Protoporphyrin IX (PPIX) production

Scale-up in *Escherichia coli*

By the University of Alberta iGEM team in Collaboration with the University of Calgary iGEM team

Introduction

The following report is based on the study and preliminary findings about PPIX scale-up by the University of Calgary (UofC) iGEM team members, given to the University of Alberta iGEM team (UAlberta) for review and further analysis. This study being reviewed was created for a chemical engineering course term project and was based on the UAlberta iGEM 2018 project. The report given to us from the modelling UCalgary subteam and addresses the scale-up of PPIX production, building on the University of Alberta iGEM 2018 project APIS (the Antifungal Porphyrin-based Intervention System). APIS aims to treat *Nosema ceranae* in *Apis mellifera* (Honey Bees) to decrease mortality rates due to fungal infections. In the following report, the UAlberta team has reviewed the microbial process of PPIX production scale-up in *Escherichia coli*, detailing the microbial fermentation process; moreover, our recommendations address these issues rather than the reactor design and market business model. The UAlberta team is still interested in continuing the work on the APIS project to complement our 2019 project, the Beetector, offering a complete solution against *Nosema ceranae* to beekeepers.

Platform strain

E.coli is a gram-negative facultative anaerobic bacteria; moreover, the strains being used by the University of Alberta iGEM team in 2018 were DH10B and BL21 DE3 (iGEM UAlberta). Not only was *E.coli* chosen for its lab trained qualities and well-known description, a major advantage with this bacteria is the presence of effective efflux pumps, making it easier to obtain our product of interest (Turlin *et al.* 2014).

In the UAlberta 2018, the heme biosynthesis pathway for the *E. coli*, chassis was genetically modified to allow for the overproduction of PPIX (iGEM UAlberta).

