In-dye-go
Report

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***Abstract*—Dyes are everywhere around us, from the ones used in laboratories to the ones beautifying our clothes. Most of these dyes are chemically synthesized and some of them may contain ingredients derived from carcinogenic petrochemical derivatives. Natural alternatives are also present and should be considered as a future field of study because they can present many advantages regarding the ones we are used to. Of course, they have their own limitations such as the huge quantities required, as well as the lack of durability of the colors over time. In addition, a wider use of this kind of technology would present its own set of limitations, such as the costly creation of new facilities and the possibility of contamination. In this project this problem was approached from a proof-of-principle perspective, using synthetic biology and biodesign principles.**

Using a genetically modified *Escherichia coli* that contains the Trimethylamine Monooxygenase (TMM) gene, the indigo color was produced with the aid of tryptophan. Different genetical engineering/ cloning methods were used to this aim (plasmid extraction, restriction digestion, ligation, polymerase chain reaction, electrophoresis and transformation). Following this, biodesign principles using 3D printed shapes were applied. The final goal of this project was to prepare a genetically modified bacteria able to produce durable and environmentally friendly dye thanks to the introduced TMM gene and its interaction with tryptophan.

***Index Terms*—TMM gene, plasmid, tryptophan, cloning**

I. Introduction

Coloring of textiles is a common practice nowadays introduced to add customer value, increase the appeal and the desire of clothing. Initially natural dyes were used, and these were soon replaced by synthetic and chemically produced dyes as they were more efficient and effective. Most of these dyes are produced by large scale companies that use petrochemical sources which are toxic and could contribute to pollution of the nature if not handled carefully. These dyes can also cause harm to factory workers who handle them, which often results in respiratory problems (Mojahede et al, 2014. [1]). In addition they may be harmful to consumers. Due to this, the idea to create sustainable, environmentally friendly alternatives was born.

A current alternative on the market, that preceded synthetic dyes and continued to coexist with it at a smaller scale, is natural dyes made from fruits and vegetables (Samanta and Konar, 2011. [2]). While natural dyes are less harmful to the planet, they come with their own limitations. First, they are less economical as big quantities of raw material is required for their production, in addition to their seasonal availability. Second, textiles colored using natural dyes are not very resistant to wash, and they fade over time. These limitations and many more drive the need for more sustainable, durable and safe dyes.

A relatively new field of research called synthetic biology opens the doors to solve such problems. With the use of genetic manipulation tools and organisms that provide ”production factories”, solving such problems can now be approached differently.

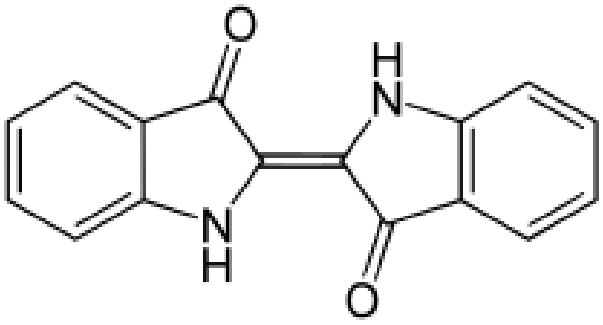

Fig. 1. Indigo molecule

A recent project sponsored by the Bettencourt iGEM team in 2014. was named *The Smell of Us* [3]. One of the subtopics covered under this project was named *something fishy*, which discovered that a specific gene unexpectedly produces indigo color. The initial goal of the project was to treat a physiological condition that is the product of a rare genetic disorder named Fish Odor Syndrome. This fishy smell is the result of the absence of the FMO gene which breaks down trimethylamine (TMA). This leads to TMA accumulating in sweat, saliva and urine causing a strong foul odor. A gene that is found in *Ruegeria pomeroyi*, named trimethylamine monooxygenase (TMM), was seen to perform the same functions as the human FMO gene, and therefore was introduced into *E. coli* to develop a product to help FMO deficient patients. The idea was to produce a cream or a spray that can be used on skin of patients to help treat this condition. Upon the introduction of the TMM into *E. coli*, an indigo color was observed in the presence of tryptophan. Tryptophan is an amino acid which is often used in biosynthesis. Tryptophane is broken down by an enzyme present in *E. coli* named tryptophanase.

