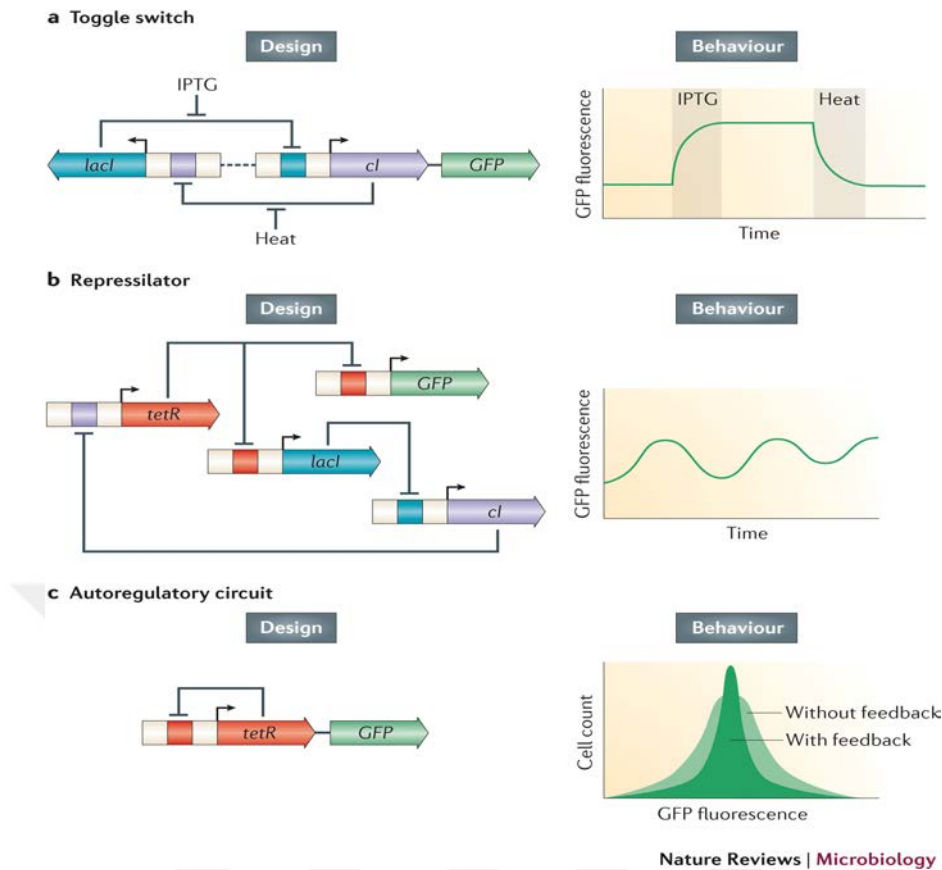


Figure 1. 1: 3 examples of the synthetic genetic circuits created during the early years of Synthetic biology. They are some of the fundamental versions. **A** is toggle switch, designed in a way that 2 transcriptional regulators work antagonistically and only one of them is active at a given time and once the system is activated with an input signal persists for a long time. **B** represents a repressilator design and result. It is a system where 3 repressors work in conjunction and repress each other and finally the third one actually repressing both GFP and the first repressor. In this way there is an oscillation of GFP, when the active repressor changes GFP signal is present or absent. **C** represents an autoregulatory circuit where the noise of the system is dampened by introduction of a negative feedback where TetR is repressing its own transcription. (Adopted from Ref. 1 with permission of Nature Publishing Group)

The emerging future aspect for synthetic biology when it was developing was to actually feed, heal and fuel humans in much more efficient way by manipulating already existing systems. Although much of the studies remained at the proof of concept stage, the field actually had some real life applications such as antimalaria phosphonate natural product.[6] To further emphasize the complexity of the method in present day, it is now possible to even engineer metabolism or a pathway, meaning creating a synthetic pathway for production of a natural substance.[7], [8] The opiate pathway synthesis[9] in yeast and studies on ethanol production and conversion of cellulose into alcohol based molecules [8] are only a few of the examples of how synthetic biology paves the way to its promise of feeding, fueling and healing us.

1.2 CATALYSIS AS A SUBJECT OF SYNTHETIC BIOLOGY

Chemical reactions are a crucial part of almost all aspects of life from molecular cellular events such as transcription, to processing of hydrocarbons which are essential for continuity of life.[10]–[12] Common key characteristic of chemical reactions is catalysis.[13]–[15] Organisms use enzymes to catalyze reactions in cells and these events inspire many applications in various fields such as medicine, food industry, and fabrication of fundamental materials.[16]–[19] This inspirations lead to the fundamental application called biocatalysis.[16]–[19] It is a process involving transformations of chemicals with enzymes as catalysts. This process is mostly environmentally friendly, relatively cheaper than industrial counter parts and due to enzymes' selective nature very precise.[10], [18], [19] Although biocatalysis comes with great advantages, it has some pitfalls. Main pitfalls are yield of enzyme, longevity, and modularity of the system, meaning parts of the system cannot be exchanged freely.[20]–[22] Utilization of enzymes at first required purification of

them. In early applications enzymes were harvested directly from their sources such as organs of animals, and parts of plants etc.[20]–[22] However the yield is low for large scale applications and marketing.[23]–[25] Recombinant DNA technologies tackled this problem by making it possible to produce enzyme of interest using a suitable host in a cheap and large scale way.[10], [20], [23], [25]–[28] Second problem is longevity of the enzymes. Conditions for the reactions generally harm the enzymes and they require continuous replenishment. [29]–[32] To overcome this obstacle, there are a few ways one of which is using whole cells as biocatalysts.[33]–[37] This approach provides molecular factories in a way, to replenish the degraded enzymes in the system and it is called whole cell biocatalysis.[32]–[37] Final issue, more of feasibility related one, is the modularity of the system. Today it is possible to produce enzymes in large scales with engineered hosts but this requires specialized equipments such as bioreactors so limits the applicability.[38]–[41] This issue is addressed with some researchers by utilizing protective materials to sustain cells or enzymes on desired surfaces.[29], [30] One of the applicable materials is biofilms.[42]–[44] Since using bacteria is common practice for recombinant protein production, utilizing their naturally occurring fibers, biofilms, for preservation purposes is nothing but logical. In summary although biocatalysis is a wondrous process, it still has room for growth from different perspectives. [18], [22]

1.3 *E.COLI* BIOFILM: CURLI PROTEINS AND THEIR ASSEMBLY

Escherichia coli is one of the best studied hosts, optimized for recombinant protein production.[45]–[48] It is a gram negative bacteria which has many modified strains for different applications.[48], [49] These bacteria synthesize proteins called curli and form an extracellular network in the environment they grow, under stressful

conditions.[50]–[54] These curli proteins consist of 7 types in *E. coli*; csg A, B, C, D, E, F, and G. They are transcribed from two operons, csgBAC and csgDEFG.[55]–[58] CsgA is the major curli subunit and csgB is minor curli subunit, and they are secreted to the extracellular environment with the help of other curli proteins and form fiber network.[57], [58] (Figure 1.2)

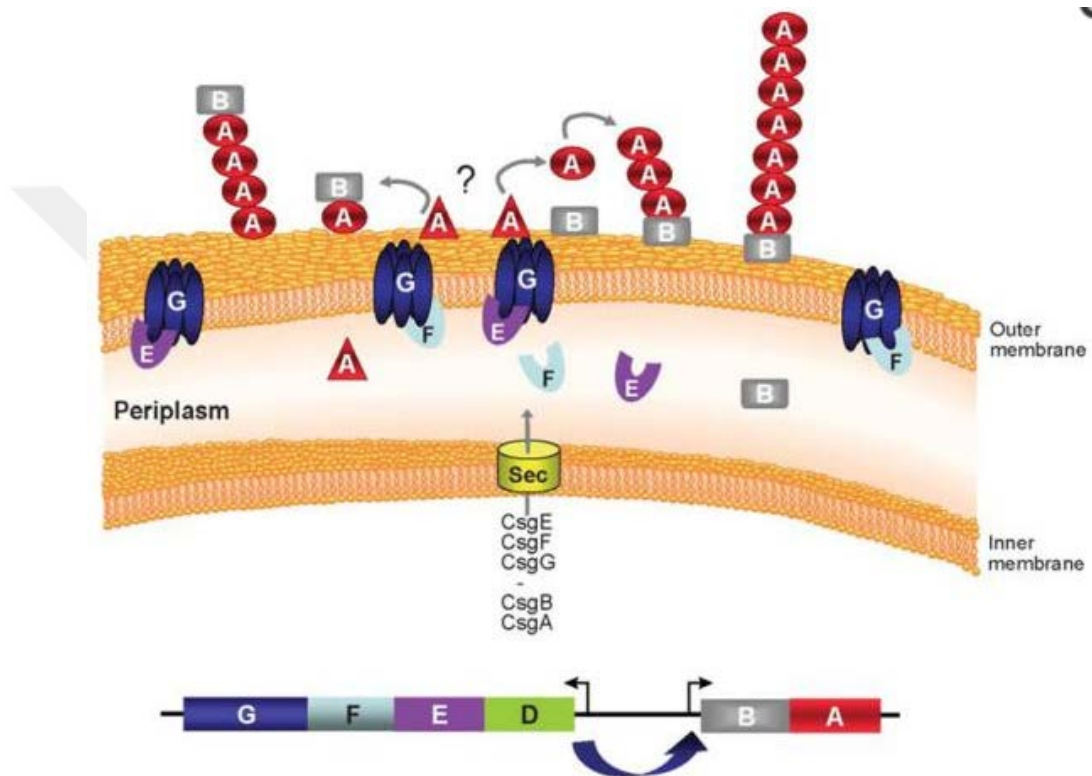


Figure 1. 2: Curli Biogenesis and Assembly is depicted. CsgA and CsgB proteins are secreted to the ECM due to their sec signal sequence. CsgB is nucleator factor here forming a platform on membrane for oligomerization of CsgA and, CsgG is forming channels for secretion. CsgF and E are helping with transportation. (Adopted from Ref 52 without any special permission requirement.)

Basically this network is for increasing survivability and exists in many enterobacteriaceae but studies over the years showed that it has more than a couple

functions.[58]–[62] It can help form 3D structures to increase survivability in many harsh conditions, it can increase pathogenicity, it is also important as a sole structure since involvement of the similar structured plaques in Alzheimer's and Parkinson's diseases.[63]–[66] These fascinating characteristics gained attention from several researchers and led to engineering of these networks for other purposes.[67] They can be used to cover surfaces to make them accommodate desired materials, have catalytic parts to create bio reactive surfaces and they can even be engineered to have conducting properties.[32], [68]–[70] Also their mostly discovered biogenesis and secretion(Figure 1.2) make them great candidates to be used in biocatalysis applications as supporting materials or even as an active part of the system.

1.4 CONVENTIONAL RIBOREGULATORS AND TOEHOLD SWITCHES

Synthetic biology applications and systems sometimes include regulatory elements at transcriptional or translational level. They are generally RNA based. These elements are important because regulating a system only at the activation of transcription initiation actually restricts the variety of parts that can be used in it. Because it is mostly dependent on repressors or activators and only a limited number of these regulators are identified and even less are characterized. This limitation causes a bottle neck during the design of synthetic systems. However as a solution some scientists started to use regulator elements that are showing their effect dependent of RNA, which opened the possibility to regulate a system both at transcriptional and translational level.[71], [72] Then with developing technologies, riboregulators came in to play. Using RNA elements that are forming different secondary structures to restrict the translation by preventing ribosome binding and changing its shape only when there is complementary and thermodynamically more favourable RNA

sequences are around opened a variety of other possibilities in synthetic biology.[73]–[75] These are now one of the widely used elements of synthetic biology, riboregulators. Their design usually involves the RBS and start codon where RBS is usually in the secondary structures formed by RNA and start codon is located just at the end of those structures.[4] Therefore even though these parts opened up new ways to design synthetic systems there is still some limitation to riboregulator designs because of this RBS element and secondary structure forming limitations. However the study on these regulators are still continuing and recently one of the research groups actually engineered riboregulators in depth to achieve the most flexible sequence independent kind of riboregulators called toehold switches.[4] In this study they checked the thermodynamics of the antisense RNAs, called trigger[4] in this study, to check their binding to the secondary structure forming RNAs, called switches[4] in this study. They tried to change the design of switches and take the RBS out of hairpin structures to give it sequence based flexibility during the designing period unlike the conventional riboregulators. (Figure 1.3) Within two rounds of engineering the sequence and testing, they actually presented a novel design where the regulation is very tight and the signal induction is up to 653 fold increased. [4] They can even be used in a polycistronic and multiplex manner where multiple switch RNAs are transcribed from one construct and form a one long RNA where multiple secondary structures are forming and they are successfully opened upon trigger binding.[4] This design provides flexibility because now that there is no crucial element in the secondary structure of switch RNA so the sequence can virtually be anything that can form a secondary structure to block translation.

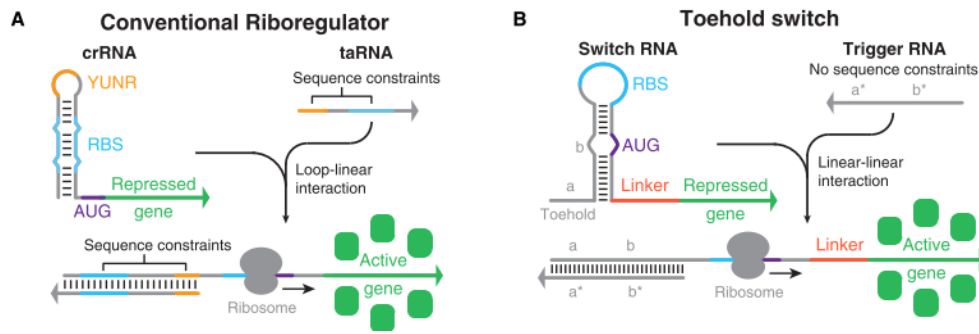


Figure 1.3: Illustration of the Toehold switches and conventional riboregulators. **A** shows the conventional riboregulators where RBS is in the hairpin structure which limits the design whereas **B** depicts the toeholds switch where RBS is actually not in the hairpin structure but in the loop so sequence limitation is gone. (Adopted from Ref 4 with permission from Cell Publishing Group)

1.5 AIM OF THE STUDY

In this study, we are showing two different models of biocatalysis with utilization of *E. coli* curli network to provide increased longevity and modularity. We are using the enzyme alkaline phosphatase (ALP) for all biocatalysis processes in this study due to its easily assessed activity through its commercially available substrate PnPP. Also this enzyme, after being produced, transported to the periplasmic space and this provides convenience in using whole cell approaches since its substrate can pass through outer membrane and be processed. Also this enzyme can be utilized future applications requiring phosphatase activity such as biomineralization.[76] Inspired by the methods to overcome sustainability issue of biocatalysis,[37] we are firstly focusing on whole cell biocatalysis and calling this approach from now on as whole cell approach. To further improve this approach and increase its longevity we are also utilizing *E. coli* curli network. (Figure 1.4) For this purpose we used a system

where the host, *csgA* knock-out *E. coli*, forms curli network by producing anhydrotetracycline (aTc) induced *csgA* from one plasmid and produces arabinose induced alkaline phosphatase (ALP) enzyme from another plasmid. This approach showed that linking enzyme production with controlled biofilm formation significantly increased enzyme activity and prolonged its duration compared to using wild type (WT) cells. Encouraged by the results we moved on to try different type of systems. Secondly we wanted to focus more on biofilm engineering to improve our biocatalysis system and calling this approach from now on as engineered biofilm approach. (Figure 1.5) For this approach we fused ALP to *csgA* and make the same host produce its curli network with enzyme coupled fibers this time. We showed that enzyme coupled curli fibers showed significant ALP activity and preserve this activity for a similar duration to the whole cell system. We have then aimed to couple the first two systems to riboregulators called toehold switch for more controlled production.[4] This design contains *csgA* and ALP enzyme induced with aTc and arabinose respectively. Toehold switches works in a way that regulates expression in between transcription and translation. Switch fused enzyme RNA is forming a hairpin due to designed switch sequence and it can only be opened by trigger RNA which is thermodynamically favourable by switch sequences and complimentary to them.[4] This way we aimed to reduce the leakage in our system to further increase the enzymatic activity but it is only tested with GFP in our strains and constructs and it showed significant increase, almost 50 fold. All in all we showed that biocatalysis longevity and efficiency can be increased by engineering and utilizing biofilms along with enzymes and cells.

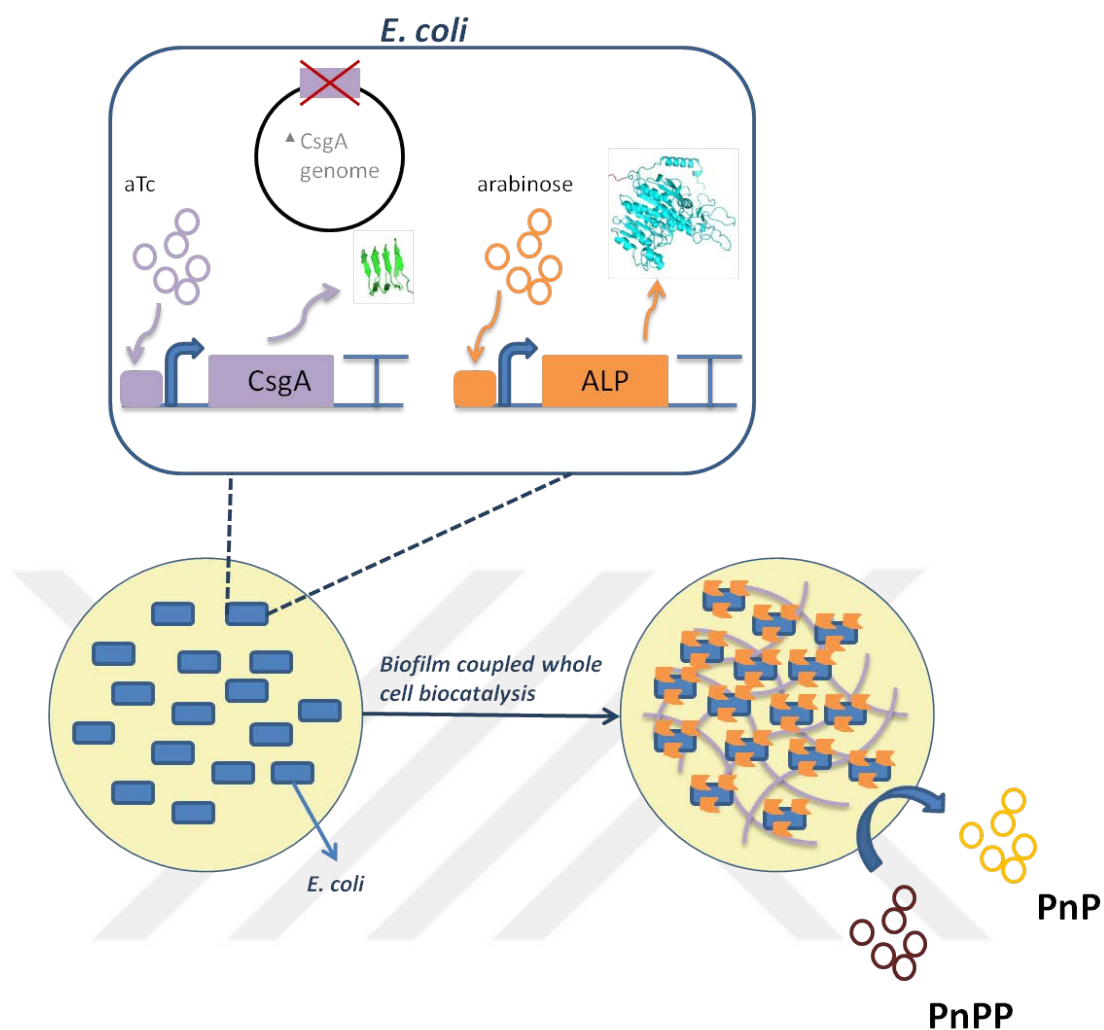


Figure 1. 4: Representation of the whole cell approach where cells are first induced to form a biofilm network for different periods of time. Trying to assess the effect of biofilms formed for different durations, cells are then induced to produce enzymes and their enzymatic activity is measured by PnPP.

