Synthetic Ink: Using Synthetic Biology Techniques to Produce Biological Pigments in *Escherichia coli*

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Printer ink is a costly commodity that is used on a daily basis. The current pigments used to colour conventional inks may be costly and environmentally harmful to produce. Our goal is to produce biological pigments by putting the genes for different pigment biosynthesis pathway proteins into *Escherichia coli*. We will create four genetic constructs, each being responsible for producing either black, cyan, magenta or yellow pigments. Eventually, constructs would include genes in one plasmid for production of multiple colored pigments. Constructs will also be tested under the control of promoters of varying strength, with the objective being to achieve different color combinations and ratios. Pigments will be purified from bacterial culture after production and incorporated into an ink mixture.

Keywords: Synthetic biology, iGEM, ink, bacteria, pigment, printing, *Escherichia coli*

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Watch a video introduction by the authors at
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Ink is a fluid or viscous substance that is colored by dyes or pigments and used for coloring a surface to create an image or imprint. In North America, ink is used on a daily basis for both domestic and commercial purposes, as it is found in common products such as pens and printers to carry out functions such as writing, printing or painting. Large companies that require extensive amounts of printing, such as publishing companies, newspapers, and schools, are the facilities that use the highest quantities of ink.

There are two primary forms of coloring used in printers: toner and ink. They both contain pigments or dyes as colorants. However, the composition of both substances differs greatly from one another, making them non-interchangeable. Toner is a dry powder made of plastic particles, a colorant and a polymer that adheres to the paper once melted to its surface with heat. In contrast, ink is a liquid made specifically of a resin, a solvent and a colorant that can either be fully absorbed by the paper or adhered to the surface of the paper by the resin and dried once the solvent has evaporated. Our main objective is to produce pigments that can later be used to color by different appliances, chiefly ink. A pigment is a dry insoluble substance that when mixed with a solvent, colors the solution; however, the pigment particles remain suspended in the solvent and do not dissolve.

The production of ink is a five-step method that begins with the manufacturing of the varnish, the thick substance that will act as the body to the ink. The varnish is cooked resin combined with diluents and oils to create offset inks or solvents to make liquid inks. Pigments are then added to the varnish to be dispersed evenly in a process known as premixing. The processing of the pigment particles and varnish is what creates the intense color and glossy finish of the ink as the pigment is more evenly distributed. The ink is then transferred to a variety of different mills to undergo grinding, a method by which the ink is continuously processed and pressed between three rotating drums to further disperse the pigment. Finally, the pigment's viscosity is adjusted with dilution into a thinner or solvent and any indispensable additives are incorporated to give a high-quality finish to the ink.

Almost every person in North America uses ink on a daily basis and the majority of it is found in printers. The problem with this conventional product is the price. Ink costs between $0.59/mL and $3.40/mL, while cartridges for printers range from $20-50+ (Consumer Reports, 2017). In comparison, gasoline costs approximately $0.00128/mL in Canada (Canada Gasoline Prices, 2017). Current production of ink is also detrimental to the environment. Synthetic organic pigments are obtained from coal tars and other petrochemicals, some made by the burning of these compounds (Wigmore, 1972). In addition,polychlorinated biphenyls (PCBs) are still used in some pigments today. The Environmental Protection Agency (EPA) considers PCBs to be a probable human carcinogen. Continued exposure to these chemicals by accidental ingestion, dermal absorption or inhalation can cause chronic ailments and possibly changes in blood and urine (Grossman, 2013). This affects workers as they are exposed to these chemicals on a regular basis. The volatile organic compounds (VOCs) in ink can also negatively impact both the environment and humans. They evaporate quickly into the atmosphere causing air pollution, which can increase the risk of heart disease and lung cancer as well as an increase in ground-level ozone.

Ink manufacturers in places like China and India have been shut down due to the environmental impacts of the production of their product and its components, including pigments. This can negatively impact the economy, because fewer manufacturers will be creating ink, leading to lower production. A lower production of ink and rising demand can influence ink prices to increase, possibly decreasing the demand and, ultimately, sales (Savastano, 2011).