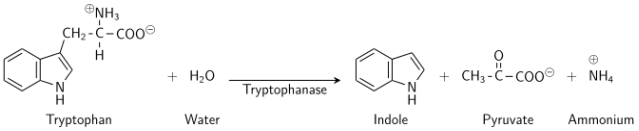

Fig. 2. Tryptophan being broken down into indole

As is shown in Figure II, indole is produced from this reaction, and then broken by the TMM gene to produce isatin. Because isatin is a highly unstable molecule, it binds with another isatin molecule and creates indigo, an organic compound with specific blue color.

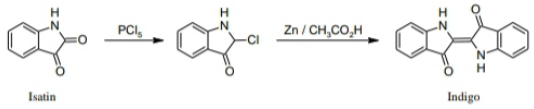

Fig. 3. Isatin molecules creating indigo

In this project synthetic biology and biodesign principles were used to recreate this indigo color by introducing the TMM gene into a wild type *E. coli*, and then used dye textiles in order to demonstrate how bacteria could be the future of sustainable dyes. The project was started with the transformed *E. coli* from which the plasmid with the gene of interest was extracted, replicated and then reintroduced into a wild type *E. coli*.

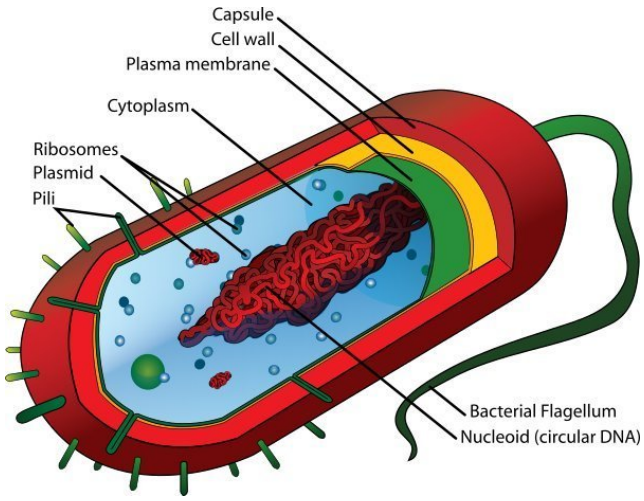

Fig. 4. Illustration of *E. coli* cell. Alongside genomic DNA, bacteria oftentimes carry additional plasmid DNA

Research question: Can genetically modified *E. coli* be used to produce a natural clothing dye?

Hypothesis: *E. coli* with the introduced TMM gene produces indigo dye in the presence of tryptophan, and can be used to dye the cloth.

II. Materials and Methods

For this experiment the Turbo NEB strain of *E. coli* was used, in addition to the pSB1C3 plasmid with resistance to chloramphenicol. The project was started with an already transformed *E. coli* (containing the TMM gene).

A. Monarch Miniprep

Firstly, 100 mL of LB liquid medium was prepared by mixing 1g of tryptone, 1g of NaCl and 0,5g of yeast extract, with 100 μL of 35mg/ l of chloramphenicol with distilled water. This medium is used for the growth of *E. coli* because it provides the needed nutrients. 1.5 mL of the medium was put into each of the four Eppendorf tubes, a colony of transformed *E. coli* with the TMM gene was added in each one and they were left overnight to incubate on the mini shaker.

The next day the *E. coli* had grown enough to proceed with the Miniprep protocol (Monarch Plasmid Miniprep Kit) in order to extract the plasmid with the TMM gene. To start with the protocol, the bacteria with LB medium were centrifuged for 30 seconds at 13000 RPM (this speed was used for every centrifugation during the plasmid isolation). After this step, the supernatant was discarded leaving the bacterial pellet at the bottom of the Eppendorf tube. The pellet was resuspended afterwards with 200 μL of the Plasmid Resuspension Buffer (B1) and inverted by hand until it turned light pink. This buffer contains RNase (Ribonuclease) which degraded RNA in bacterial cell so that the DNA resulting from the lysis could be easily extracted. Next, 200 μL of Plasmid Lysis Buffer (B2) was added to the Eppendorf tube. The tube was inverted 5-6 times and incubated at room temperature for one minute. This buffer helps to dissociate lipid components of the bacterial membrane. That allows plasmid and other cell components to spill into the solution.