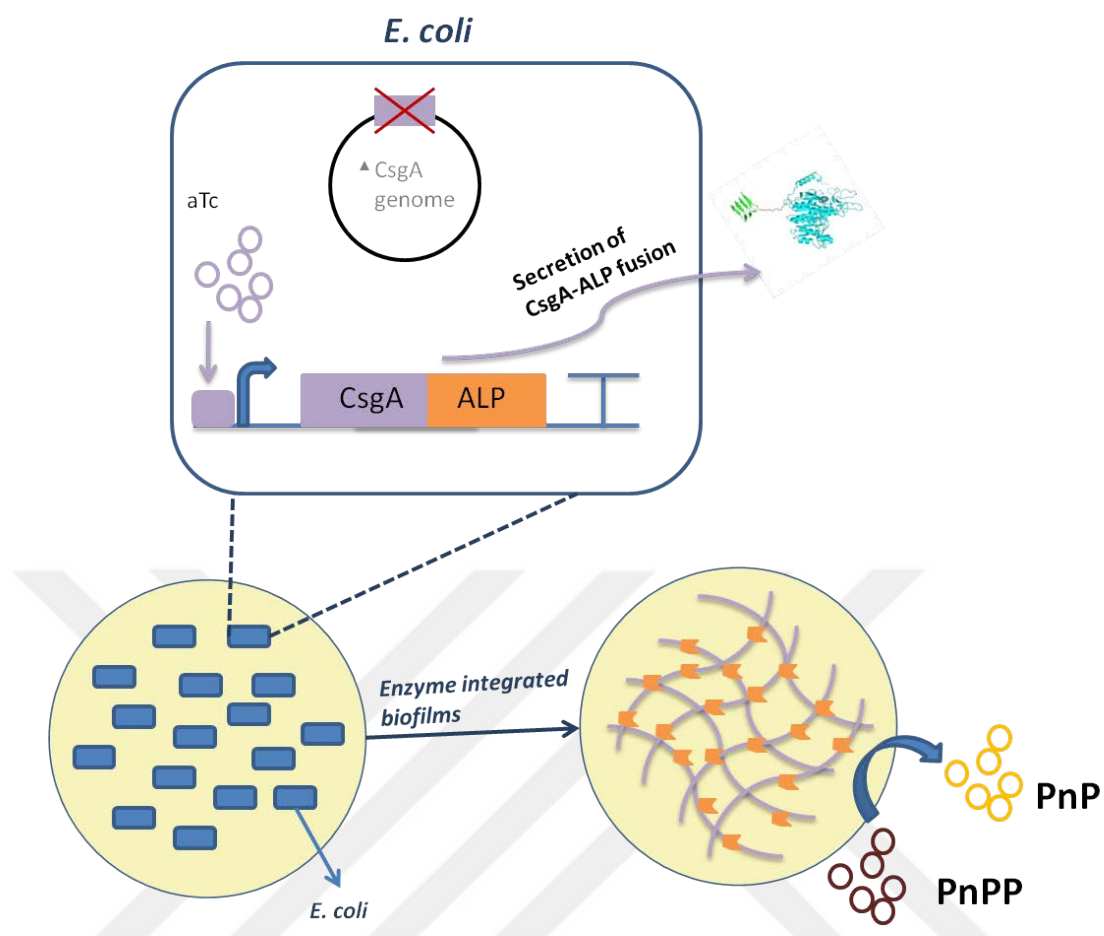


Figure 1. 5: Representation of the engineered biofilm approach, where ALP enzyme is fused to CsgA protein and during the formation of biofilms for different periods of time, ALP enzyme is also increasing in amount on the surface where biofilm is forming. Then the enzymatic activity is measured with PnPP.

CHAPTER 2 EXPERIMENTAL SECTION

2.1 CELL STRAIN, GROWTH AND MEDIUM CONDITIONS, TRANSFORMATIONS

E. coli MG1655 strain with pro expression cassette and CsgA knock-out is used as host for expression of all biofilm related constructs since we don't want wild type CsgA to interfere with our expression. Also because we want to strictly control the biofilm formation so if a WT cell is used, it would form biofilm even without our induction, up to a point thus we are using CsgA knock-out cells. This strain has the pro cassette which represses the expression of genes downstream of tet operator and therefore useful to us. Toehold switch calibration experiments with switch-GFP construct are done with *E. coli* BL21 DE3 strain. This strain utilizes T7 DNA polymerase and has it under regulation by Lac operon thus usefull for inductions with IPTG. That is why switch and trigger constructs are cloned downstream of T7 promoter in these initial calibration experimentes.

All growth are done in Luria-Bertani (LB) and changed in to M63 minimal medium for experiments involving biofilms. Toehold calibration experiments are done with LB only. LB used in growth is as follows; 10 g NaCl (Sigma-Aldrich), 10 g Tyrpton (Sigma-Aldrich), 5 g Yeast Extract (Sigma-Aldrich) dissolved in 1L of water and autoclaved. M63 is purchased from Amresco as powder and prepared as manufacturer instructed.

Transformations for clonings are done with DH5alpha competent cells since it has higher efficiency. Competent cells are prepared with chemical competent cell preparation protocol as follows; cells are grown overnight at 37 °C then diluted 1:100 into new LB medium and grown until 0.2-0.5 OD. Then they are chilled on ice for 10

minutes and centrifugated at 3000g for 10 minutes at 4 °C and resuspended in chilled TSS buffer and aliquoted as its seen fit and stored at -80 °C. TSS buffer recipe is

5g PEG 8000

1.5 mL 1M MgCl₂ (or 0.30g MgCl₂*6H₂O)

2.5 mL DMSO

Add LB to 50 mL

And aliquote and store at -20 °C. After constructs are verified with sequencing, they are transformed in to their respective hosts; for fusion and CsgA and ALP constructs it is CsgA knockout DH5alpha mg1655 with pro cassette strain and for toehold constructs it is BL21 DE3 strain.

Transformation is done by putting 100-150ng of desired DNA in to competent cell containing eppendorf and waiting for 20minutes on ice. Then shocking it at 42 °C for 45 seconds to make it take the DNA in and putting it on ice for 2 minutes. Then 400ul of LB medium is added on to it and incubated at 37 °C for 45 minutes. Then centrifugated at 3000g for 10 minutes and approximately 50-100ul of LB left and rest is discarded. Then cells are resuspended in remaining LB medium in the tube and spread on to LB agar plates with respective antibiotics in them and grown overnight at 37 °C.

2.2 CLONING CONSTRUCTS

All clonings are done using Gibson Assembly method and are shown in Figure 3.1. Gibson Assembly mix is made in laboratory by following this recipe;

320 μ l 5X ISO buffer

0.64 μ l of 10 U/ μ l T5 exonuclease

20 μ l of 2 U/ μ l Phusion polymerase

160 μ l of 40 U/ μ l Taq ligase

Add water to 1.2 ml

Aliquot 15 μ l for 1 reaction.

5X ISO buffer is as follows: 3 ml of 1 M Tris-HCl pH 7.5, 150 μ l of 2 M MgCl₂, 60 μ l of 100 mM dGTP, 60 μ l of 100 mM dATP, 60 μ l of 100 mM dTTP, 60 μ l of 100 mM dCTP, 300 μ l of 1 M DTT, 1.5 g PEG-8000, 300 μ l of 100 mM NAD, Add water to 6 ml and aliquoting.

Genes are amplified with PCR from previously available constructs in the laboratory. aTc inducible CsgA construct with cmR gene is taken from Ebuzer Kalyoncu, a fellow group member. Primers are designed for Gibson Assembly method (The Appendix Table A.1), and for toehold parts, trigger 1 and switch 1 synthesized as oligonucleotides by Sentagen Company. For Gibson Assembly protocol to work amplified inserts need to have 15-30 bp overlap with the backbone or other parts that are being cloned. That is why primers have varying lengths of bases that are not complimentary to the insert at first look but they are in fact complimentary to the backbone or adjacent parts. Gibson Assembly protocol is as follows; 50ng backbone, either amplified by PCR or digested with restriction endonuclease enzymes to make it linear, is mixed with equal concentration of insert DNA piece in 15ul of Gibson Assembly reaction mix. Then if the mixture is not already 20ul it is completed to

20ul. Then Incubated at 50 °C for 1 hour and then the mixture is transformed in to DH5alpha competent cells and left to grow on agar plates at 37 °C for 16 hours. Then colonies are picked and sent to sequencing to GENEWIZ Company to be validated. To clone ALP gene in to Arabinose inducible pBad vector with ampR gene downstream of pBad promoter, primers in the table 1 for ALP amplification are used. PCR is done 62 °C annealing temperature with 1 minute elongation period in a cycle. pBad vector is amplified with 66 °C annealing temperature and 2 minute elongation period. Then they are cloned as Gibson Assembly protocol suggests. CsgA-ALP fusion construct is cloned by amplifying inserts CsgA and ALP with 59 °C and 60 °C annealing temperatures respectively and 1 minute elongation period and, amplifying pZa backbone with cmR gene and downstream of tetO operator to make it aTc inducible, at 67 °C annealing temperature and 2 minute elongation period and following cloning protocol. Amplification of CsgA adds linker sequences. Trigger 1 oligonucleotide is amplified in PCR with 54 °C annealing temperature and 30 seconds elongation time then cloned in to pet22b backbone downstream of T7 promoter upstream of T7 terminator. Pet22b backbone for this construct is amplified at 65 °C annealing temperature at 2:30 minute elongation period and has ampR gene. Switch 1 part 1 and part 2 are put into PCR reaction at 56 °C annealing temperature and 30 seconds elongation time to make them 1 double stranded DNA piece first. Then amplified at 62 °C annealing temperature and 30 seconds elongation time and cloned in to pZa vector with cmR gene downstream of T7 promoter and upstream of GFP. GFP in this constructs is amplified from a construct taken from Behide Saltepe, a fellow group member, at 59 °C annealing temperature and 1 minute elongation period and both backbone, GFP and switch 1 double stranded DNA piece are cloned

simultaneously in Gibson Assembly reaction. All the maps for constructs are shown in The Appendix Figures A.1 to A.5 respectively as aTc inducible CsgA in pZa backbone, arabinose inducible ALP in pBad vector backbone, aTc fusion of CsgA and ALP in pZa backbone, Trigger part in pet22b backbone, Switch-GFP part in pZa backbone. Also they have been aligned to their respective sequences in Geneious Software and illustrated in The Appendix Figures 9 to 18. Switch 1 part, CsgA gene, Switch 2 part and ALP gene inserts are cloned in to pZa backbone downstream of proD promoter again with Gibson Assembly method in this order. Parts and genes are amplified with PCR along with pZa backbone. pZa backbone had proD promoter added while being amplified. T_m values are as described in Table A.1.

2.3 USING NCBI BLAST TOOL AND GENEIOUS SOFTWARE TO CHECK SEQUENCE

ALIGNMENTS OF CONSTRUCTS

NCBI Blast tool is used to check the results of Sanger Sequencing of my constructs by aligning them to the designs. To do that, when on the website <https://blast.ncbi.nlm.nih.gov/Blast.cgi> choosing “Nucleotide Blast” option will open the page. While there, choosing the option “Align two or more sequences” it is possible to align any given two sequences. Using “Highly similar sequences” option will restrict mismatches and align only high percentage of complimentary sequences. After that all constructs and their sequence results are aligned with their design sequences to validate the clonings using geneious software. (The Appendix Figure A.7, A.8, A.9, A.10).

2.3 SYNTHETIC BIOFILM NETWORK FORMATION AND ENZYME PRODUCTION

All biofilm forming cells are grown in Luria-Bertani (LB) Broth medium at 37C overnight and diluted 1:100 in to LB again on the experiment day and grown until

OD600 value of 0.6 at 37°C. Then cells are precipitated at 3000g for 10 minutes and changed in to M63 minimal medium and supplemented with %0.2 glucose and appropriate antibiotics and aTc (1:1000 V:V ratio) to induce biofilm formation. They are then distributed in to 24-well cell culture treated plates (Corning, Sigma-Aldrich) as 2ml per well to form curli network at 30°C without shake for 3 to 28 days depending on the experiment. For CsgA and ALP separate construct containing experiments, whole cell experiments, it was 3 to 28 days. Every time point, had 6 biological replicates. Cell medium with aTc is changed every three days. On each time point, after the incubation period is over, mediums on samples are discarded and samples are washed 3 times with water to discard non-sticky cells. Then samples are provided with same medium again but this time instead of aTc, they include %0.2 arabinose to induce ALP production. They are then left to incubate at 37 °C without shaking for 3 hours. For CsgA-ALP fusion protein experiments time points were 3 days, 5 days, 7 days, 9 days and 14 days. Samples again had 6 biological replicates and again induced with aTc (1:1000 V:V ratio). These experiments did not have secondary induction period since ALP is fused to biofilms, after the wash samples goes straight to PnPP assay for activity measurements.

2.4 ALP ENZYMATIC ACTIVITY MEASUREMENT WITH PNPP ASSAY

PnPP reaction buffer which has the following recipe; 0.1M Glycine (VWR), 1mM MgCl₂ (Merck), 1mM ZnCl₂ (Merck), pH set to 10.4 with NaOH. 0.5mM Pnpp added on to samples after the wash steps on time point days, just enough to cover the bottom of wells (600 ul) and leaving plates to incubation at 37°C without shaking for 2 hours. Then absorbance is measured with M5 plate reader at 405nm.

2.5 SEM AND TEM SAMPLE PREPARATION AND PROCESS

For every sample in each experiment, 1 sample is set aside for SEM preparation. For this purpose, silicon wafer pieces are left in the well where cells are forming biofilm. During this process they formed their biofilms on wafers too. On the time point days, wafers are collected and washed with water just like experiment samples. Then they are incubated in %2 glutaraldehyde solution for fixing purposes for 1 hour in room temperature. Then are washed with %25, %50 and %75 ethyl alcohol for 3 minutes and then with %100 alcohol 3 times for 10 minutes each. The last %100 alcohol is left on sample and samples are dried in critical point dryer (CPD) to preserve cell structure better. Then they are visualized with SEM.

For TEM sample preparation, each sample set aside for SEM, after wafers are collected, surface of those samples in well of 24-well plate are scraped and solved in PBS. Then from those samples 20 ul is dropped on nickel TEM grids and incubated for 5 minutes. This process is done to the surface of grid covered with carbon film. (opaque face) Then grids are washed by putting them face down on droplets of water. Then grids are dropped on %2 uranyl acetate droplet for about 20-30 seconds for negative counter staining of samples and again washed 3 times on water droplets.

2.6 CRYSTAL VIOLET STAINING AND ANALYSIS

Crystal violet staining is done with %0.1 crystal violet in water solution. Each time point of whole cell approach had extra set of 6 biological replicates for these experiments. Each sample, after the biofilm forming medium is discarded and samples are washed, treated with 400ul of crystal violet solution for 15 minutes in room temperature. Then they are washed 3 times with water and left to drying on bench. After that they are visualized by ChemiDoc instrument (Bio-rad). Images are analyzed with imageJ program. They are turned in to 8 bit black and white, used

threshold to calculate the stained areas and percentage of them over whole well area is calculated and student's t test is applied for each time point and sample.



CHAPTER 3

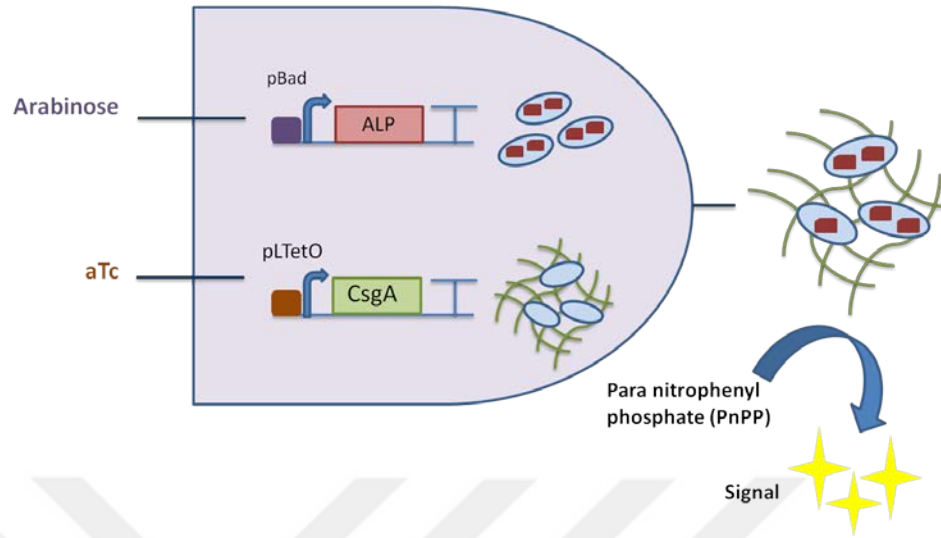
RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Building synthetic circuits

To test the synthetic cellular system approaches and RNA based regulator, toehold switch, firstly the circuits are built. Circuits are built by cloning the respective genes and parts under the conditions explained in the experimental part. Genes are amplified from already existing constructs unrelated to this work, through PCR with conditions stated in the experimental section for each gene. Backbones for each construct are also amplified as explained. Only the toehold parts, trigger and switch, are ordered as oligonucleotides first, then amplified via PCR for addition of overhangs in accordance with the Gibson Assembly conditions explained in the experimental section. After each amplification step via PCR, products are run on %1 agarose gel for pieces bigger than approximately 300-400 bp and run on %2 agarose gel for pieces smaller than that since resolution restrictions that we could get from the gel and comparison to the DNA ladder restrictions are starting approximately at that length. For smaller pieces 50 bp DNA ladder (NEB) is used and for others 2 log DNA ladder (NEB) is used. Firstly, the whole cell approach circuits are built. CsgA gene in pZa backbone with cmR gene is a courtesy of fellow group member Ebuzer Kalyoncu. This construct's is cloned by him and the Figure 3.1 shows both circuit design of whole cell approach and CsgA and ALP construct design along with agarose gel electrophoresis results for PCR product of CsgA gene amplification.

A

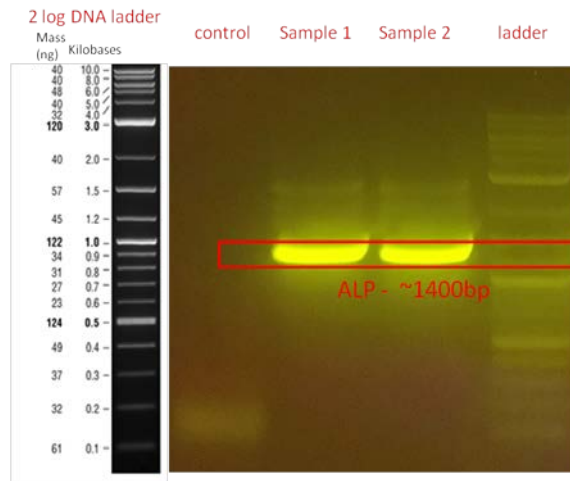


B



Figure 3. 1: **A** shows the circuit design of whole cell approach and design of CsgA construct and ALP construct. **B** shows agarose gel electrophoresis results (courtesy of Ebuzer Kalyonucu) of PCR products from CsgA amplification. In box shows the two bands from two reactions of CsgA amplification around 450 bp where they are expected and control column has no band as expected. Sample 1 and 2 are same reactions prepared in different tubes get a higher yield of PCR product. 2 log DNA ladder is used to see size of bands.