We propose to produce biological pigments that can be used in ink at a lower cost and smaller environmental impact than current sources. We have identified pigments that can be produced in *Escherichia coli* that correspond to the four main colors in ink cartridges: black, cyan, magenta and yellow. They are melanin, indigoidine, anthocyanin and zeaxanthin, respectively. We have decided to produce these pigments because there has been success in producing these pigments in the past (Misawa et al., 1990; Cabrera-Valladares et al., 2006; Lagunas-Muñoz et al., 2006; Yan et al., 2008; Lim et al., 2015; Xu et al., 2015). Using synthetic biology techniques, genes corresponding to pigment biosynthetic pathways from a variety of organisms will be engineered for expression in *E. coli*. Pigment production will be followed by purification to separate the pigments from the bacteria, which will then allow the produced pigments to be incorporated into ink.

Systems Level

This project will produce biological pigments in bacteria that will then be harvested. This is done by finding four pigments that correspond to the four colors in ink cartridges (magenta, cyan, yellow and black) and producing them in bacteria. The pigments used are from other organisms and are all produced naturally. This is the difference between our ink and other inks. The colorants used by other ink producers come from chemical synthesis as well as combustion. The way these pigments will be formed is by putting the genes involved in the pigments' biosynthetic pathways into *E. coli*. The genes that code for the pigments will be placed into a plasmid and transformed into *E. coli* BL21(DE3), which will then express the gene and produce the pigments, which in turn can be purified. Purified pigments will be mixed into a solvent with a resin to form ink.

Device Level

All genetic constructs from pigments were designed to be standardized, using the same promoter, a ribosomal binding site and a terminator, cloned into the plasmid pSB1C3. pSB1C3, containing pigment biosynthetic genes, will be inserted into *E. coli* BL21(DE3). E.coli BL21(DE3) contains a small construct in the genome, containing a gene that codes for a T7 RNA polymerase behind the LacI promoter, which can be induced by IPTG addition to the cell culture. This T7 RNA polymerase will be placed in the construct behind an IPTG inducible promoter. All pigment-producing constructs are under the control of the T7 promoter, which is recognized by the T7 RNA polymerase. Additionally, the BL21(DE3) strain of *E. coli* is engineered to be deficient in the proteases Lon and OmpT, which is ideal for overexpression of foreign proteins such as those involved in pigment biosynthesis.

Parts Level

The promoter that will be used is a T7 promoter that will be recognized by the T7 RNA polymerase. This means the cells can be grown and begin pigment production only when the addition of IPTG to the culture induces the production of T7 RNA polymerase. The ribosomal binding site that will be used is one that is naturally made in E. coli, meaning the bacteria's ribosomes will recognize it and complete the translation process. The terminator will be a T7 terminator which will stop the T7 RNA polymerase and end transcription.

Black Pigment (Melanin)

Melanin will be used for the black pigment, and the melA gene from *Rhizobium etli* will be used to produce this pigment (Figure 1). The melA gene codes for a tyrosinase which converts acid L-tyrosine into dopaquinone, which then polymerizes into melanin (Lagunas-Muñoz et al., 2006). Melanin production requires L-tyrosine and copper supplements in the media. Copper is needed for supplementing the media because it is a cofactor for the tyrosinase, and in melanin biosynthesis, tyrosine is a

precursor (Cabrera-Valladares et al., 2006). Constructs containing the melA gene have been previously submitted to the iGEM Parts Registry (Cambridge iGEM 2009, Stanford Brown iGEM 2016, BBa_K274001).

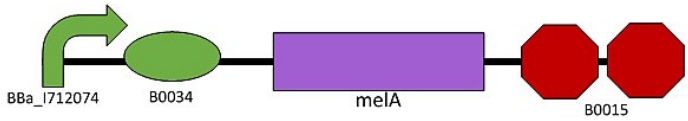

Figure 1. Construct for melanin. The part BBa_I712074 is the T7 promoter in the iGEM parts registry, B0034 is an E. coli rbs, melA is the gene for Melanin tyrosinase and a double terminator from E. coli is included to stop transcription. The MelA gene has been codon optimized for expression in E. coli. This construct will be cloned into pSB1C3.