After Resuspension and Lysis buffer, the third, Plasmid Neutralization Buffer was added. This buffer keeps pH neutral and allows DNA strands to renature. The Eppendorf tube was, again, inverted until the color (yellow) was uniformly neutralized. The solution was left at room temperature for two minutes and later centrifuged for 2 to 5 minutes. The supernatant was carefully transferred to the spin column and again centrifuged for 1 minute. This column has a special silica membrane which filters our plasmid from other cell structures. With this step finished, the plasmid stayed on the column and the flow-through was discarded. The column was then re-inserted into the collection tube. Afterwards, 200 μL of Wash Buffer 1 was added to the column (washes salt leftovers from the buffer). It was centrifuged for 1 minute.

Subsequently, 400 μL of wash Buffer 2 were inserted in the column which was then centrifuged for 1 minute. The column was then transferred to a clean microfuge tube. The last step was to add 30 μL of DNA Elution Buffer. The tube was left for 1 minute and then spun also for 1 minute. At the end, in the tube there is a colorless liquid which contains purified plasmid DNA.

The result of this process was the plasmid extracted from the *E. coli*.

B. Restriction digestion

Restriction digestion (RD) is a process of cutting DNA molecules into smaller pieces. It was done three different times so as to cut the genetic material when it was required. The first time, the extracted gene from the MiniPrep was cut and as a result the TMM gene was extracted from the plasmid, splitting it into the gene of interest (TMM) and the plasmid backbone. The second RD was used to get rid of any extra nucleotides left behind from primers after PCR. This will ensure that only nucleotides of the restriction enzymes sticky ends would be present to ensure an effective and oriented ligation later, so the gene could attach to the plasmid. Finally, the third RD was done to linearize the plasmid backbone (which was originally a

circular structure) which was then used for the ligation. In this process, 34 μL of the specific genetic material required in each occasion, 4 μL of buffer (NEB + BSA 2.1) and 1 μL of both enzymes used (*Xbal* and *PstI*) were added. Two different enzymes (one that would attach to one side of the gene and the other to the other side) were needed so the gene would always be well oriented in the plasmid and make sure that when it would be expressed in *E. coli* it would be done in the right direction.

The result of this process was: a) extracted TMM gene from plasmid; b) sticky ends on the PCR TMM gene; c) a linearized plasmid backbone.

C. Polymerase chain reaction

Polymerase chain reaction (PCR) is a method used to make copies of a specific DNA segment. PCR works on the principle of relatively sudden temperature changes. It has three stages: denaturing, annealing and extending. These three stages differ from each other based on temperatures.

For the first stage (denaturation) the temperature must be above $90°$C, specifically $94°$C. This causes melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

For the second stage (annealing) the temperature is lowered to 50-60°C, specifically $55°$C. This allows for the annealing of the primers to each of the single-stranded DNA templates. Generally, two different primers are included in the reaction mixture, one for each of the two single-stranded complements containing the target region (gene of interest, TMM).

For the third and final stage (extension/elongation) the temperature is increased to $72°$C for most types of DNA polymerases, including the one used in this project. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, joining the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where *n* is the number of cycles. The cycle begins with an initalization step (during which the temperature rises to the denaturation stage temperature) and ends with the final hold step (during which the reaction chamber is cooled down to $4°$C for an indefinite amount of time). The number of cycles in this project was 30 in total.

In this project PCR was used to amplify the sample of the gene of interest (TMM). The sample that was used in this project consisted of 25 μL of the JumpStart REDTaq ReadyMix mixed with 15 μL of MiniPrep DNA as well as 5 μL of both primers.