A



B



Figure 3. 2: **A** shows the agarose gel electrophoresis result of PCR product from ALP amplification for cloning in to pBad backbone. 2 bands from samples prepared exactly the same to get more DNA product after PCR, are seen around 1400bp as expected and control sample produced no products represented by no band on the gel. **B** shows the agarose gel electrophoresis results of amplification of pBad vector through PCR. Band around 4000bp is the expected pBad backbone amplified. 2 log DNA ladder is used in both, to determine band lengths. Inboxes show the bands indicating products from PCRs.

ALP construct is cloned as seen in Figure 3.1, design of ALP construct. Amplification of ALP gene and backbone, pBad vector, is shown by running the PCR products on agarose gel. (Figure 3.2 A and B) ALP construct is verified by digesting and running on agarose gel to see digested ALP band and backbone at respectively 1400 bp and 4000bp approximately. (Figure 3.3)

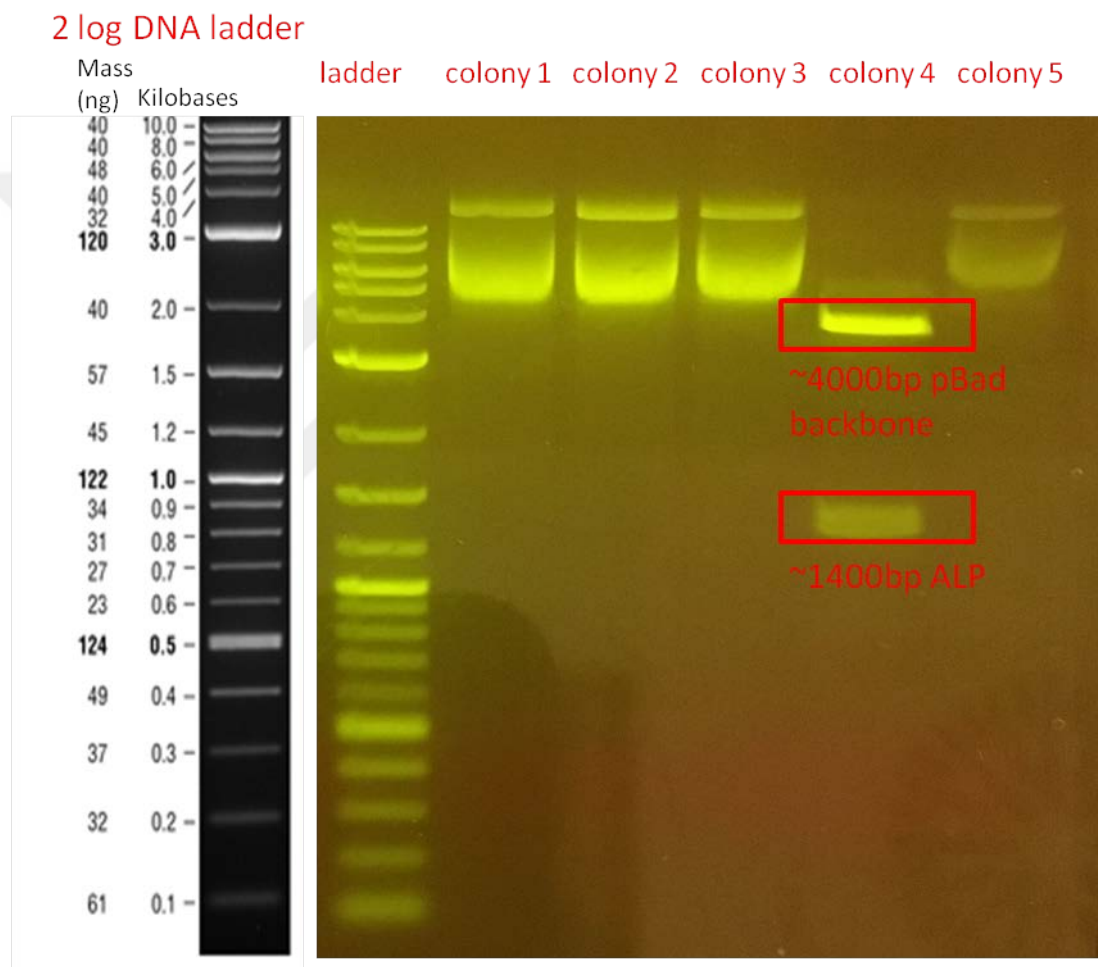


Figure 3. 3: Agarose gel electrophoresis results of ALP constructed isolated from colonies picked after cloning. Constructs are digested with KpnI and XhoI enzymes and run on gel and only the 4th colony is verified seen by ALP band, around 1400bp, and backbone band, around 4000bp. 2 log DNA ladder is used to determine the lengths of bands. Inboxes show the bands indicating products from PCRs.

For the engineered biofilm approach, circuit design and constructs are shown in Figure 3.4, 3.5 and 3.6. Firstly CsgA and ALP genes are amplified by PCR and run on agarose gel for separation and isolation from the reaction. Then backbone, pZa, is amplified with conditions explained in experimental section and all three products, CsgA, ALP and backbone are used to build the fusion protein construct through Gibson Assembly method.

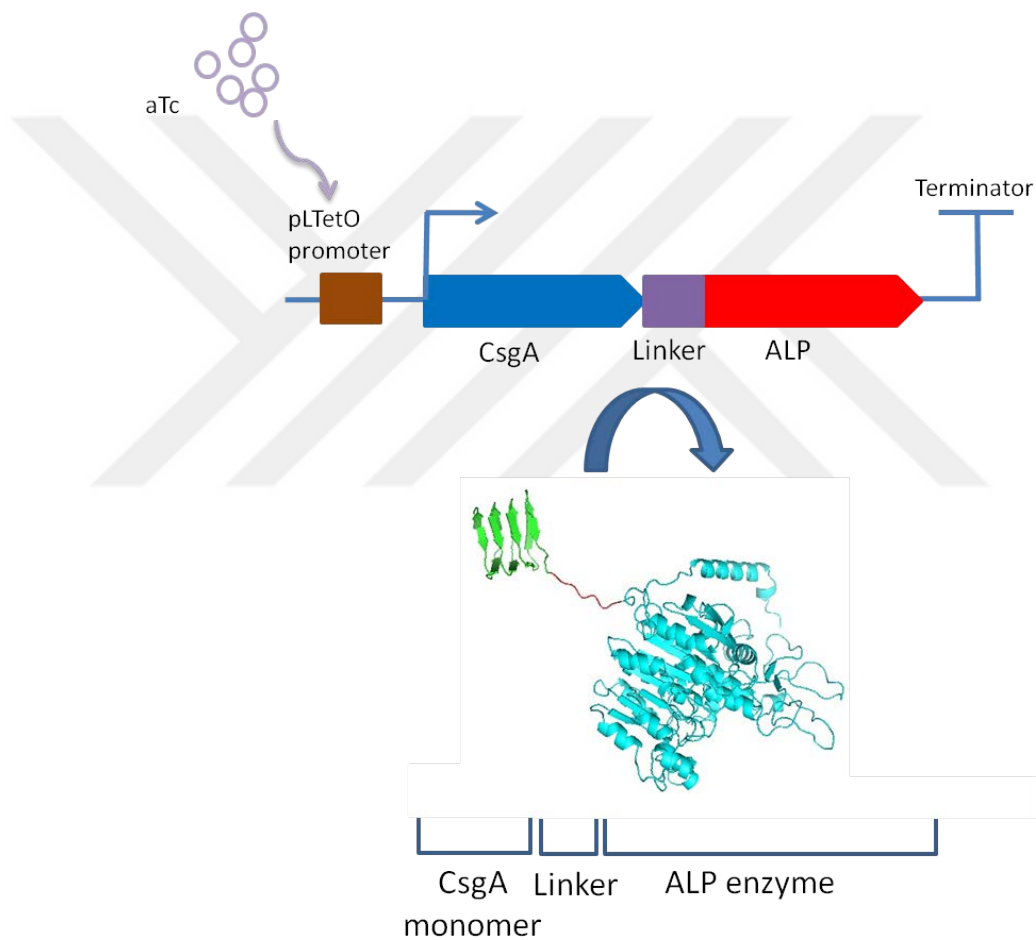


Figure 3. 4: The design of construct for fusion of ALP and CsgA for engineering of biofilm and computationally predicted product of the construct.

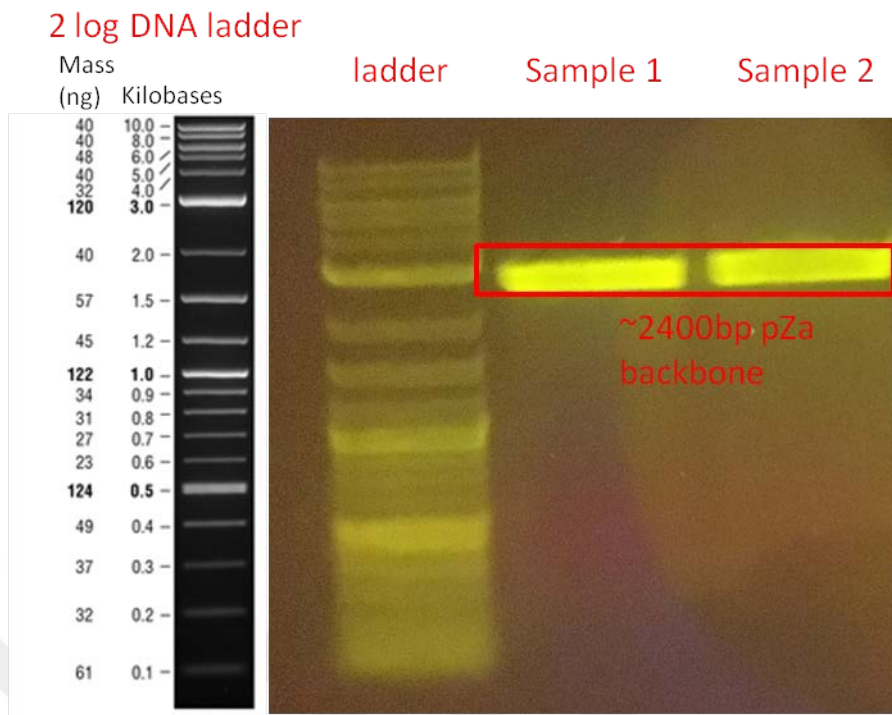
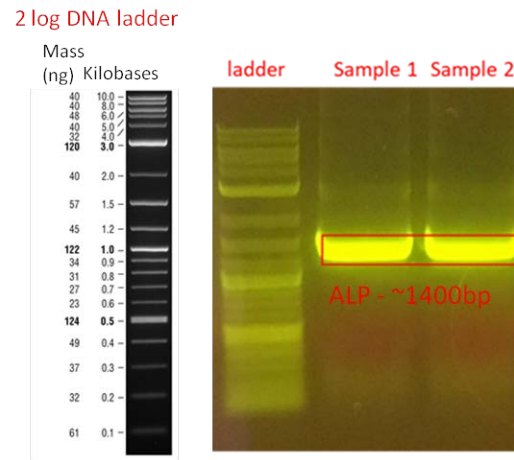


Figure 3. 5: **A** shows the design of construct for fusion of ALP and CsgA for engineering of biofilm. **B** shows the agarose gel electrophoresis results of PCR product from amplification of pZa backbone for fusion protein cloning. Bands are seen around 2400bps which expected from the pZa backbone with tet operators and no insert. Samples are exactly the same in preparation, just to get high yield of PCR product there are two. 2 log ladder is used for determining the length of bands. Inboxes show the bands indicating products from PCRs.

A



B

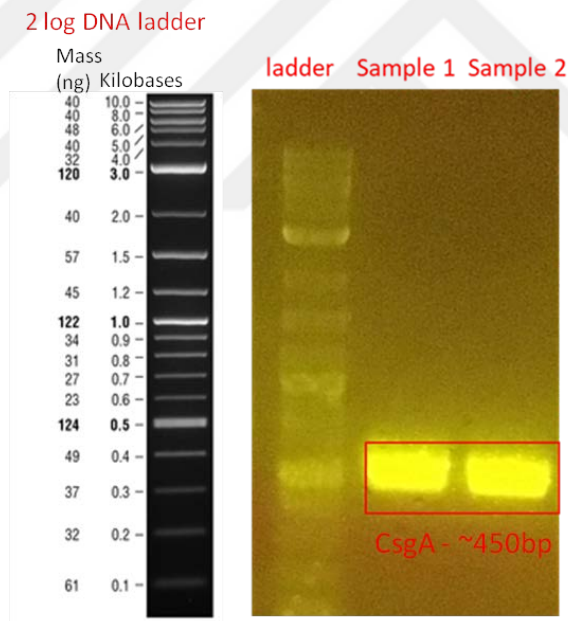


Figure 3. 6: **A** and **B** shows the agarose gel electrophoresis results of PCR products from ALP and CsgA amplification respectively. For ALP the bands are seen around 1400bps, and for CsgA around 500bps as expected. 2 log DNA ladder is used for both to determine lengths of bands. Inboxes show the bands indicating products from PCRs.

Toehold switch riboregulator parts are ordered as oligonucleotides and amplified via PCR using the noted primers and conditions in The Appendix and experimental section. Figures 3.7, 3.8 and 3.9 are showing the design of toehold switch constructs and gel electrophoresis results of amplifications of Trigger part and pet22b backbone; switch part, GFP part and pZa backbone. Trigger is cloned in to pet22b backbone downstream of T7 promoter and switch part is cloned in to pZa backbone downstream of T7 promoter, upstream of GFP gene. Design requires BL21 DE3 strain due to T7 promoters. T7 polymerase production in this strain of cells can be induced by IPTG and otherwise closed thus toehold GFP system is inducible by IPTG.

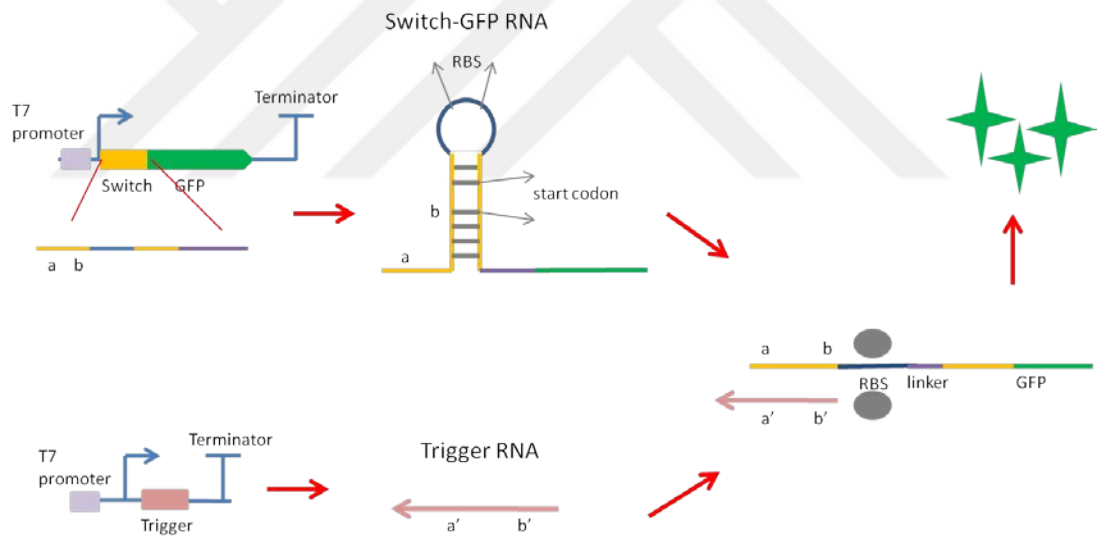
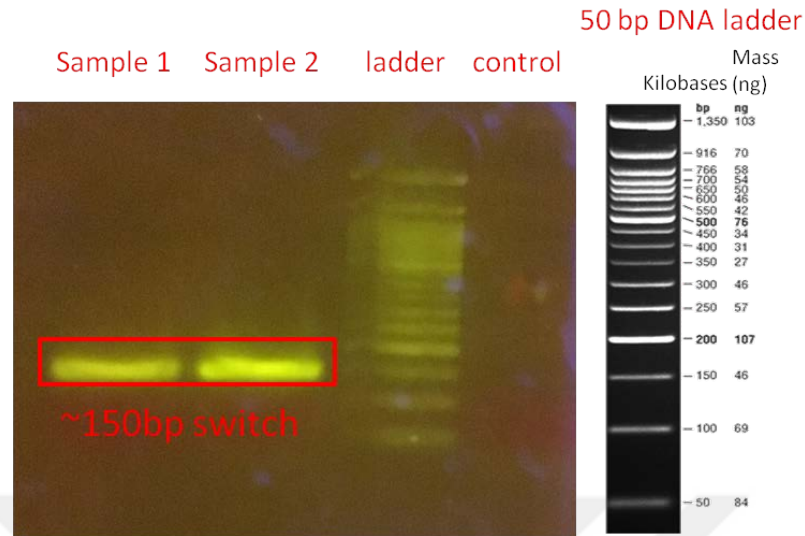


Figure 3. 7: Design of toehold constructs where switch part and GFP are cloned in succession in to pZa backbone and trigger is cloned in to pet22b backbone. T7 promoters are used since they are inducible by IPTG in BL21 DE3 strain.

A



B

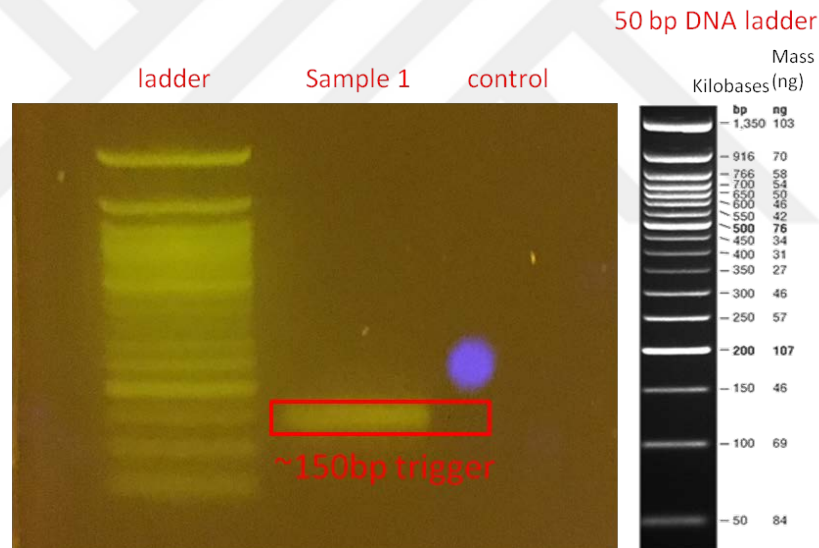
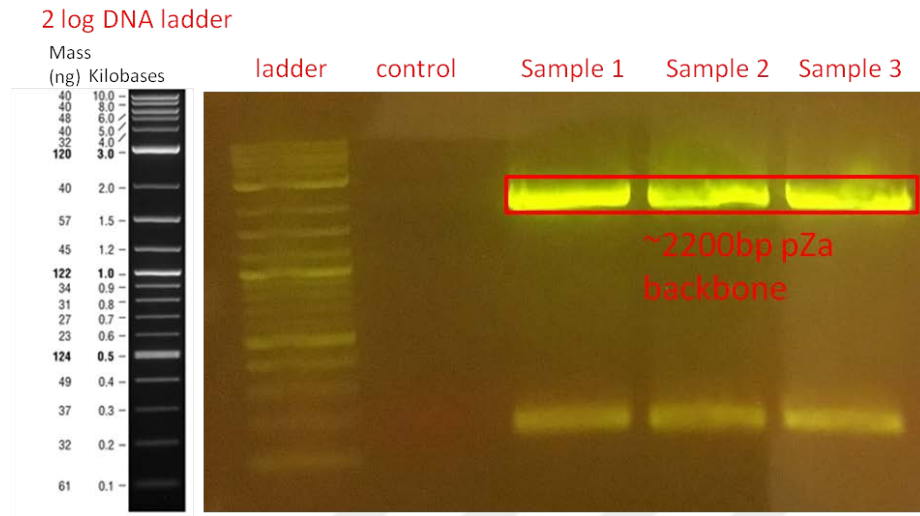


Figure 3. 8: **A** and **B** shows the agarose gel electrophoresis results of switch and trigger parts respectively, amplified through PCR using the oligonucleotides of these parts as templates. Both were expected around 150bp and seen on gel. Due to small size of pieces, %2 gel is used and 50bp DNA ladder is used for size determination. Bands are shown with inboxes. Samples in each gel image are exactly the same with each other in preparation, only there to get a higher yield of PCR product.