Magenta Pigment (Anthocyanin)

The magenta pigment will be anthocyanin, a pigment commonly found in plants. It is produced using the following genes: dfr, which codes for dihydroflavonol 4-reductase (*Anthurium andraeanum*); f3h, which codes for 3B hydroxylase (*Malus domestica*); ans, which codes for anthocyanin synthase (*M. domestica*), and 3gt, which codes for 3-o-glucosyltransferase (*Petunia hybrid*) (Figure 2). The *E. coli* membrane transporter yadH has also been shown to export anthocyanin from the cell (Yan et al., 2008; Lim et al., 2015). The dfr, f3h, ans and 3gt genes constitute a pathway that converts eriodictyol into anthocyanin. Overexpressing the yadH transporter in *E. coli* has been shown to increase anthocyanin yields (Lim et al., 2015). A key reason for choosing to produce anthocyanin as opposed to a different pigment is that it can change color according to the pH level of its environment (Yan et al., 2008). This may allow us to have more versatility in making ink of different colors and shades, simply by adjusting the pH of the solvent. The efficacy of this method has yet to be determined. Previous constructs for anthocyanin production have been submitted to the iGEM Parts Registry (BBa_K1497023).

Cyan Pigment (Indigoidine)

The cyan pigment will be indigoidine and it will be produced by proteins encoded by the indB and indC genes from *Streptomyces chromofuscus*. The indB gene codes for a putative phosphatase and the indC gene codes for indigoidine synthase. Together, these enzymes convert L-glutamine into indigoidine (Yu et al., 2012) (Figure 3). It has been previously shown that indC alone can produce indigoidine, but the inclusion of indB expression in the system will increase yields significantly (Xu et al.,2015). A previous part for IndC has been submitted to the iGEM Parts Registry (BBa_K1152013).

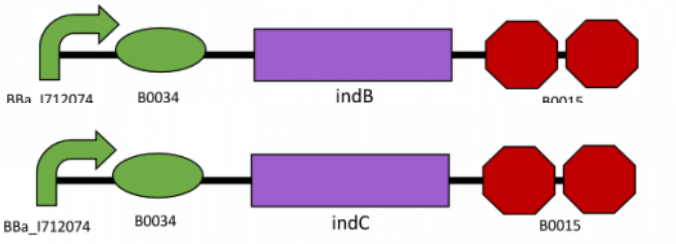

Figure 3. The indB and indC constructs that will be inserted into cells to convert L-glutamine to indigoidine. IndB encodes a putative phosphatase and indC encodes an indigoidine synthase. The control elements in the construct are the same as shown in Figure 1.

Yellow Pigment (Zeaxanthin)

The yellow pigment will be zeaxanthin, and it will be produced by a modified carotenoid biosynthesis pathway. The genes used will be crtY and crtZ, which come from the organism *Pantoea ananatis*. The crtY gene encodes a lycopene cyclase and crtZ codes for beta-carotene hydroxylase (Sedkova et al., 2005, Misawa et al., 2007). We will attempt to skip the first steps of the carotenoid biosynthesis pathway to reduce the number of foreign proteins introduced into *E. coli* by supplementing the media with lycopene (Figures 6 and 7). Previous parts for the B-carotene hydroxylase have been submitted to the iGEM Parts Registry (BBa_I742157).