The result of this process was 2^{30} copies of the gene of interest (TMM).

D. Ligation

Ligation is a process of joining DNA strands with the help of an enzyme. It is an essential step in the process of cloning. In this project it was used to ligate the linearized plasmid backbone (from the third RD) with the gene of interest (after PCR and the second RD) which was done using an enzyme called T4 DNA ligase.

The reaction mixture included 2 μL of the linearized plasmid backbone, an equimolar amount of *Xbal* and *Pstl* digested fragment (less than 3 μL), 1 μL of T4 DNA ligase buffer, 0.5 μL of T4 DNA ligase and enough water to make the sample 10 μL in total. This sample was ligated for 30 minutes at $16°$C and then heat killed for 20 minutes at $80°$C.

The result of this process was a plasmid with the TMM gene inserted.

E. Electrophoresis

Electrophoresis is a method for analysis of macromolecules and their segments based on their charge and size. Horizontal gel electrophoresis uses an agarose gel as its medium. This gel is prepared by mixing 0.6 mL of concetrated buffer, 29.4 mL of distilled water and 0.23g of agarose. This solution is heated until it reaches transparency and then poured into a tub with a comb and left to solidify. This method was used to compare the samples from the steps listed above.

The camples were: the extracted plasmid (after MiniPrep), the extracted TMM gene (after RD1), the amplified TMM gene (after PCR), the TMM gene with sticky ends (after RD2), the linearized plasmid backbone (after RD3), the plasmid with the TMM gene (after ligation), the circular plasmid backbone without TMM gene, the extracted plasmid (after miniprep2). The last two samples were used for comparative purposes.

To prepare the samples, 3 μL of loading dye, 1.8 μL of SYBR Safe and 13.2 μL of the sample were needed.

The result of this process is further discussed under *Results* and *Discussion*.

F. Transformation

Transformation is the process of genetical alteration of a cell resulting from a direct incorporation of exogenous genetic material. This was the final step of the cloning portion of the project.

To start the process, 0.5 mL of bacteria in 50 mL of LB need to be cultured overnight. They are then grown to A600 of 0.5 - 0.7 and centrifuged for 5 minutes at 8000 rpm in sterile JA-17 tubes. Next, the sample is resuspended in 5 mL of ice-cold $CaCl_2$. At this stage, the sample can be vortexed and, if necessary, left on ice for several hours.

In the following step, the sample is distributed between three Eppendorf tubes (1.5 mL each) and centrifuged for 30 seconds. Then, each pellet is resuspended with 0.5 mL of ice-cold $CaCl_2$. Vortexing is not recommended at this step, so to resuspend the pellets the tubes are lightly tapped. After the resuspension, 50 μL is pipetted into single-use Eppendorf tubes.

To complete the process, 1 μL of DNA (in this case, MiniPrep DNA) and 2 μL of ligation mixture needs to be added to each sample. The samples are then incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds and then cooled on ice again for 5 minutes. The final step is to grow the samples in 1 mL of LB for 1 hour at 37°C and then plate.

The result of this process is a plasmid with the TMM gene in a bacterial cell. Chloramphenicol resistance is used to select the successfully transformed bacteria.

G. Lysogeny broth

Lysogeny broth (more often referred to as LB) is a nutritionally rich medium primarily used for growing bacteria. It comes in two basic forms: liquid and gel. The only difference in the preparing of the two is the agar as an added ingredient when making the gel LB.

To make the liquid LB, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 2 mL of NaOH (pH 7.2). This will make 1 L of liquid LB.

H. Biodesign

Biodesign is a method in which living things (such as bacteria) are used to produce art or products. It was the last step of this project.

The first step was to design the patterns which would be filled with the bacteria. Inkscape program was used to compose the designs and a 3D printer was used to print the patterns. The dimensions of the pattern were 10 cm by 10 cm because that was the approximate size of the Petri dishes used.