A



B

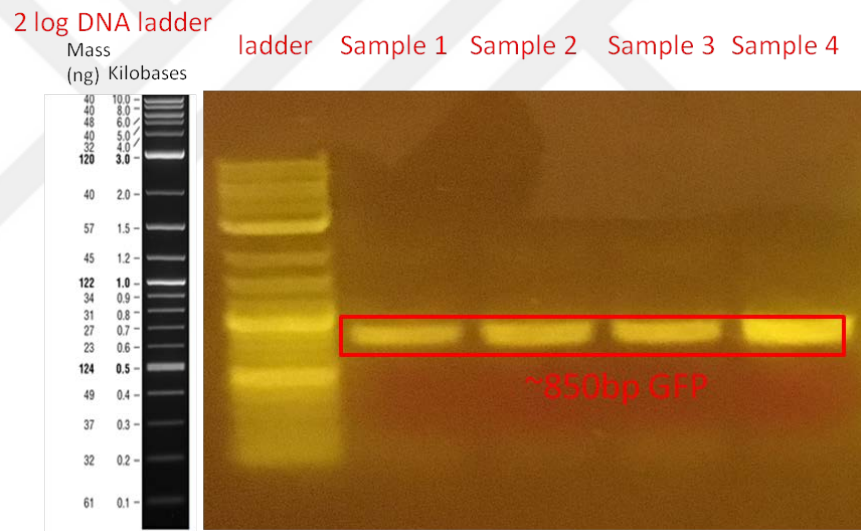


Figure 3. 9: **A** shows the gel electrophoresis results of amplification of pZa backbone to be used in toehold construct clonings. **B** shows the same results for GFP. Both are size determined by 2 log DNA ladder. GFP is seen around 850 bps as expected due to additions of hangovers in accordance with Gibson Assembly protocol. pZa backbone is seen around 2200bps expected due to lack of tet operator and insert. Band are shown with inboxes. Samples in each gel image are exactly the same with each other in preparation, only there to get a higher yield of PCR product.

Toehold integrated ALP and CsgA constructs are built by Gibson Assembly method. CsgA is regulated by switch 1, ALP is regulated by switch 2 thus they are opened to translation upon meeting with trigger 1 and trigger 2 respectively. Switch parts and CsgA and ALP genes are on one vector, pZa backbone and they are downstream of proD promoter. This promoter is constitutively active thus producing CsgA and ALP constantly but since they have switch parts upstream of them, their translation is not occurring. Trigger containing construct produces trigger 1 upon aTc induction, since it is downstream of aTc inducible promoter, and trigger 2 upon arabinose induction since it is downstream of pBad promoter. Therefore upon inductions and trigger part productions, translation of CsgA and ALP can continue. Only the switch, CsgA and ALP gene containing construct is built in time for this thesis. Genes and pZa backbone is amplified with respective primers (Table A.1) and run on 1% agarose gel as seen in Figure 3.10 then cloned with Gibson assembly and verified by sequencing. Result of sequencing is aligned with the design sequence (Figure A.10) in Geneious Software.

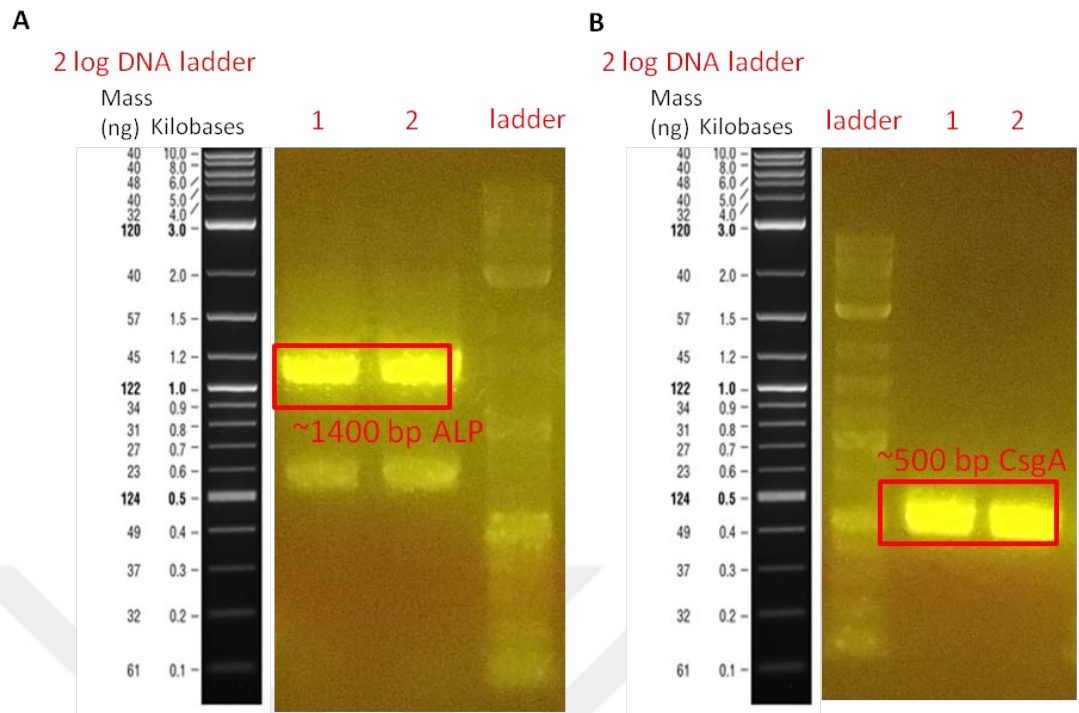


Figure 3. 10: **A** and **B** shows the agarose gel electrophoresis results of ALP and CsgA gene amplifications while adding switch 2 and switch 1 respectively through PCR. 2 log DNA ladder is used for size determination. Bands are shown with inboxes. Samples 1 and 2 in each gel image are exactly the same with each other in preparation, only there to get a higher yield of PCR product.

3.1.2 Whole cell system with curli network

First set of experiment was to test whole cell approach mentioned in the introduction where whole cell biocatalysis is coupled with biofilm to increase the viability of cells and immobilize them on the surface to achieve greater efficiency and longevity. Biofilms in the experiments are formed as explained in experimental part and over 28 days period. On the time points measurements of ALP activity are done with PnPP assay. Firstly CsgA knockout cells transformed with aTc inducible CsgA construct and arabinose inducible ALP construct are needed to be tested for their biofilm forming properties. Firstly crystal violet staining is done to check preservation and immobilization capability of biofilms. (Figure 3.11) From this result it can be seen that biomass is significantly higher in biofilm forming samples compared to other even compared to uninduced biofilm forming samples for each time point. Also a decreasing trend is seen here after 14 days, implying the stability of cells or biofilm is debunked after that point.

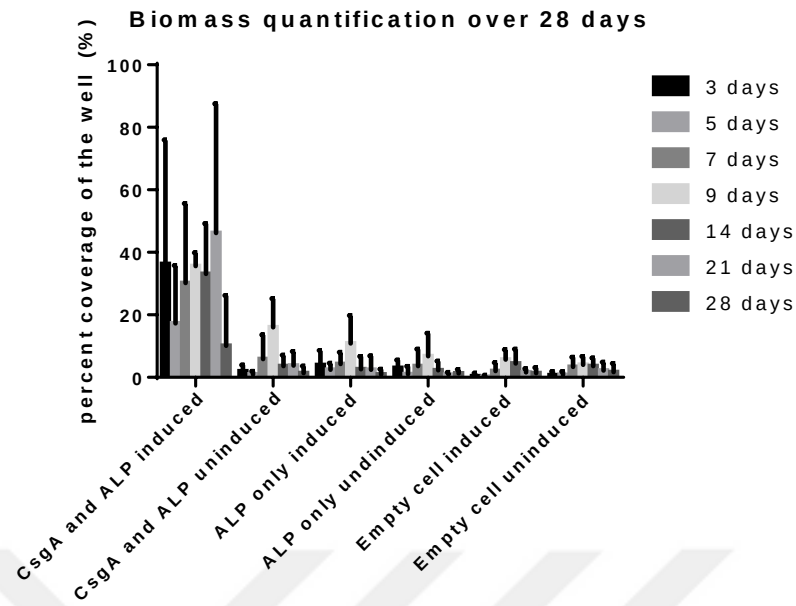


Figure 3. 11: Crystal Violet staining quantification for Biomass comparison of whole cell approach experiments. CsgA and ALP labeled samples are cells transformed with both constructs and biofilm formation is induced or not. ALP only labeled samples are cell transformed with only ALP construct and induced with aTc or not. Empty cells are not transformed with anything and only control. Percent of stained area over whole well area is represented here. CsgA and ALP labeled samples (double transformed) are significantly higher than other samples for all time points. (p value < 0.05 student's t test.)

After this, SEM imaging is used (Figure 3.12, 3.13, 3.14) and biofilms and morphologies of cells are visualized. Control groups consisting of non transformed cells and transformed with only ALP constructs are also visualized. It was seen that all double transformed samples formed biofilms as expected. The biofilms are starting to appear around day 3 as white little fibers surrounding the cells and then over time, up to 14 days they are even more widely dispersed and making cells form clusters on the surface. After 2 weeks time though it was seen cells are starting to lose stable shapes they were presenting at the beginning suggesting biofilms are not able to protect them after this point. To be sure that the fibers are there, TEM is used to see fibers up close. (Figure 3.15) It was seen there are actually fibers surrounding the cells.

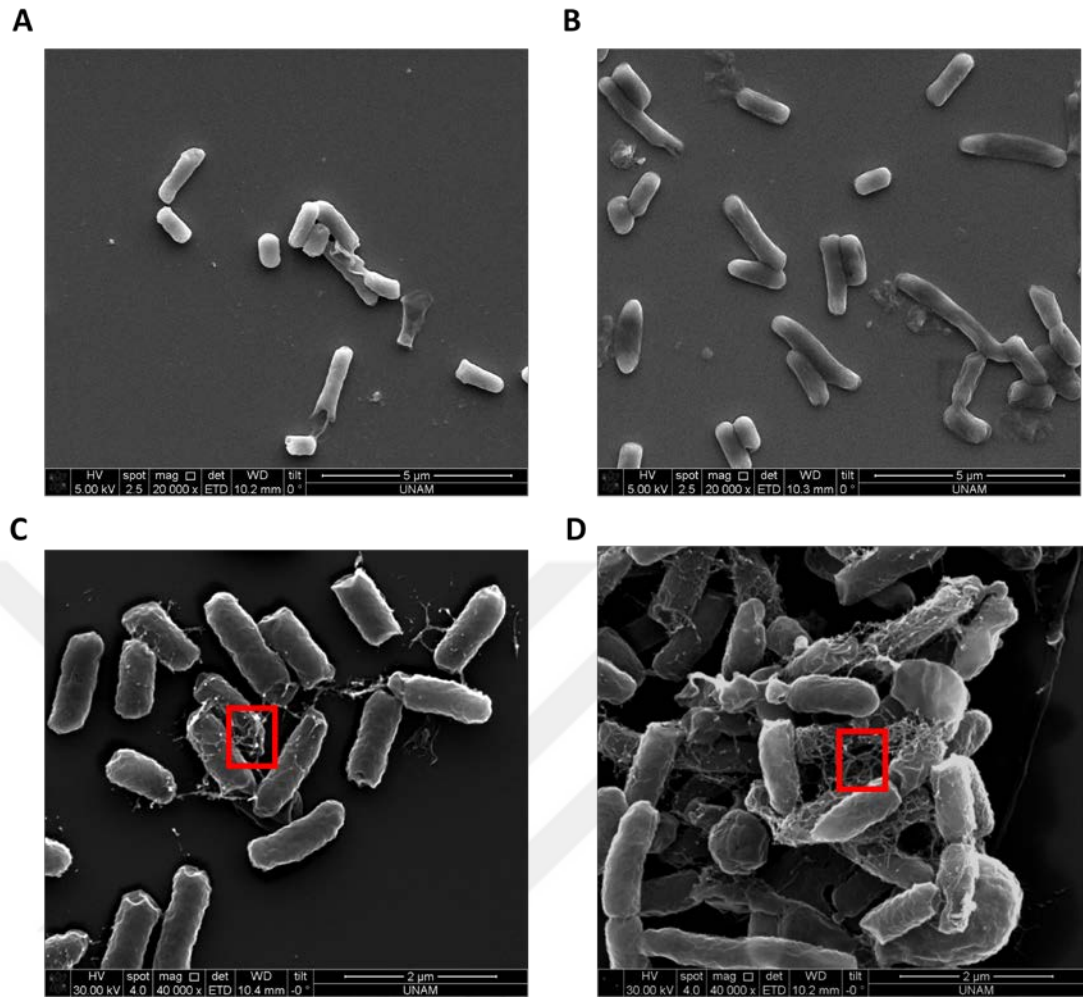


Figure 3. 12: SEM images of whole cells non-transformed (A) transformed with only ALP construct (B), only CsgA (C) construct or with both 2 constructs (D). Cells are induced with aTc and incubated for 3 days. Biofilms are formed in double transformed cells and only CsgA transformed cells. Images are taken by SEM after sample preparation on silicon wafers. Scale bars of SEM images are on each image respectively and magnifying amount is also stated on the images. Red inboxes shows biofilms.

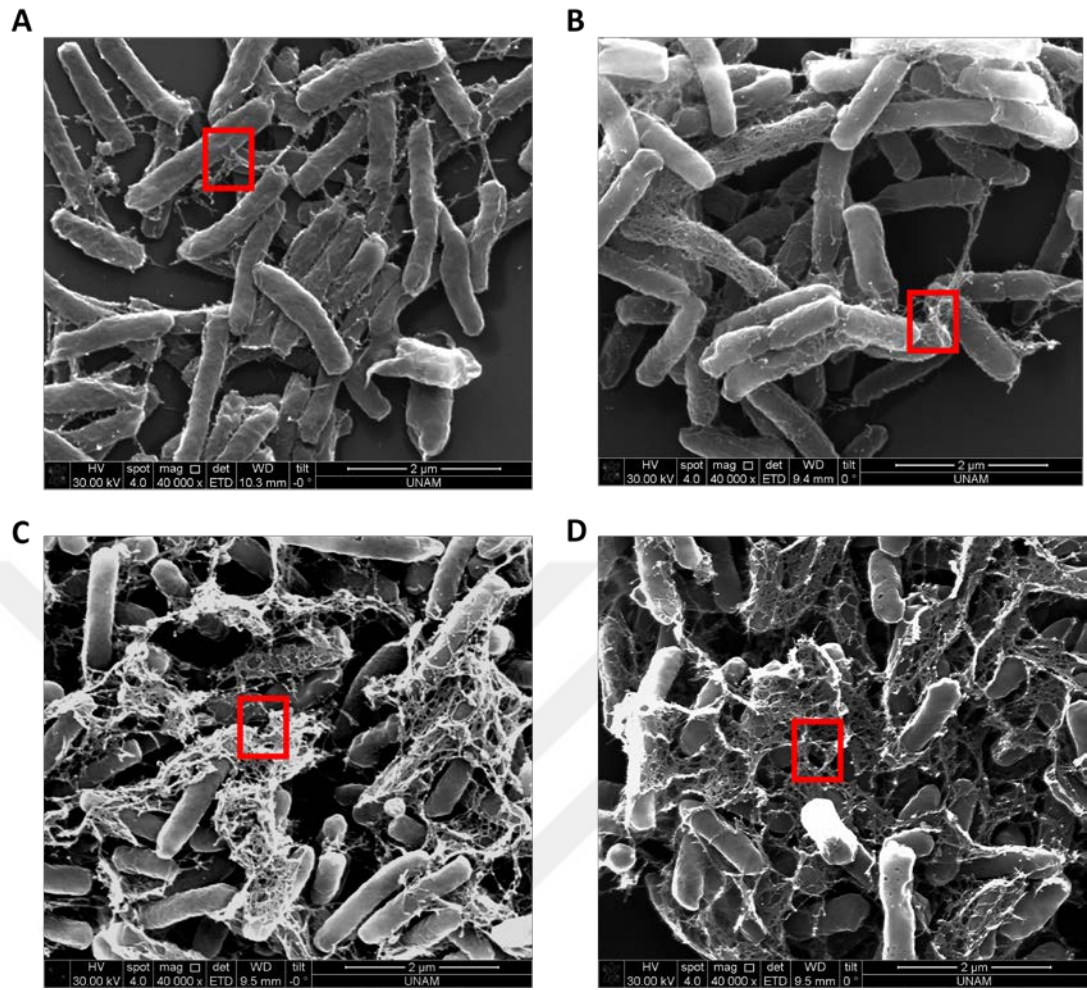


Figure 3. 13: SEM images of whole cells transformed with ALP and CsgA constructs, producing CsgA upon aTc induction. Biofilms are formed in 5, 7, 9 and 14 (A to D respectively). Images are taken by SEM after sample preparation on silicon wafers. Scale bars of SEM images are on each image respectively and magnifying amount is also stated on the images. Red inboxes shows biofilms.

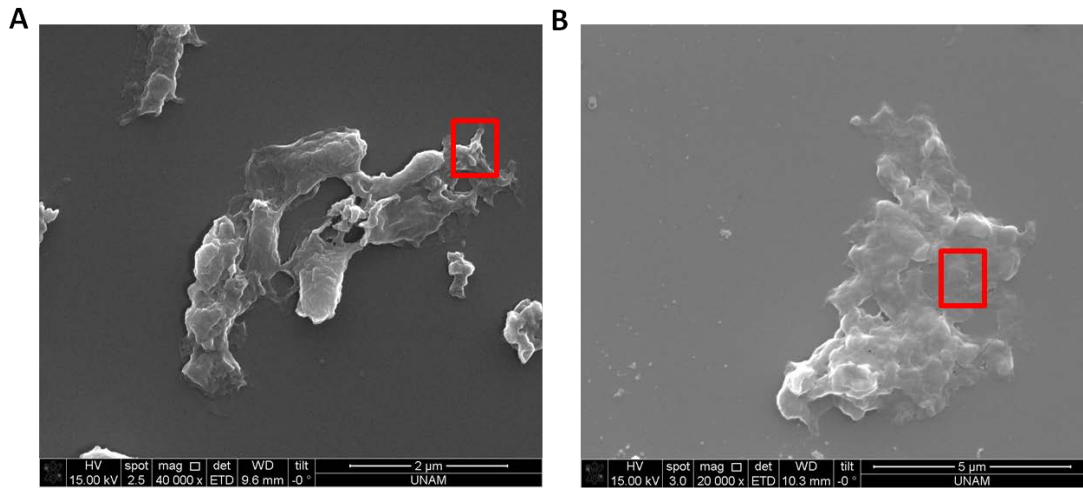


Figure 3. 14: SEM images of whole cells transformed with ALP and CsgA constructs, producing CsgA upon aTc induction. Biofilms are formed in 21 and 28 (A and B respectively). Images are taken by SEM after sample preparation on silicon wafers. Scale bars of SEM images are on each image respectively and magnifying amount is also stated on the images. Red inboxes shows biofilms.