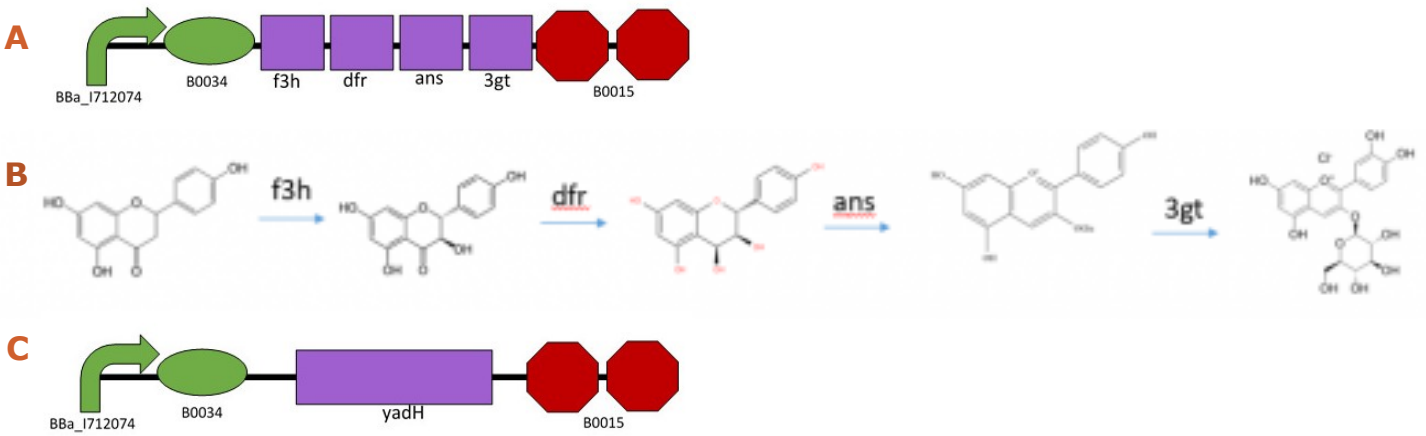

Figure 2. Constructs that will produce and export anthocyanin. (A) The operon contains the same promoter and ribosomal binding site as Figure 1 and contains the genes that are in the pathway (B) that convert eriodictyol into the anthocyanin.The dfr gene is from Anthurium andraeanum, f3h and ans are from Malus domestica, and 3gt is from Petunia hybrid. (C) Construct to express the E. coli transporter yadH that will export anthocyanin outside of the cell. The control elements are the same as shown in Figure 1.

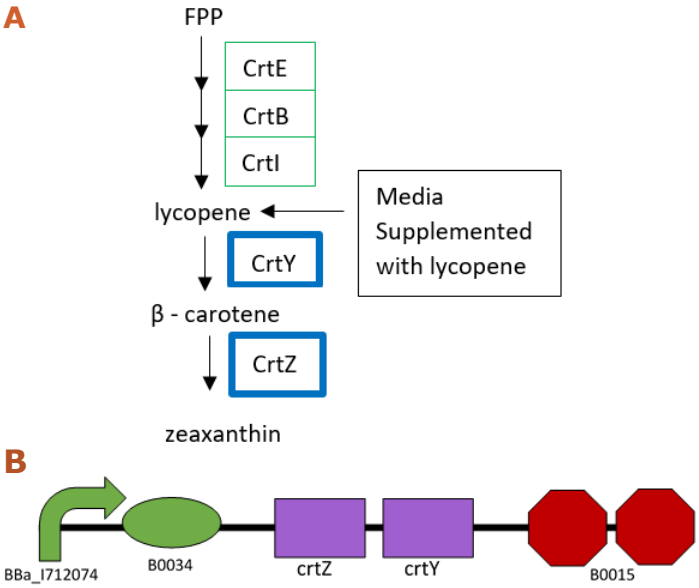

Figure 4. The carotenoid synthesis pathway and associated operon. (A) We propose supplementing the media with lycopene in order to bypass the first three steps and produce only CrtY and CrtZ in E .coli. (B) The construct that will allow the cells convert lycopene in to zeaxanthin includes the genes encoding a beta carotene hydroxylase and a lycopene cyclase. The control elements of the construct are the same as shown in Figure 1.

Safety

The safety of this project includes a variety of categories, including lab safety. To prevent harm to the lab participants, a number of aspects were looked into. Lab tables were wiped with ethanol before and after lab sessions and a Bunsen burner was used to sterilize the air in the immediate area, while also ensuring accurate lab results. Personal Protective Equipment (PPE) was used in the lab, such as lab coats and glasses. Antibiotics were included in the media used for the bacteria to prevent the growth of unwanted bacteria during trials. In addition, the lab technicians were trained prior to project development in order to ensure accurate results, as well as to prevent any injuries resulting from human error. To increase the safety of our product, the final product will undergo purification to ensure only the pigments will be left behind, with no live bacteria. This will be done through liquid-liquid extraction.