The bacteria must be grown overnight in a liquid LB with added chloramphenicol (concentration 1:1000). The Petri dishes with the gel LB with added chloramphenicol also must be prepared. The next step is to equally spread 150 μL of non-diluted bacterial culture onto the Petri dish and incubate for 8 to 12 hours.

The following step is to place 150 mL of gel LB with chloramphenicol (concentration 1:1000) in a larger Petri dish. The pattern is then placed on top of the gel LB in such a way that the gel fills the holes of the pattern and left there until the gel LB solidifies (this should take 10 to 15 minutes). The bacteria should then be spread with a spreader stick so that it covers the holes of the pattern. A sterile textile (preferably silk) is then placed on top of the pattern and fixed into position with a ring of plexiglass.

This should be incubated for a minimum of 8 to 12 hours and 24 hours for a more intense color. Once the bacteria produce the satisfying intensity of color, the plate should be removed from the incubator, put in acetic acid (vinegar) mixed with a concentrated water salt solution in a 1:3 ration for 1-2h for fixation. Following this, the sample is sterilized in a 70% ethanol and washed in water.

The result of this process is a pattern made by a bacteria producing dye.

III. Results

A. Electrophoresis Result

The expected results would have shown a difference in size and charge between the extracted plasmid sample (tube 1) and the extracted TMM gene sample (tube 2). The sample with plasmid would ”stay” near the negatively charged end of the gel and TMM gene samples would ”travel” to the positively charged end. The actual results after gel electrophoresis showed faded bands that are all in the same row. Under the ultraviolet light bands weren't correctly shown and with that didn't have the difference between samples. We tried this process two more times and didn't get new results. The only difference is that in the third attempt we added SYBR Safe for better results. For our project this didn't provide better results under UV lights. It is believed that SYBR Safe wasn't reliable because we added it in the samples and not in the gel. Overall, the correct bands on the gel weren't shown due to insufficient amount of DNA and missing dye agent.

B. Transformation Results

Following all the previously described procedures, three different samples were plated on a tryptophan (2g/1000 mL) and Chloramphenicol (1:1000) rich agar plate.

1) Ligated and transformed bacteria sample: Unable to detect blue color, in addition a few colonies with pink and yellow color were observed on this plate.

2) Miniprep and transformed sample: 24 hours after plating this sample on a tryptophan rich medium, bacteria were observed which shows that it was transformed (it had the antibiotic resistant gene), but blue color was only detected 48 hours later.

3) TMM bacteria (the one we started with) for comparative purposes: Blue color was observed growing on an agar plate. It is worth noting that when this bacteria was transferred to a liquid medium it did not produce the blue color was expected.