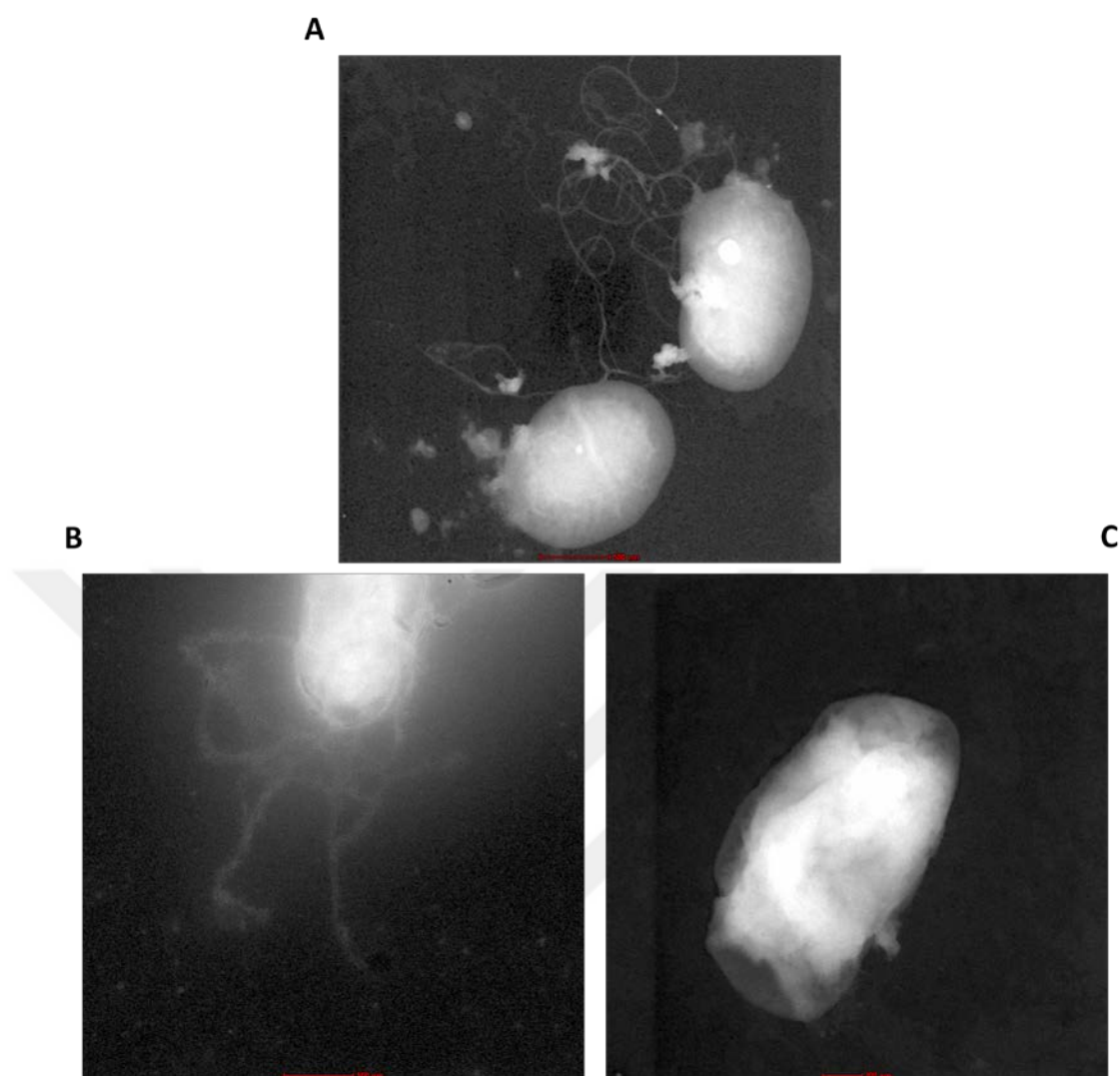


Figure 3. 15: TEM image of fibers formed by cells that are double transformed (**A**) only CsgA transformed cells (**B**) and non transformed cells (**C**). Scale bars are 500nm.

After confirming the presence and preservation capability of biofilm, ALP activity of the samples on each time point is measured by PnPP assay. (Figure 3.16) It is seen that, ALP activity is showing a trend in harmony with biomass quantification and increasing up to 14 day but from that point on decreasing again implying destability in biomass or biofilm.

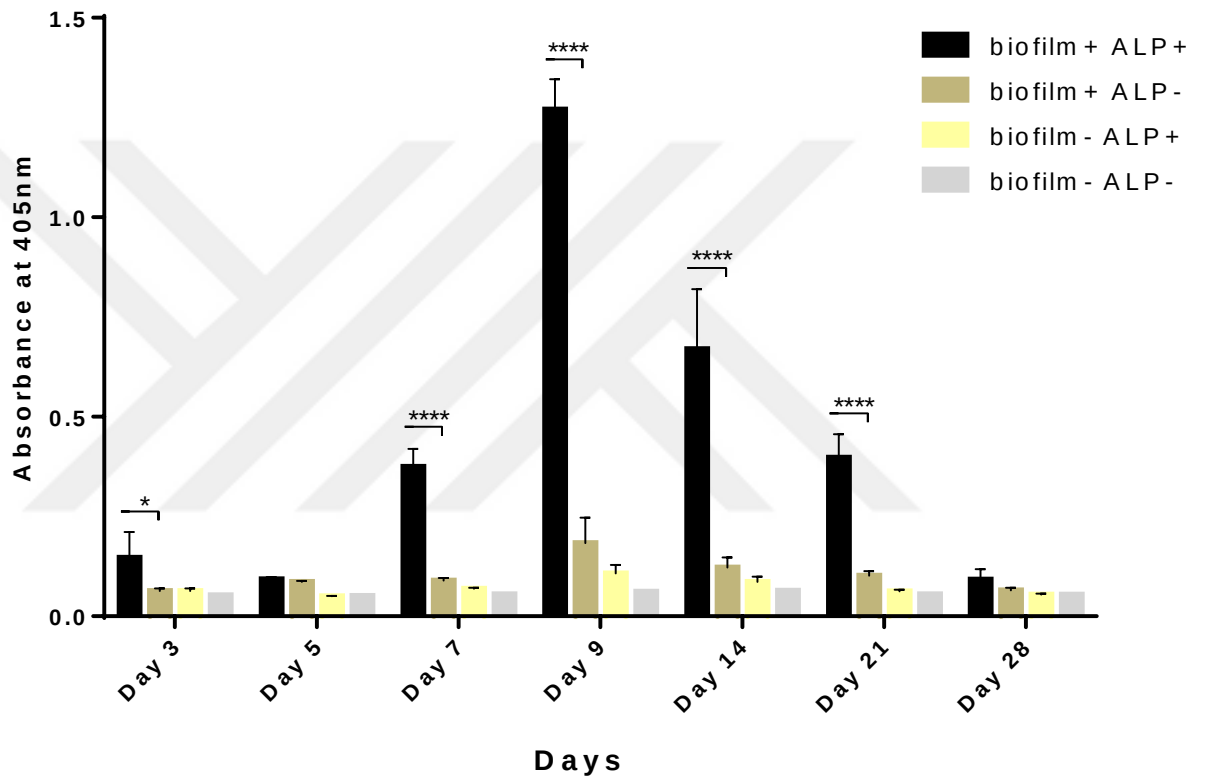


Figure 3. 16: ALP activity of biofilm forming CsgA knockout cells transformed with Arabinose inducible ALP construct over 28 days period. ALP activity is measured by PnPP assay. T test is used for significancy appointment (p value < 0.05 *, p value < 0.001 ** and p value < 0.00001 ****)

3.1.3 Enzyme coupled curli network

Engineered biofilm approach is tested with CsgA knock-out cells that are transformed with fusion protein CsgA-ALP and produce the protein upon aTc induction. Experiments in this section involves forming of biofilms with fusion protein expressing cells alongside with control cells that are not transformed or transformed only with ALP construct. Biofilms are formed in 3, 5, 7, 9 and 14 days and their structure is again visualized by SEM and the fiber presence is confirmed TEM imaging. (Figure 3.17 and 3.18 respectively) It is seen that, similar biofilm formation and cell clustering is present similar to whole cell approach experiment with the distinction of fiber structure difference where they are seen with little clumps on them via TEM imaging most probably ALP fusion.

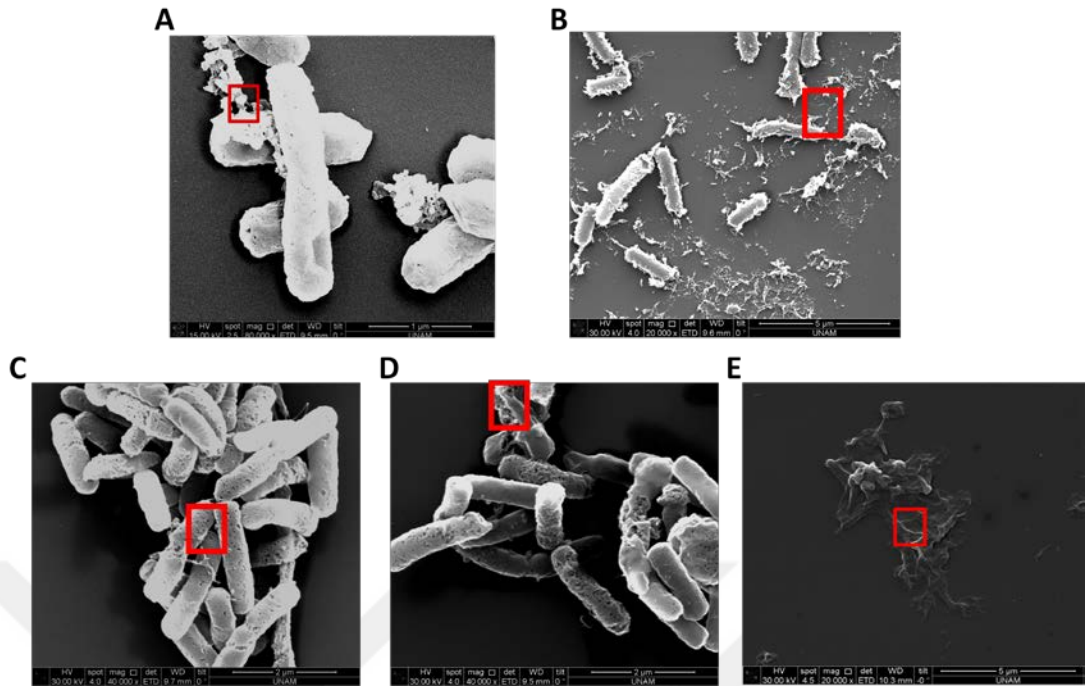


Figure 3. 17: SEM images of CsgA knockout cells transformed with CsgA-ALP fusion protein and expressing it upon aTc induction. Biofilms are formed in 3, 5, 7, 9 and 14 days (A to E respectively). Images are taken by SEM after sample preparation on silicon wafers. Scale bars of SEM images are on each image respectively and magnifying amount is also stated on the images. Red inboxes shows biofilms.

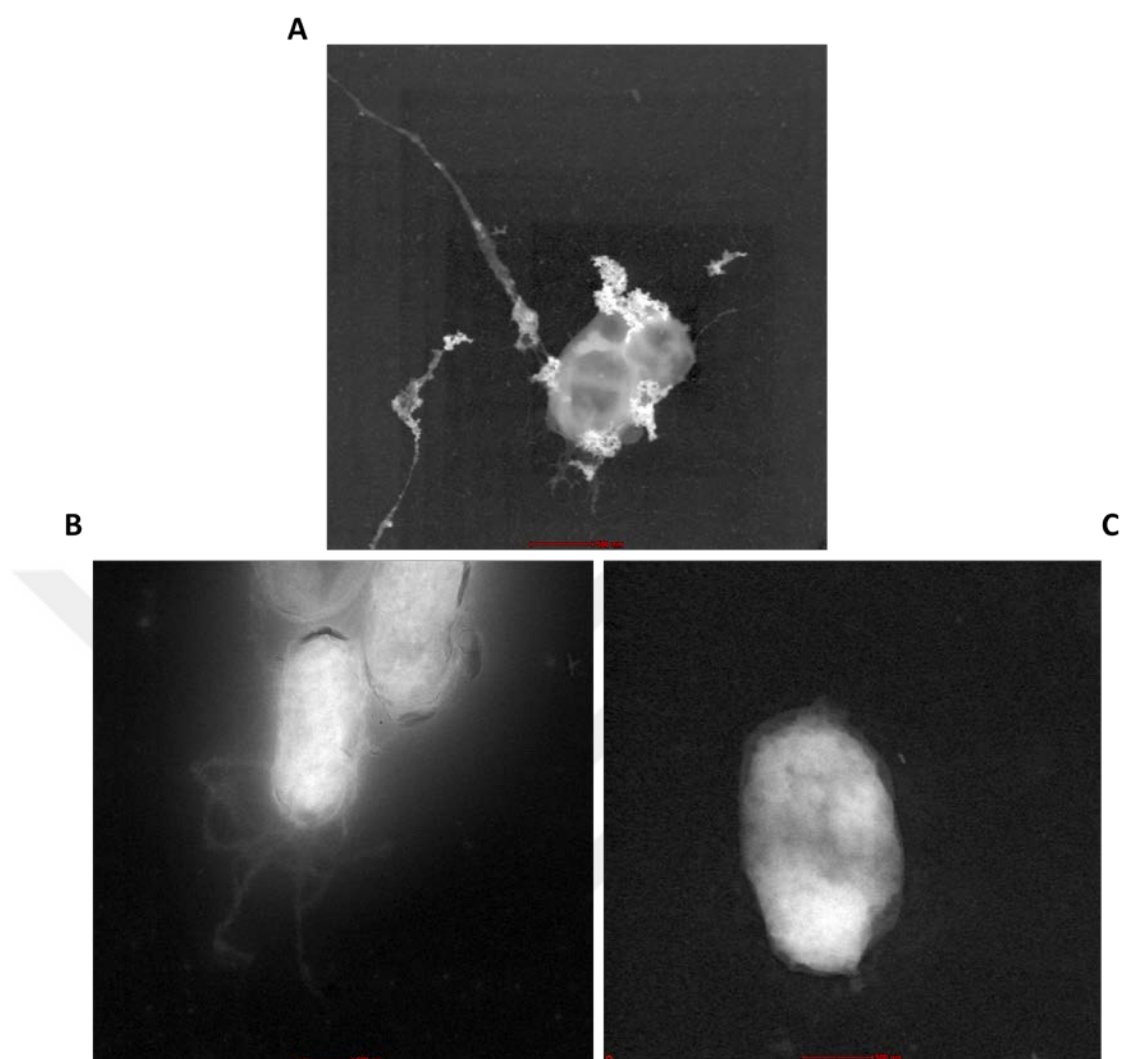


Figure 3. 18: TEM image of fibers formed by CsgA-ALP fusion protein expressing CsgA knock-out cells (**A**) only CsgA transformed cells (**B**) and non transformed cells (**C**). Scale bars are 500nm.

Biofilm formation is stopped at the pre-determined time points of 3, 5, 7, 9 and 14 days and their ALP activity is measured for each time point. (Figure 3.19) It is seen that ALP activity again has a trend of increasing up to 14 days of biofilm forming. It is actually even higher in general and in each time point compared to whole cell approach experiment indicating even further stability and increase by engineering biofilms for biocatalysis.

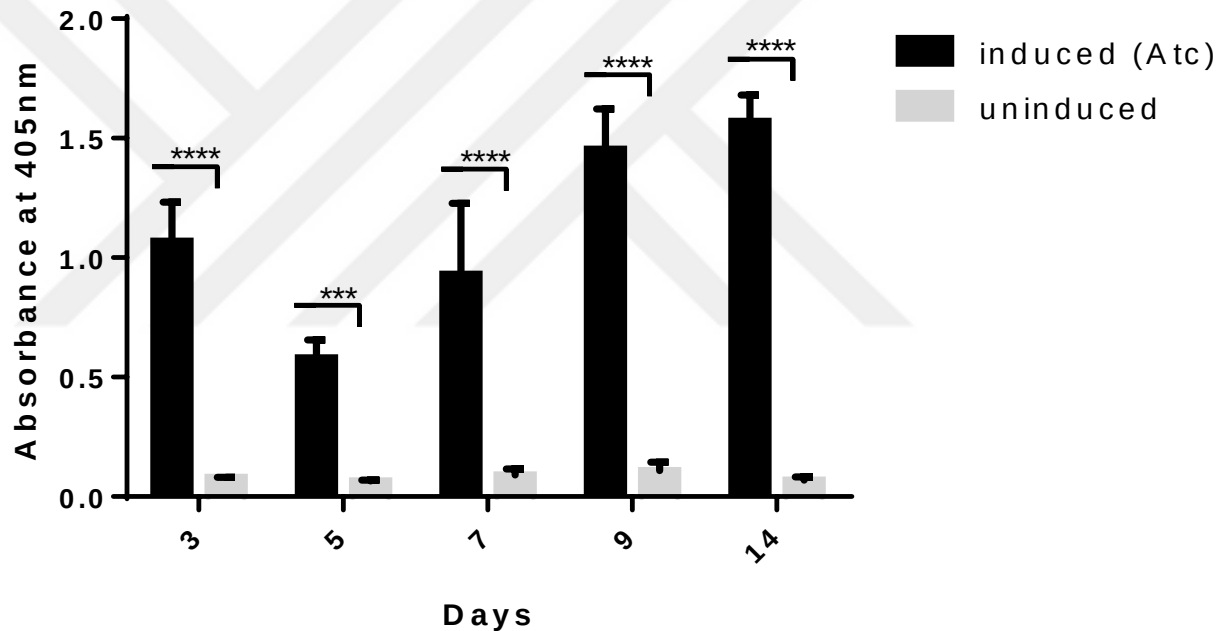


Figure 3. 19: ALP activity of csgA-ALP fusion fiber producing cells after forming curli network over 14 days time span. ALP activity is measured by PnPP assay. (t test is used for significance, p value < 0.05 *, p value < 0.001 ** and p value < 0.00001 *****)

3.1.4 Toehold switch regulated whole cell biocatalysis

Biocatalysis is successfully stabilized, sustained and longevity of it increased over 2-3 weeks of time with utilization and engineering of biofilms. However there is still leakage in the system where control cells are also forming negligible amount of biofilm and showing a background enzymatic activity. (Figure 3.16) To address this issue and further improve the presented approach, implementation of toehold switches for tight control and even further increase in expression of biofilm proteins and enzymes upon induction, is suggested. To test the efficiency of toehold switch with our strains and construct, firstly the set of experiments where toehold switch is regulating the GFP expression is done. For this experiment a construct containing switch-GFP gene and another construct containing trigger part are transformed in to BL21 DE3 strain. These constructs are IPTG inducible. To compare the results of double transformed cells, as a control BL21 DE3 are transformed with only switch-GFP but not with trigger. For the experiment both cells are induced with 0.1mM and 1mM IPTG and results are showing that double transformed cells with switch and trigger constructs are showing significantly higher GFP signal then their uninduced and only switch construct containing counterparts. (Figure 3.20) This almost 50 fold increase shows that toehold switch is in fact a very tight regulator as expected.