Discussions

The purpose of SynthetINK is to produce four unique pigment colors: black, cyan, magenta and yellow. This will be accomplished by creating four genetic constructs, each being responsible for producing a pigment. In order to determine the effectiveness of the constructs, a variety of experiments will be carried out. For example, it is crucial to know the quantity of pigments made, as well as the amount of protein produced by the constructs.

Quantity of Pigment Produced

To learn how much pigment is being produced, the proteins in the constructs will be overexpressed in *E. coli* and the proteins should produce pigments, resulting in colored bacterial cultures. Once overexpression is completed, it will be followed by a liquid-liquid extraction of the pigments to remove them from the cells and media (Venil et al., 2013). Extracted pigment will then be dried and weighed. In addition to this, a spectrophotometric analysis can also be used to determine the concentration of the pigments.

According to papers previously researched on pigment production, the wavelengths of light under which the respective pigments can be detected are known. Spectrophotometric data for melanin specifically could not be found. However, the wavelength for tyrosinase is 475 nm (Lagunas-Muñoz et al., 2006). We can use this value to calculate the concentration of melanin. At a pH of 1.0, the wavelength by which anthocyanin can be detected is 520 nm. In addition, the team might also research into the wavelengths of the intermediate (dihydroflavonol), which is 290 nm, and the by-product (flavonol), which is 360 nm (Yan et al, 2008). Indigoidine can be detected at a wavelength of 612 nm (Cude et al, 2012). Finally, zeaxanthin can be detected at a wavelength of 320 nm (Rodriguez-Amaya et al., 2001).

Protein Production

Samples of cell culture will be taken throughout the overexpression for SDS-PAGE analysis to determine if and how much protein is being produced by the constructs. The band intensity is indicative of the amount of protein present. This provides a qualitative means of checking the protein production of the constructs.

Determining the amount of pigment and protein produced by the bacteria will allow experimenters to discover any problems within the constructs. For example, if one construct was producing a small amount of pigment, as well as small amounts of protein, experimenters can infer that the low protein production was causing the drop in pigment production. As well, by learning of the shortcomings of the construct, experimenters can take the necessary steps to improve the design of the constructs to prevent the same problems from happening in the future.

Producing the Ink

Once there is confidence in the constructs' functionalities, the pigments will be purified by a liquid-liquid extraction, allowing the pigment to be separated from the bacteria. Then, the pigments can be mixed with the various other components found in an ink mixture, such as solvents. We will mix the pigments with either acetone or isopropanol as the main solvent. Currently, ink producers use either acetone or ethyl acetate as a solvent. However, it has also been found that glycerol can be used as a solvent in place of ethyl acetate (Venil et al., 2013).

Compared to current ink manufacturers, our ink production is rudimentary. While currently the production of ink is a five-step process, the production of our ink takes the form of three steps. After creating the pigment, we will take the dried material and add it to the solvent (acetone). Then, we will incorporate Gum Arabica as the resin. We have chosen this particular resin for convenience. This will complete the ink mixture and allow it to be used for a number of purposes, such as pen ink.

Future Direction

In order to expand the versatility of SynthetINK, the idea is to make a variety of different colors of ink. This can either be done by physically mixing two different pigments together or by incorporating multiple constructs into one plasmid to produce a new color of pigment.

At the end of the experimentation process, the hope is to create a prototype to showcase the ink. For example, by taking a fountain pen, introducing SynthetINK into it, and seeing how well it shows up on different surfaces.

Another idea is to try photobleaching SynthetINK as well as the ink that is found in current markets. Seeing the effect of ultraviolet rays on the two different kinds of inks for a certain time interval will allow the quality of the inks to be compared to each other.

Advantages

The largest benefit in this project is the ability to interchange parts. All of the genes for the different pigments can be interchanged in the constructs and plasmids. In addition, the magenta pigment, anthocyanin, changes colors at different pH levels, allowing the possibility of slightly changing the color with acids and bases.

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