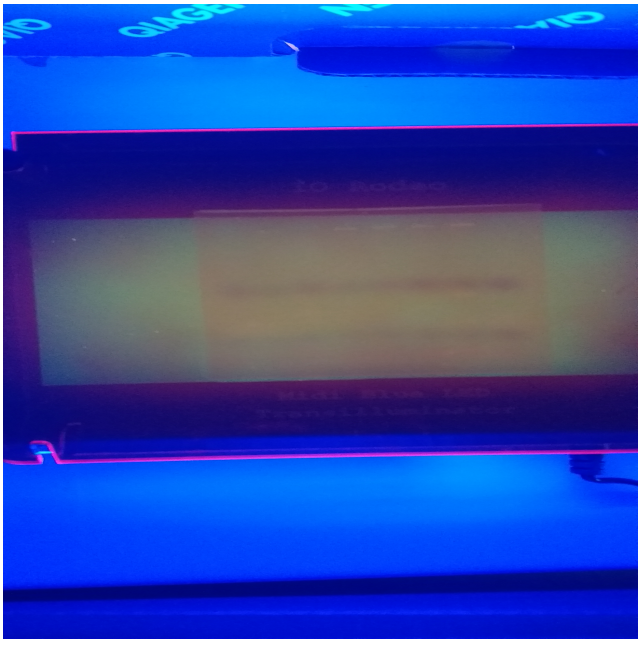

Fig. 5. Gel electrophoresis results under UV light

Fig. 6. Petri dish with ligated plasmid sample

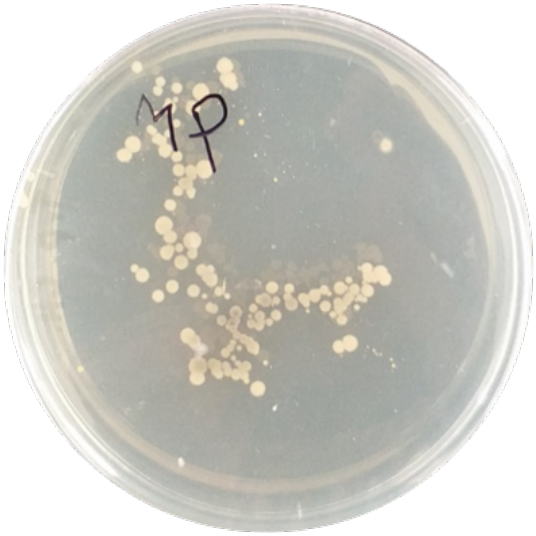

Fig. 7. Petri dish with miniprep sample

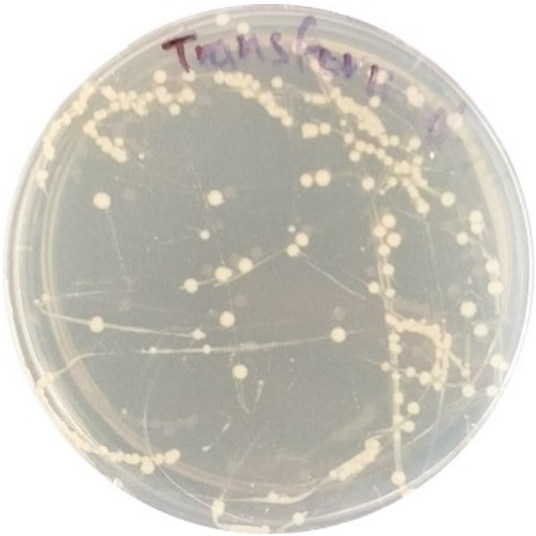

Fig. 8. Petri dish with transformed *E. coli* sample

Fig. 9. Original Petri dish with tryptophan

C. Biodesign Results

Using the initial bacteria with the TMM gene (sample number 3 above) some blue dye was produced on the silk textile. The intensity of the color was not as intense as anticipated and the amount of harvested bacterial yield was also lower, limiting the amount of surface area was covered with the bacteria.

The dye that was printed on the silk textile with the help of the bacteria remained on the cloth with the same intensity even after being washed with acetic acid 1:3 ration (vinegar solution), 70% ethanol, and destilled water.

Fig. 10. Biodesign results (1)

Fig. 11. Biodesign results (2)

IV. Discussion

Because this project was a multi-step one, there are many elements to be considered before concluding why there was a difference between the expected and actual results.

First of the possible factors is the over dilution of the DNA samples. During the miniprep, one of the final steps was to add "more than 30μl" of the DNA Elution Buffer and the incorrect interpretation was that, if there is only an established minimum, 200μl can be used. This was far too much and caused the sample to be over diluted, therefore making it unusable.

Another possibility (one that is not caused by human error) is that there was something wrong with either the enzymes that were used or with the bacteria itself. As enzymes work on the 'lock and key' principle, there is a possibility that either end somehow malfunctioned and therefore caused the whole mechanism to fall apart. As for the bacteria, there is a possibility that it was either contaminated to begin with or got contaminated along the way, causing it to react with chemicals that it was not meant to react with. Such chemicals include yeast extract (from the LB) or ice-cold $CaCl_2$, which is what might have happened with the sample of the ligated plasmid (the appearance of pink and yellow pigments).

The following two possible reasons both revolve around tryptophan. One of the possibilities is that tryptophan is more effective on bacteria when it is in a liquid LB rather than in a gel LB. Most molecules have an easier time moving through gas and liquid rather than through gel or solids, which means that the tryptophan might have been more effective if it had been introduced into a liquid medium and had had time to affect the bacteria before being transferred into a gel medium. The other possibility is that the environment in which the tryptophan was introduced was not optimal. The hypothesis is that the optimal temperature for tryptophan to dissolve is not room temperature, perhaps even the fact that the temperature was not constant (varied during the day).