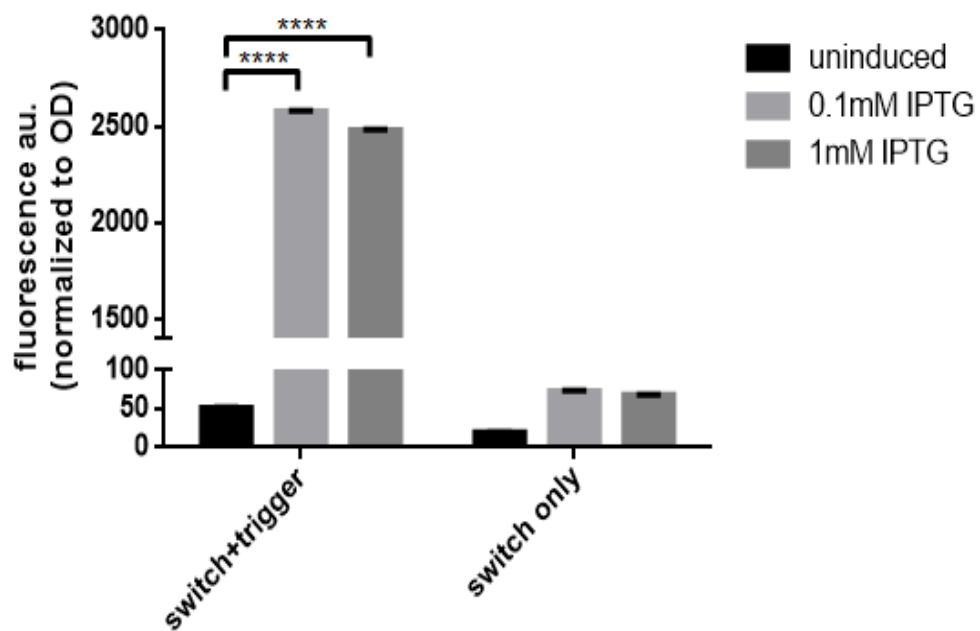


Figure 3. 20: GFP signal upon induction with IPTG. Cells containing both trigger and switch-GFP constructs show significantly higher signal. (t test is used for significance, p value < 0.05)

Dramatic increase from the test of toehold switches with a design of our own, lead us to our next approach in using synthetic systems to manipulate biocatalysis. Constructs are cloned as explained in the experimental part. However trigger part containing plasmid couldn't be built in time for this thesis. Switch 1 CsgA and Switch 2 ALP containing plasmid is built and verified by sequencing. (Figure A.6 and A.10)

3.2 DISCUSSION

Using whole cells to catalyze enzymes is now a common way but still lacks the longevity due to viability issues of the cells.[18], [21], [22] To overcome this we utilized *E. coli* curli network and at first let the cells to form an optimal environment for longer durations of survival. We let the cells form their network for different periods of time and checked their enzymatic activity by para-Nitrophenylphosphate (PNPP) which is substrate of ALP and it absorbs light at 405nm wavelength upon being processed by ALP. Cells producing CsgA upon aTc induction clearly forming a fine network of curli fibers, and it can be seen under scanning electron microscopy (SEM) and transmission electron microscopy (TEM). (Figure 3.12-3.15)

They are coming into clusters covering themselves with fibers mostly. (Figure 3.12-3.15) Different incubation times lead to similar network formation but not quantifiably by SEM. We are seeing effects of incubation time when we measure enzymatic activity. (Figure 3.16) Cells, after their respective incubation times, are washed and again incubated with PNPP this time for 2 hours and absorbance at 405nm wavelength shows significant increase in ALP activity. This activity is increased in samples incubated for up to 14 days and starts to decrease after that point until the 28 days incubation but still stays significantly higher than the uninduced cells up to 21 days incubation time. (Figure 3.16) This suggests that biofilm formation protects and keeps the cells alive up to 3 weeks with their protein production abilities intact, because ALP is induced in each sample with arabinose, after the incubation time set for the network formation. The decrease in enzymatic activity might point to the loss of viability for the cells which explains the enzymatic activity at levels of uninduced cells after 28 days incubation. However we still see

fibers in these samples (Figure 3.12-3.15). Since this design is based on whole cell biocatalysis, even though the fibers are intact, losing their protein production abilities, cells wouldn't show enzymatic activity. This revelation further supports our second design.

Engineering biofilms is another widely focused subject[42], [68] but engineering them for enzymatic activity is rather vague at this point due to focus on the enzyme of interest's specific reaction in each study. We are using ALP to engineer curli network of our host and using easily reachable conditions for its activity, we show that engineering curli network for enzymatic activity indeed increases activity and longevity. Fusing ALP to csgA and inducing it with aTc in our hosts, we again let the cells form their network during different incubation times. Cells producing enzyme coupled CsgA are again forming structures similar to the cells producing WT CsgA in Figures 3.12-3.15 and cover their surfaces with fibers. They also form different structures, fiber like formation but with clumps on them (Figure 3.17 and 3.18). This might happen due to the presence of ALP fused to them, ALP's tertiary structures might have changed these structures. We are seeing these structures with all samples, incubated for 3 to 14 days but we are seeing their effects again by measuring the enzymatic activity after the incubation. We showed that formed curli network has indeed fair enzymatic activity and it sustains this activity up to 2 weeks. (Figure 3.19) Levels of activity starting at 3 day incubated network are even higher than the whole cells and they remain higher even after 2 weeks. Our first approach showed that cells remain viable up to 3 weeks. This characteristic combined with enzyme coupled network might explain higher enzymatic activities of this approach.

Our previous approaches showed that there is some leak in transcriptional control of our system. Although we are seeing significantly higher activity in most cases, there is still some activity in the uninduced samples. For regulation of a system, there are several ways to implement. In our case we have focused on building an AND logic gate with toehold switches. These switches are designed by Green et. al. and they are mostly regulating the system between transcription and translation. Switch component of the toehold forms hairpin while the product is in mRNA level and upon meeting with its trigger component (again another RNA) this hairpin opens up and lets the translation machinery to proceed. This has been shown to provide a tight control over the system its implemented. So we wanted to use two different switches, one for CsgA and one for ALP and used their respective triggers to open up the system in the first approach. However we are stopped at the testing of toehold switches due to some complications during integration of toehold switches to the biofilm and ALP constructs. Switch 1 and trigger 1 couple is tested with GFP and showed almost 50 fold increase upon induction and near zero leakage in uninduced samples (Figure 3.20). However Green et. al. shows almost 600 fold increase with exact same switch-trigger couple. Our lack of increase in fold might be due to cell strain and GFP gene difference. They are using a degradation tagged GFP which is destroyed after some time in cell and this further decreases the background and leakage. Also they are using an RNAase deficient strain of BL21 which further increases trigger and switch-GFP RNA amount and preserve it thus gives more signal. All in all this toehold switch system still has a tight control over expression.

Another aspect of the study that can be inferred but not tested here is the stability quantification of the synthetic systems. Proposed and tested systems are showing

significant increase in enzymatic activity upon engineered biofilm implementation to the whole cell systems and biofilm fusion with enzyme directly. However, how this significant increase is achieved should also be tested, which can be done through characterization of the biofilms used in both systems. Biofilms are, as explained, expected to increase the systems efficiency and longevity through their preserving properties however detailed characterization might reveal further engineering possibilities of biofilms for implementation in to systems like the ones in this study. Some characterization approaches that were planned but couldn't be done in this study were; testing the preserving ability of biofilms against pH and temperature.

For future work actual goal was to implement this toehold system along with environmental signal sensitive promoters. This way, the system we engineered could be used in real life applications. Cells can be optimized to response to calcium amount in environment for example. Upon changes in calcium they may start forming biofilm and then a phosphatase depending on temperature for example. So In the right temperature and right calcium concentration we would have a platform that phosphorylates a substrate of interest or even form calcium phosphate crystal which implicates even bigger uses for this system. Since calcium phosphate is crucial in bone formation a future implementation of such system for bone regenerative studies should intrigue studies on this topic.

CHAPTER 4 CONCLUSION

In conclusion, two synthetic system including different approaches were created and tested for their effects on enzymatic activity included in the systems. They were designed to improve the efficiency of biocatalysis and increase the stability and longevity of it to show the synthetic systems are a viable way to improve pre existing biological processes through combination of other already existing biological processes, in this case the focus was on biocatalysis to test the synthetic systems and combination of biocatalysis with biofilms, another biological process open to manipulations. First synthetic system tested was a whole cell approach where cells are first immobilized on to a surface with their wild type biofilms and then produce an enzyme to perform biocatalysis. This approach showed that viability and enzymatic activity are significantly increased and they remain high even after 3 weeks. Second synthetic system tested was engineered biofilms approach where cells are immobilized to a surface with engineered biofilms where fibers of biofilm network also include fused enzymes to them. This approach showed that again enzymatic activity is significantly increased but biomass is not as high as whole cell approach even though the enzymatic activity is even higher. Lastly one of the significant results of synthetic biology, an engineered riboregulator called toehold switch is meant to be implemented in to two systems to further improve them and have tight control over the regulation of system. Toehold switches are tested with GFP and they showed almost 50 fold increase in signal upon induction which indicates it might increase enzymatic activity in same period of time for both approaches. All in all this study shows that by a syntethic biology approach including

combination of two biological process and engineering one of them, it is possible to improve currently available biocatalysis processes to a point where their stability and longevity is increased along with their enzymatic activity.



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THE APPENDIX

Table A. 1: Primers and oligonucleotides used in cloning of the constructs.

Primer purpose	Primer sequence	Properties
ALP insert amplification for arabionose inducible construct Forward Primer	5'TTTGGGCTAACAGG AGGAATTAAGGTACC ATGAAACAAAGCACT ATTGCACTGG 3'	Tm: 56.7 GC content: %40 Length: 55bp
ALP insert amplification for arabionose inducible construct Reverse Primer	5'ATCTTCTCTCATCCG CCAAAACAGCTCGAG TTATTTTCAGCCCCAGA GCGG 3'	Tm: 56.5 GC content: %55 Length: 50bp
pBad vector backbone amplification Forward Primer	5'CTGTTTTGGCGGATG AGAGAA 3'	Tm: 54.9 GC content: %47.6 Length: 21bp
pBad vector backbone amplification Reverse Primer	5'TTAATTCCTCCTGTT AGCCCAA 3'	Tm: 53.1 GC content: %40.1 Length: 22bp

CsgA insert amplification with linker Forward Primer	5'GATTTGGGTATTAA AGAGGAGAAAGGTAC CATGAACTTTTAAA AGTAGCAGC 3'	Tm: 50.3 GC content: %30.4 Length: 53bp
CsgA insert amplification with linker Reverse Primer	5'GTTTTCCAGAACAG GCATTTCTGGTGTCCG GTACTGATGAGCGGT CG 3'	Tm: 51.4 GC content: %58.7 Length: 47bp
ALP insert amplification for fusion to CsgA Forward Primer	5'GGTAACAACGCGAC CGCTCATCAGTACTA ACGGACACCAGAAAT GCCT 3'	Tm: 54 GC content: %55.6 Length: 48bp
ALP insert amplification for fusion to CsgA, adds his-tag Reverse Primer	5'TCGACTGAGCCTTTC GTTTTATTTGATGCCG TGGTGGTGGTGGTGG TGTTTCAGCCCCAGA GCGG 3'	Tm: 56.1 GC content: %64.1 Length: 65bp
pZa vector backbone amplification Forward Primer	5'CATGGTACCTTTCTC CTCTTTAATACCC 3'	Tm: 56.7 GC content: %42.9 Length: 28bp

pZa vector backbone amplification Reverse Primer	5'GGCATCAAATAAAA CGAAAGGCTC 3'	Tm: 55.5 GC content: %41.7 Length: 24bp
Trigger 1 insert Synthesized as oligonucleotide	5'GGGACTGACTATTCT GTGCAATAGTCAGTA AAGCAGGGATAAACG AGATAGATAAGATAA GATAG 3'	Length: 65bp
Trigger 1 insert amplification for cloning Forward Primer	5'GATCCCGCGAAATT AATACGACTCACTAT AGGGACTGACTATTC TGTG 3'	Tm: 48.1 GC content: %50 Length: 48bp
Trigger 1 insert amplification for cloning Reverse Primer	5'CCCGTTTAGAGGCC CCAAGGGGTTATGCT ACTATCTTATCTTATC TATCTCGT 3'	Tm: 46 GC content: %30.4 Length: 53bp
pet22b vector backbone amplification Forward Primer	5'TAGCATAACCCCTTG GGG 3'	Tm: 52.1 GC content: %55.6 Length: 18bp

pet22b vector backbone amplification Reverse Primer	5'TATAGTGAGTCGTAT TAATTTTCGC 3'	Tm: 50.8 GC content: %33.3 Length: 24bp
Switch 1 insert Part 1 Synthesized as oligonucleotide	5' GGGTCTTATCTTATCT ATCTCGTTTATCCCTG CATACAGAAACAGAG GAGATATGC 3'	Length: 56bp
Switch 1 insert Part 2 Synthesized as oligonucleotide	5'AGAAACAGAGGAGA TATGCAATGATAAAC GAGAACCTGGCGGCA GCGCAAAAG 3'	Length: 53bp
Switch 1 insert amplification Forward Primer	5'AGATATCGACGTCT AATACGACTCACTAT AGGGTCTTATCTTATC TATCTCGT 3'	Tm: 50.4 GC content: %39.1 Length: 53bp
Switch 1 insert amplification Reverse Primer	5'TCCAGTGAAAAGTT CTTCTCCTTTACGCAT CTTTTGCGCTGCCGCC AGG 3'	Tm: 62 GC content: %68.4 Length: 49bp

GFP insert amplification for cloning with switch 1 Forward Primer	5'ATAAACGAGAACCT GGCGGCAGCGCAAAA GATGCGTAAAGGAGA AGAAC 3'	Tm: 49.3 GC content: %42.1 Length: 49bp
GFP insert amplification for cloning with switch 1 Reverse Primer	5'CCCGTTTAGAGGCC CCAAGGGGTTATGCT ATTATTTGTATAGTTC ATCCATG 3'	Tm: 54.7 GC content: %27.3 Length: 52bp
pZa vector backbone amplification Forward Primer	5'TAATAGCATAACCC CTTGGGGCCTCTAAA CGGGTCTTGAGGGGT TTTTTGCCTAGGGGAT ATATTCCGC 3'	Tm: 50.2 GC content: %52.6 Length: 69bp
pZa vector backbone amplification Reverse Primer	5'GACGTCGATATCTG GCGAAA 3'	Tm: 54.4 GC content: %50 Length: 20bp

pZa vector backbone amplification for toehold integrated CsgA and ALP Reverse Primer	5'GCATATCTCCTCTGT TTCTGTATGCAGGGAT AAACGAGATAGATAA GATAAGACCCTGAAT ATATTATACGAGCCT 3'	Tm: 54 GC content: %30 Length: 76bp
pZa vector backbone amplification for toehold integrated CsgA and ALP Forward Primer	5'CAAATAAAACGAAA GGCTCA 3'	Tm: 58 GC content: %35 Length: 20bp
CsgA insert amplification with switch 1 part Forward Primer	5'AGAAACAGAGGAG ATATGCAATGATAAA CGAGAACCTGGCGGC AGCGCAAAAGATGAA ACTTTTAAAAGTAGC AG 3'	Tm: 56 GC content: %27 Length: 75bp
CsgA insert amplification with switch 1 part Reverse Primer	5'GTGATATCTCCTCTG TTTATGTATCACTAAA CTAAACGACAATGTA ATCAAACCTCCAGTTT AGTACTGATGAGCGG T 3'	Tm: 59 GC content: %44 Length: 78bp

ALP insert amplification with switch 2 part Forward Primer	5'ATACATAAACAGAG GAGATATCACATGAC TAAACGAAACCTGGC GGCAGCGCAAAAGAT GAAACAAAGCACTAT TGC 3'	Tm: 58 GC content: %35 Length: 77bp
ALP insert amplification with switch 2 part Reverse Primer	5'GTCTTTCGACTGAG CCTTTCGTTTTATTG TTATTTTCAGCCCCAGA G 3'	Tm: 58 GC content: %47 Length: 47bp

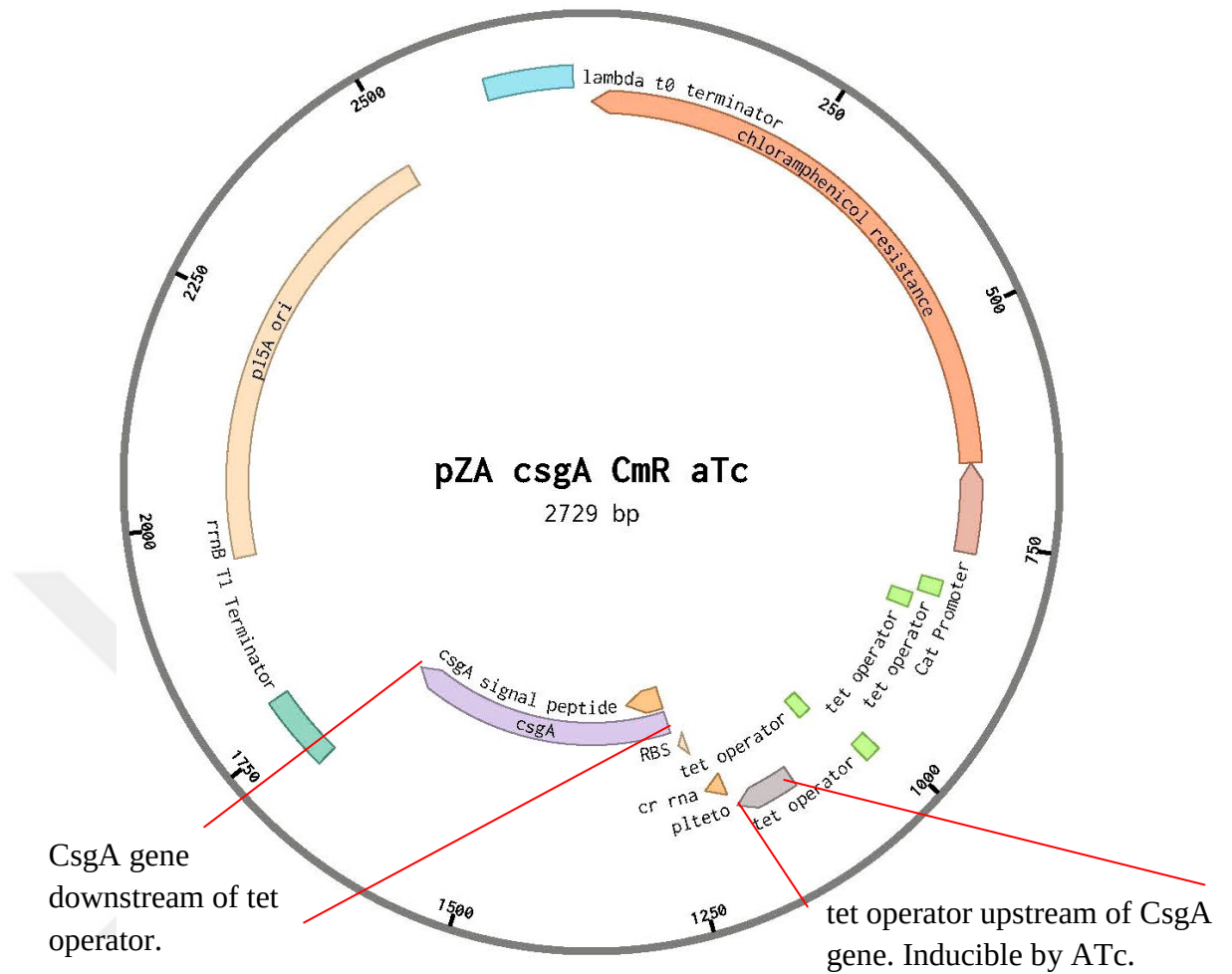


Figure A. 1: aTc inducible CsgA construct. CsgA is downstream of tet operon. Plasmid has chloramphenicol resistance gene providing a selection method. Location of the CsgA gene and operator is labeled in the figure.

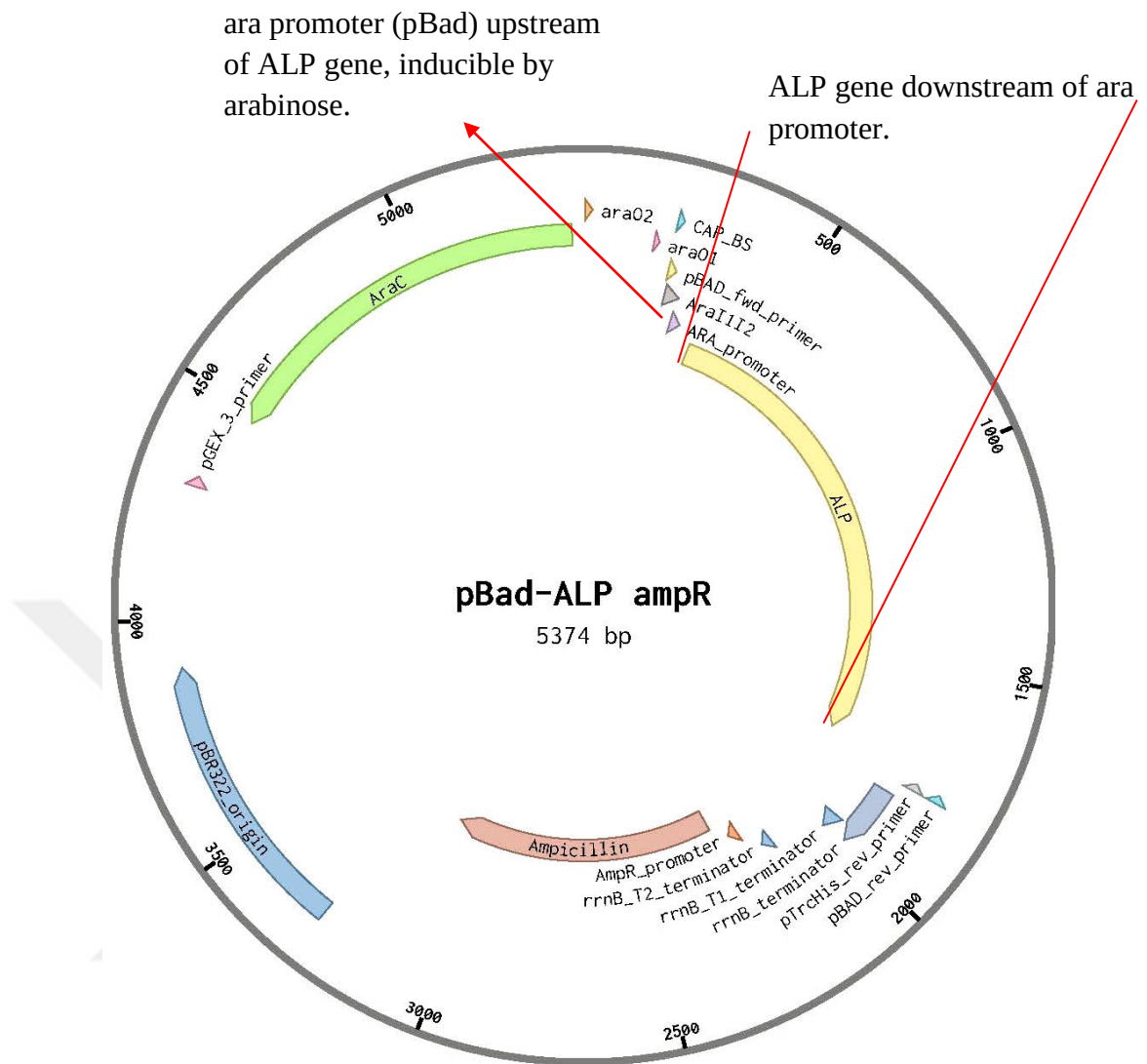


Figure A. 2: Arabinose inducible ALP construct. ALP is downstream of pBad promoter. Plasmid has ampicillin resistance gene providing a selection method. Location of the ALP gene and ara promoter (pBad) is indicated in the figure

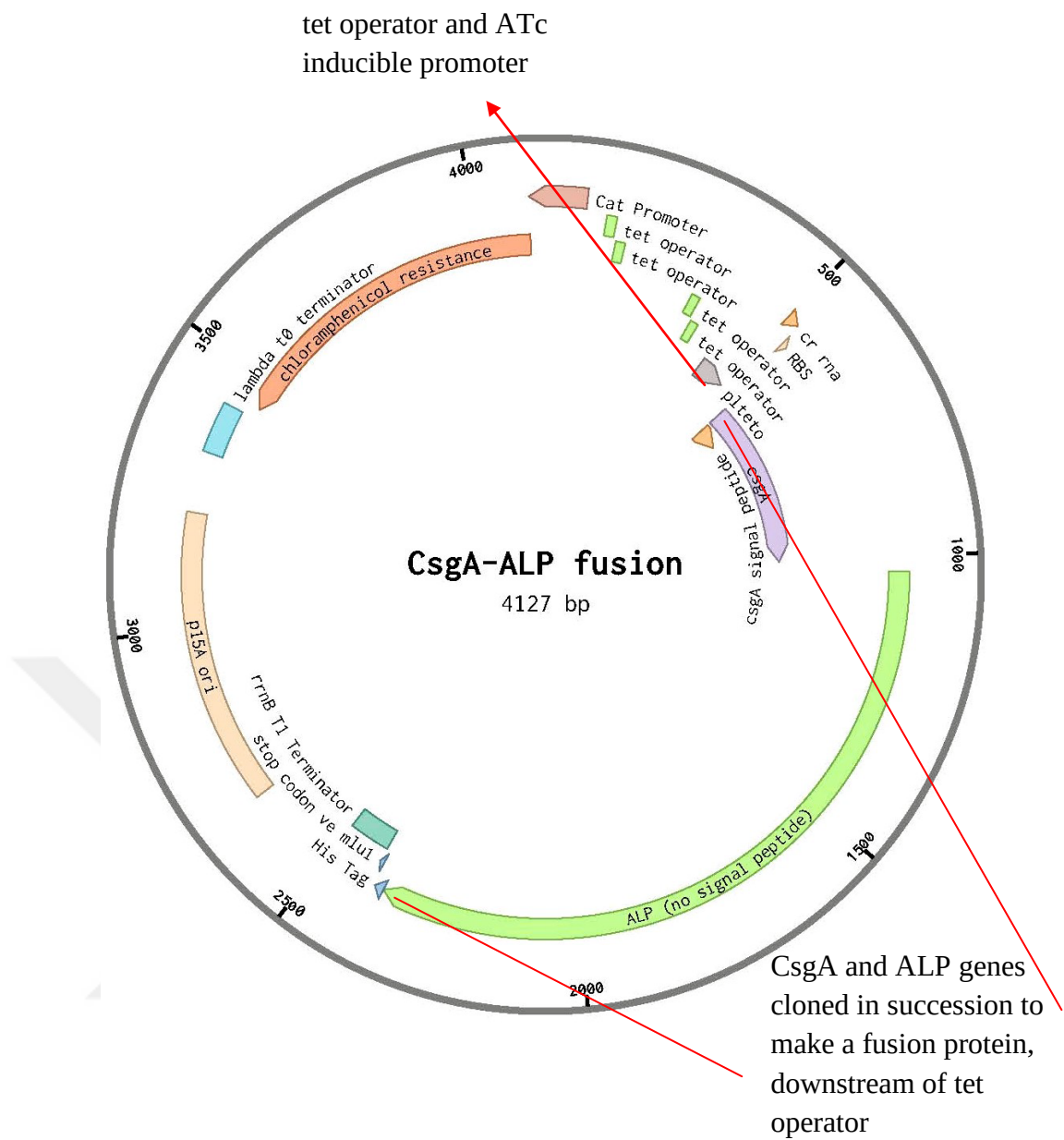


Figure A. 3: aTc inducible CsgA-ALP fusion protein construct. Fusion of genes are downstream of tet operons. Plasmid have chloramphenicol resistance gene providing a selection method.

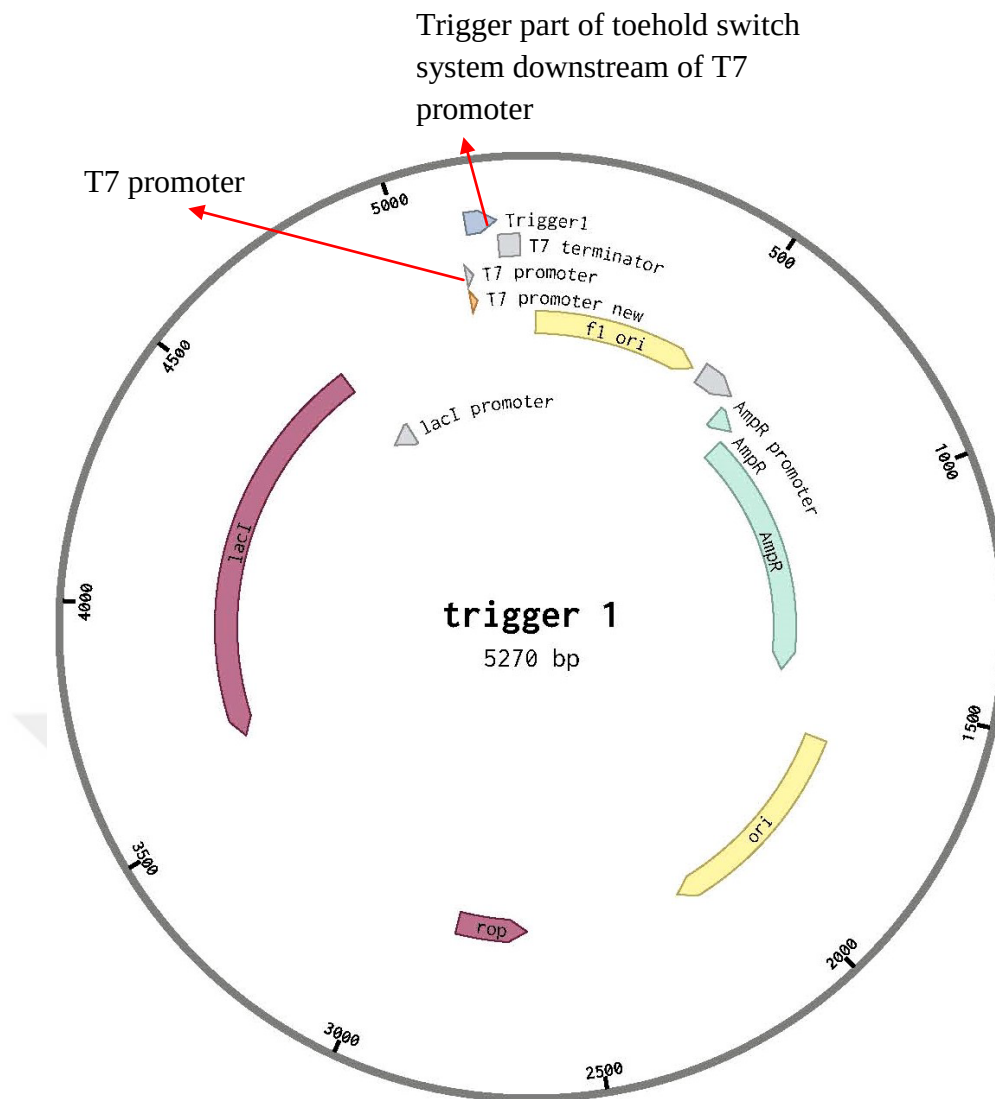


Figure A. 4: Trigger part of toehold switch design in pet22b backbone. Trigger is downstream of T7 promoter which is suppressed by regulation of intrinsic T7 expression in BL21 with lac operons and can be induced upon IPTG addition. Plasmid have ampicillin resistance gene providing a method for selection.

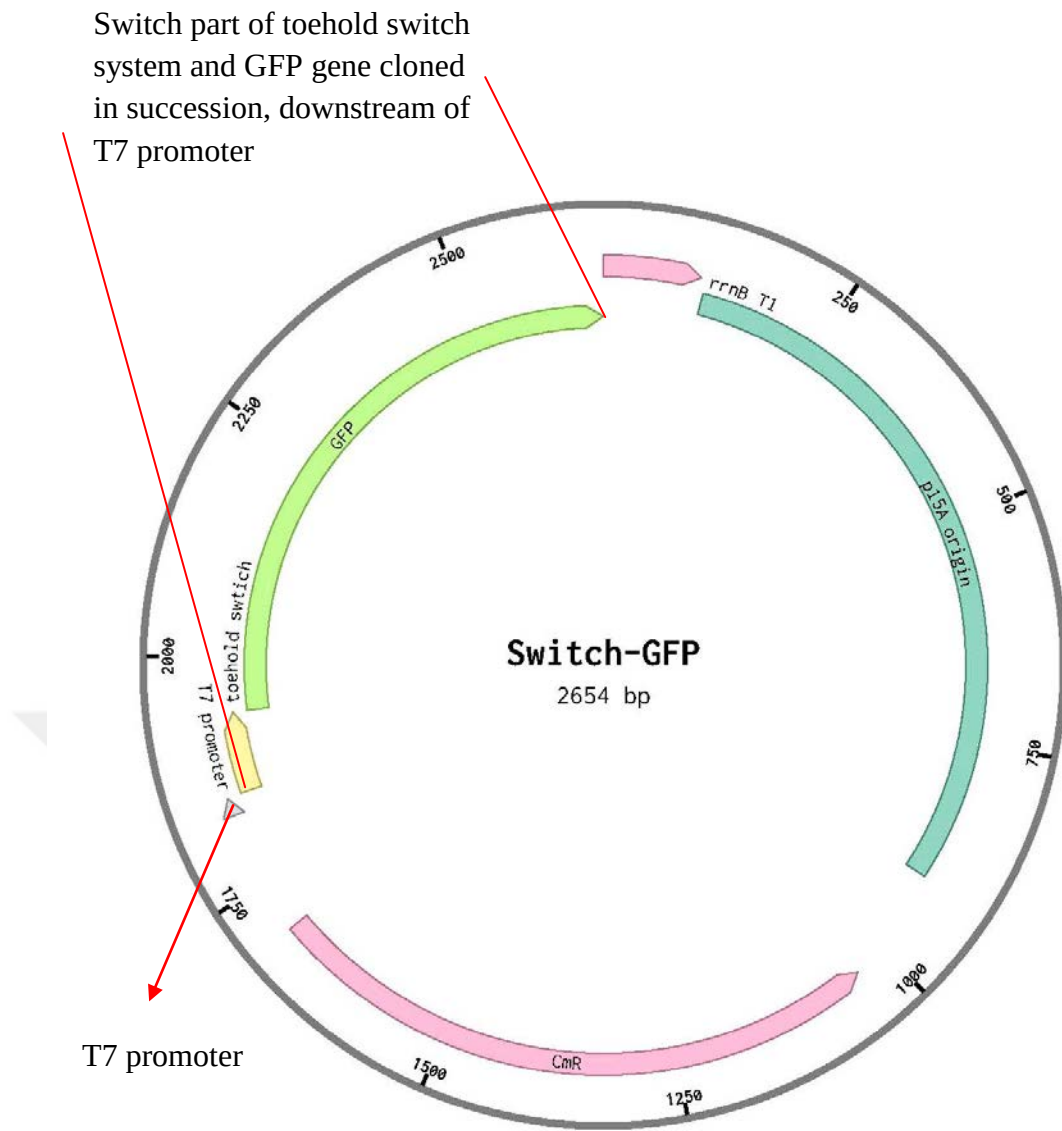


Figure A. 5: Switch part of toehold switch design and GFP gene in pet22b backbone. Switch-GFP is downstream of T7 promoter which is suppressed by regulation of intrinsic T7 expression in BL21 with lac operons and can be induced upon IPTG addition. Plasmid have ampicillin resistance gene providing a method for selection.

Switch1 and switch2 parts of
toehold system and CsgA and
ALP genes cloned in
succession, downstream of
proD promoter

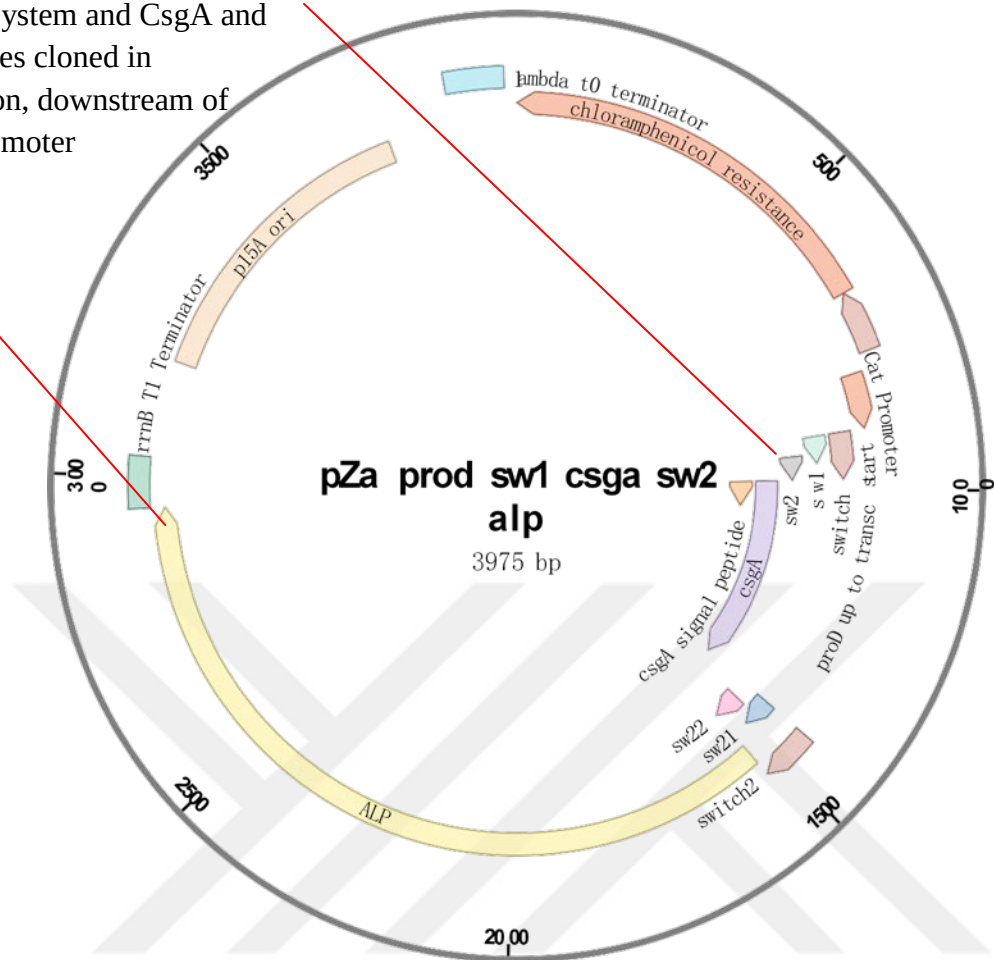
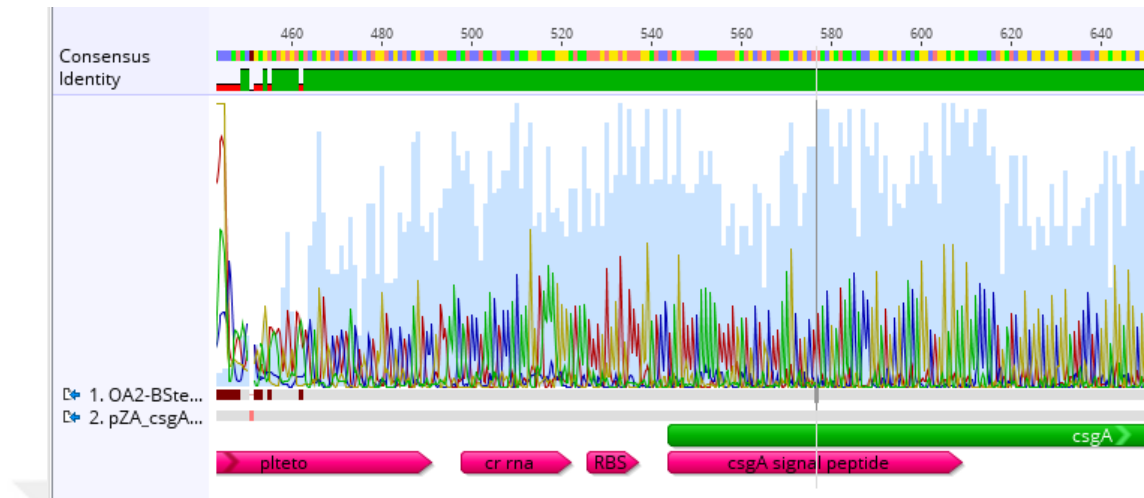


Figure A. 6: Switch part of toehold design integrated in to CsgA and ALP. Cloned downstream of proD promoter which is a constitutively active promoter. From this construct, CsgA and ALP will be expressed as one mRNA along with their respective switch parts and will not be translated until their respective trigger parts are present.