The final reason why there was a difference between the expected and actual results is the possibility of contamination which was present throughout the project. While multiple precautions were taken to avoid contamination, there is still a chance one or multiple samples were contaminated, which could have caused a domino effect kind of reaction, thus distorting the results.

A. First sample(ligated and transformed bacteria)

Bacteria was growing but did not produce a blue pigment, so we know we did not kill the bacteria . We diluted our DNA samples therefore not enough DNA was present when we did our digestion. We did the PCR with the restriction digestion sample meaning that the gene and the plasmid backbone were not together, therefore the gene was not really copied as the primers were on the plasmid backbone. Possibly something went wrong with the transformation process as it is very heat sensitive and we were using water bath. The yellow and pink colors observed on the plate could be the result of a contamination.

B. Second sample (only observed after 48 hours)

Perhaps the solid form of tryptophan on the agar plate took longer to be absorbed by the genes, maybe it is more efficient in a liquid medium. It was proved that this hypothesis was wrong because bacteria was transferred into a liquid medium with tryptophan and it did not show any result.

C. Third sample

Without tryptophan the initial bacteria that was used for the whole process produced blue dye. Maybe there could be another factor apart from tryptophan that makes bacteria express that gene.

The above listed are the factors that could have affected the results. The most likely explanation is that what caused the difference between the expected and actual results is not one individual element but rather a mix of a few. To be certain what exactly contributed to the results, the whole project would have to be repeated after extensive research and with extreme precision.

V. Conclusion

With the help of synthetic biology it is possible to create blue dye using the TMM gene and tryptophan. To achieve the goal, MiniPrep, restriction digestion, PCR, ligation and transformation were performed. After combining the plasmid and the TMM gene, it was confirmed that *E. coli* can produce a blue dye. This would not be possible without an amino acid tryptophan. Until further research, it cannot be claimed with certainty that this method is reliable for making new, unharmful dye. Of course, for this method to be implied in everyday life it would take a lot of time, money and new facilities.

A. Limitations

While the aim of this project is to produce more sustainable dyes, it might take a while until this sort of reform is even considered.

There are multiple reasons for this, the first of which is the new facilities that are needed for producing the dyes. Furthermore, the facilities would have to be planned perfectly, not only to avoid contamination of samples, but also to have an appropriate way of disposing of the dangerous and contaminated waste. This leads us to the following limitation: the potential for a bacterial outbreak.

As is the problem with synthetic dyes, bacterially produced dyes also have the potential to pollute rivers through wastewater which cant be thoroughly purified. If this isnt properly disposed of after use, it could cause an outbreak of *E. coli*. Another limitation is a result of the dye itself; the only color currently available is indigo and, while many pieces of clothing are this color, there is very little chance that the idea of bacterially produced dyes will be widely accepted and will therefore be poorly financed. However, this is only a limitation at the moment as there is a chance that further research will result in the discovery of other possible pigments. Although it might seem a technique easy to start up because it doesnt take up much space (considering the size of the bacteria), the facilities to make clothing are not yet that efficient: it does not seem simple to execute the process itself (eg. Petri dishes with agar where the cloth has to be soaked) on a large scale. Moreover, all the products that are needed to decontaminate clothing can result toxic or harmful.

The final and least problematic limitation is the material that can be dyed. While most materials do turn indigo after the process is completed, a clear pattern is only possible on those that are non-absorbent. So far, silk has proved to be the optimal material to use, while cotton has not (Indico Dye, 2018. [4]). Therefore, even though bacterially produced dyes seem to be more sustainable than synthetic and natural ones, there is more work to be done until they replace the latter.

To **conclude**, synthetic biology is a very promising and reliable field of biology for new discoveries which can help us expand human knowledge. Many people say that synthetic biology is *The Field of the Future*.

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