A) Beginning of the sequence of fusion CsgA-ALP construct



B) Second part of the fusion CsgA-ALP construct where ALP gene can be seen.

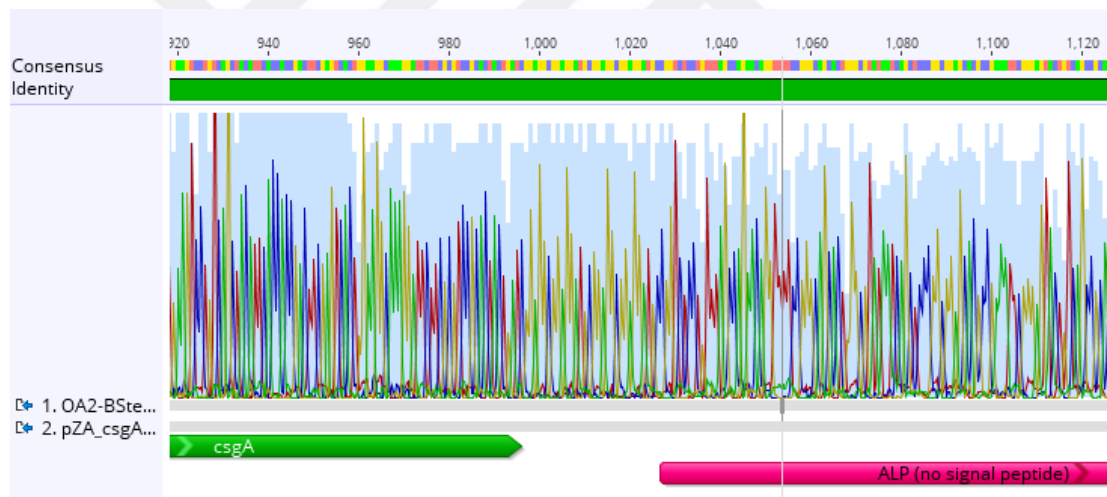


Figure A. 7: Alignment of aTc inducible CsgA-ALP fusion protein construct sequence result with its design. **A** part shows tet operons and CsgA where **B** part is the continuum of the sequence showing ALP gene.



Figure A. 8: Alignment of IPTG inducible Switch-GFP construct sequence result with its design.

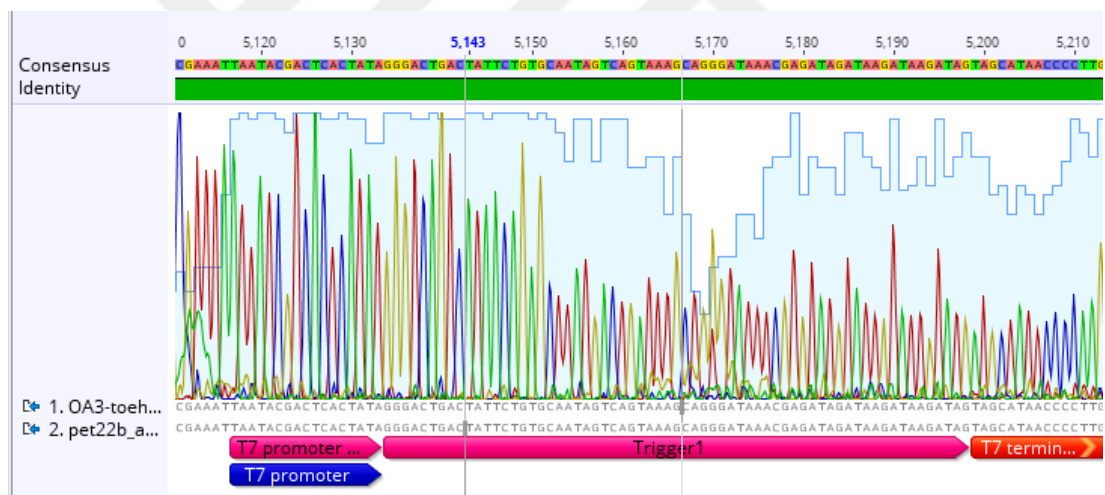


Figure A. 9: Alignment of IPTG inducible Trigger 1 construct sequence result with its design.

A) Beginning of the sequence of switch parts 1 and 2 and CsgA and ALP genes containing construct



B) Continuum of the sequence showing second part of the construct

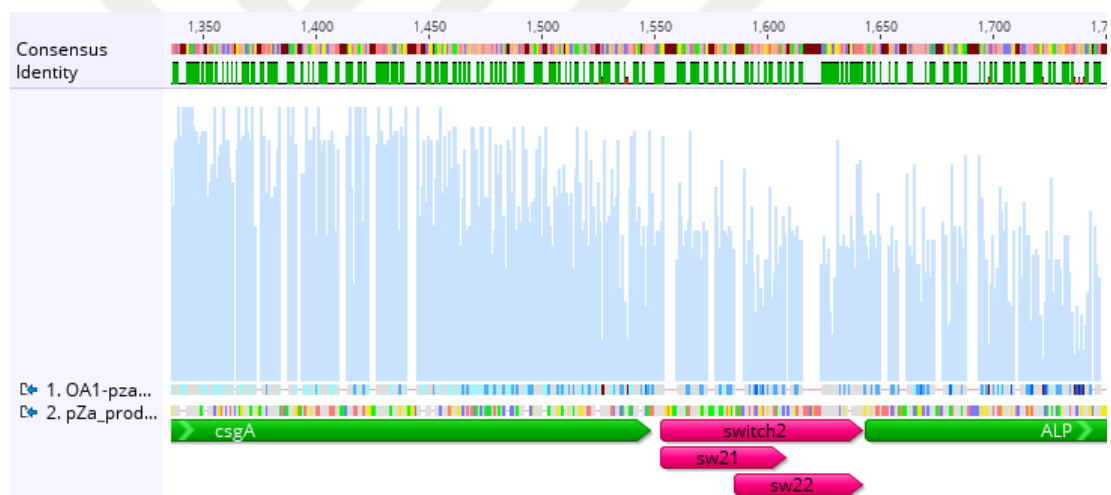


Figure A. 10: Alignment of constitutively active switch 1-CsgA-switch 2-ALP construct sequence result with its design. **A** part shows proD promoter, switch 1 and CsgA gene and **B** part is the continuum of the sequence showing switch 2 and ALP gene.

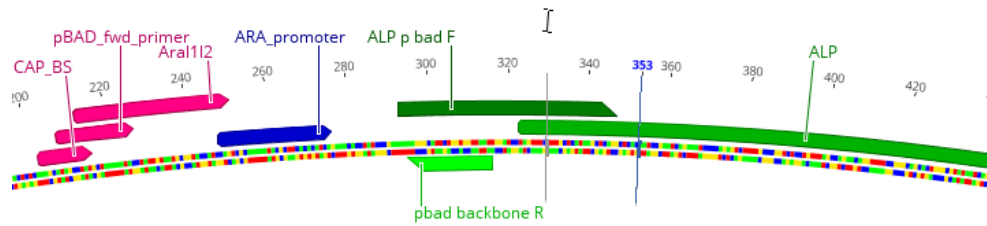


Figure A. 11: Alignment of Forward primer for ALP amplification for cloning in to pBad vector and, Reverse primer for amplification of pBad backbone on the desined sequence. Alignments are done in Geneious software.

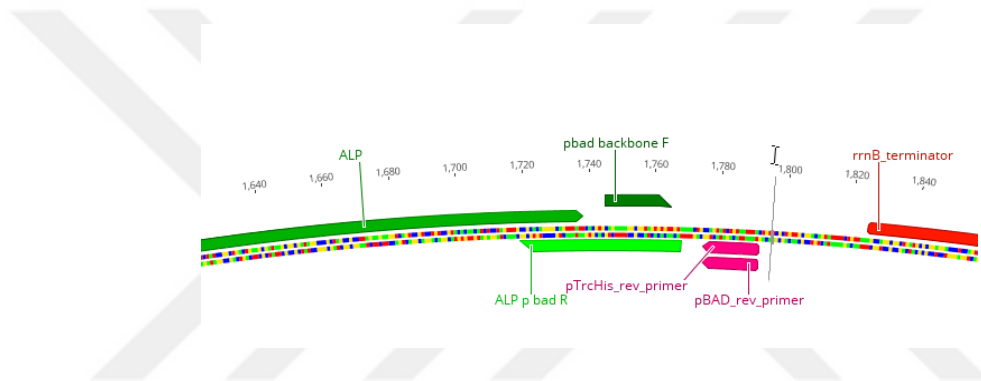


Figure A. 12: Alignment of Reverse primer for ALP amplification for cloning in to pBad vector and, Forward primer for amplification of pBad backbone on the desined sequence. Aligned in Geneious software

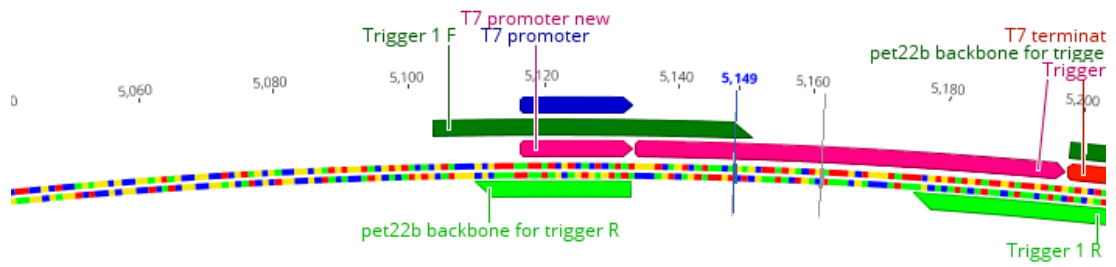


Figure A. 13: Alignment of Forward primer for Trigger amplification for cloning in to pet22b vector and, Reverse primer for amplification of pet22b backbone on the desined sequence. Aligned in Geneious software

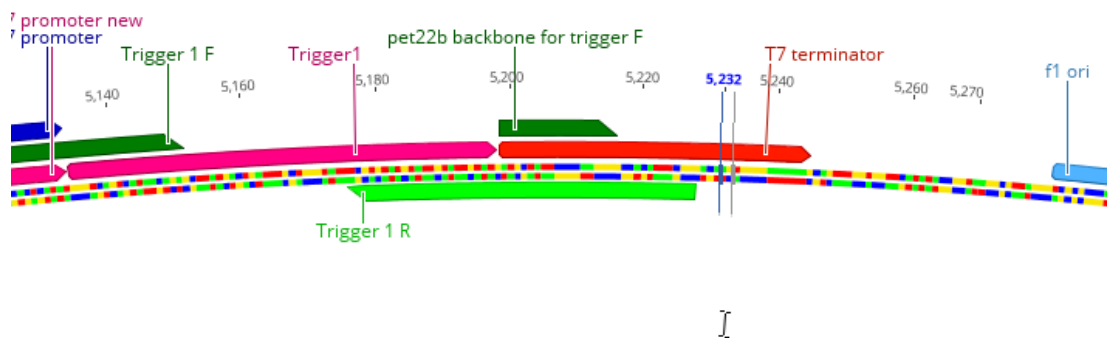


Figure A. 14: Alignment of Reverse primer for Trigger amplification for cloning in to pet22b vector and, Forward primer for amplification of pet22b backbone on the desined sequence. Aligned in Geneious software

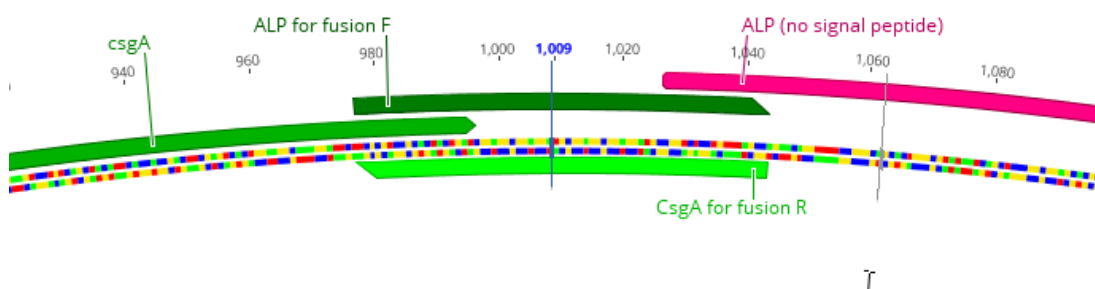


Figure A. 15: Alignment of Reverse primer for CsgA amplification for cloning in to pZa vector and fusion to the ALP and, Forward primer for amplification of ALP for cloning in to pZa vector and fusion to the CsgA on the desined sequence. Aligned in Geneious software



Figure A. 16: Alignment of Reverse primer for ALP amplification for cloning in to pZa vector and fusion to the CsgA on the desined sequence. Aligned in Geneious software

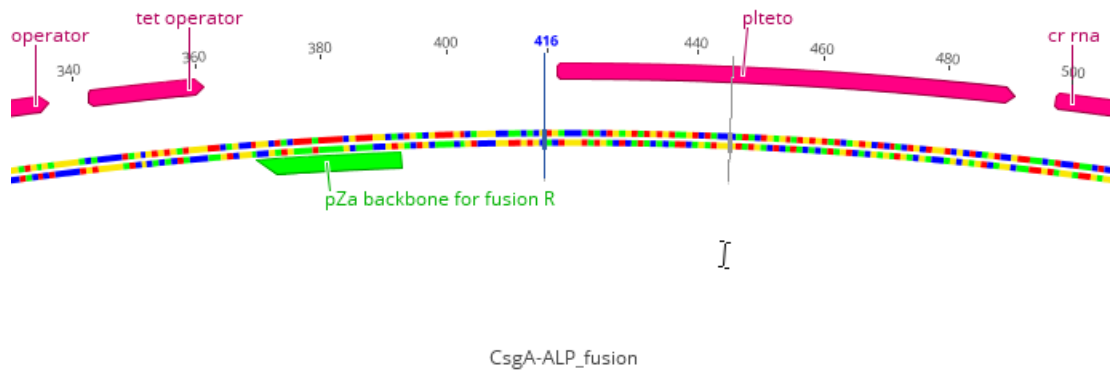


Figure A. 17: Alignment of Reverse primer for pZa backbone amplification for cloning of CsgA and ALP fusion protein on the desined sequence. Aligned in Geneious software

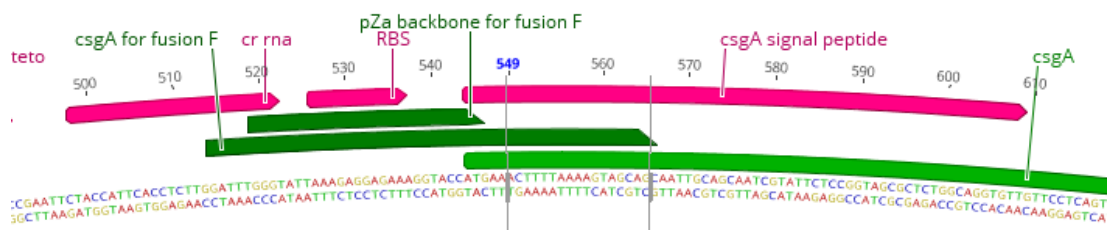


Figure A. 18: Alignment of Forward primer for pZa backbone amplification for cloning of CsgA and ALP fusion protein and, Forward primer for amplification of CsgA for cloning in to pZa vector and fusion to the ALP on the desined sequence. Aligned in Geneious software. Forward pZa backbone primer is seen as close to CsgA because actual template used for amplification of pZa backbone normally doesn't contain CsgA gene.

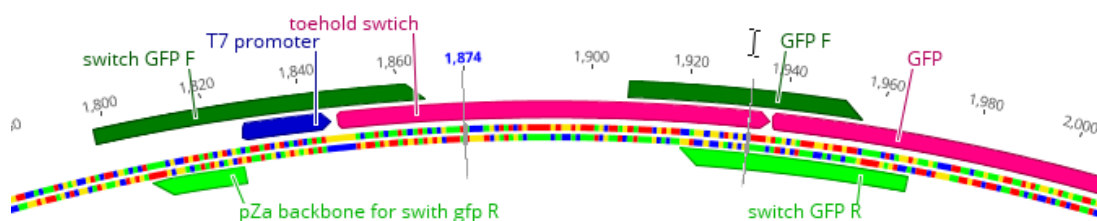


Figure A. 19: Alignment of forward and reverse primers for Switch part of toehold riboregulator amplification for cloning in to pZa backbone along with GFP gene. Alignment of forward primer for GFP amplification for cloning in to pZa backbone along with Switch part. Alignment of reverse primer for pZa backbone amplification suitable for switch and GFP cloning. Alignments are done with Geneious software

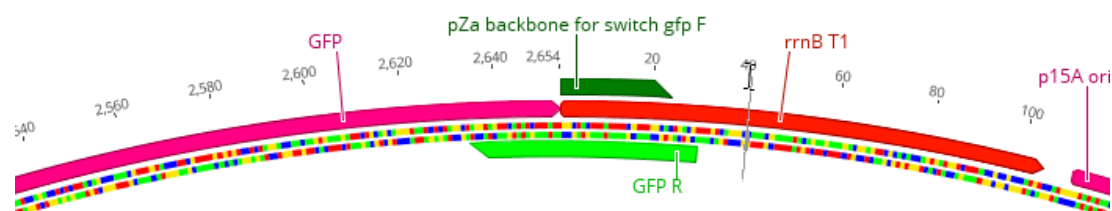


Figure A. 20: Alignment of reverse primer for GFP amplification for cloning in to pZa backbone along with Switch part. Alignment of forward primer for pZa backbone amplification suitable for switch and GFP cloning. Alignments are done with Geneious software