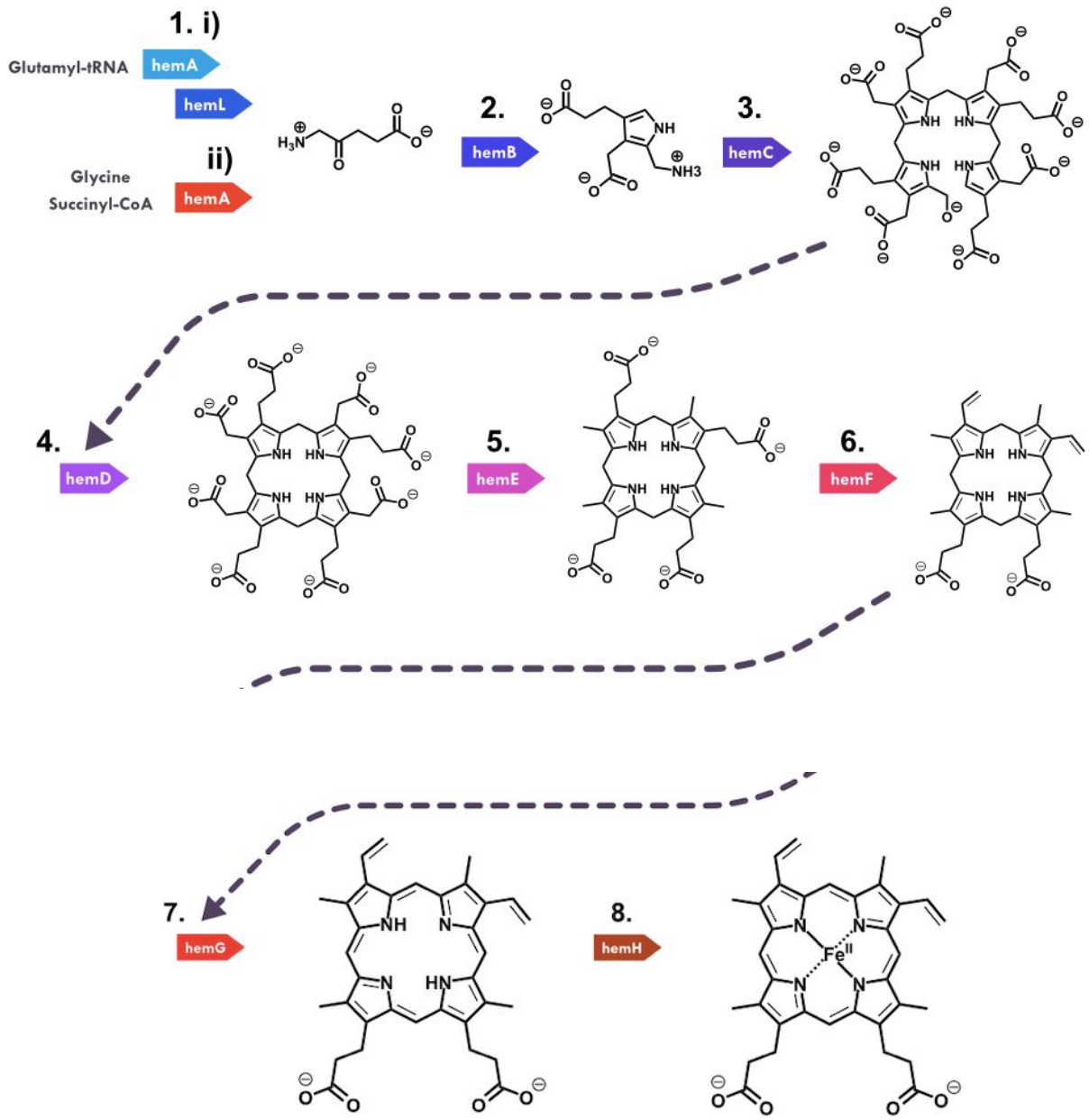

Figure 1: The two main heme biosynthesis pathways where the first step in (1.i) the C5-pathway and (1.ii) the Shemin-pathway are explicitly denoted. The remaining steps (2-8) depict the steps found in the *E. coli* endogenous pathway and its associated genes. The first compound produced in this schematic is ALA and the last step depicts heme. Our molecule of interest is PPIX is the last intermediate produced before heme.

This was achieved by introducing a plasmid containing the genes for PPIX synthesis under strong constitutive promoters, independent of the endogenous heme synthesis pathway (iGEM UAlberta). The

increase of PPIX would then be pumped out of the cell using the efflux pumps to be retrieved in the supernatant, as the solubility of PPIX is 169 mg/L at 25 °C according to the Aquasol database by Yalkowsky & Dannenfelser (1992).

The University of Calgary report did not mention what strains of *E. coli* would be used in the process.. The University of Alberta iGEM team decided to use a plasmid with the genes from the pathway on it; however, the University of Calgary report did not mention if they would continue to do this or choose a new mechanism for upscale.

Fermentation conditions

To maximize PPIX production the fermentation system needs to be optimized and scaled-up using multiple techniques and steps. Our first suggestion is to integrate the constructed plasmid into the bacterial chromosome to improve stability. After this has been done, the next step is to start an inoculum train which will then be increased by 10-fold each time to achieve the correct inoculum for each different fermenter.

The preferable fermenter for scale-up is a stirred bioreactor operating in a fed-batch regime, as it will allow for rapid growth of cell density and inhibit the crippling of the cell (Lim & Shin 2013). In the lab, pilot and industrial-scale production, temperature will be controlled to maintain a constant temperature of 37° C, the use of antifoam, pH controls of around 7, and having aeration below 10%. The chosen parameters of antifoam, neutral pH and aeration below 10% aim to promote the growth of *E. coli.* Its optimal growth pH is 7 and temperature of 37C. The antifoam is used to help stabilize the solution in the fermenter, and aeration below 10% allows for the anaerobic metabolism of the *E. coli* to take over.

Low levels of aeration also allows for mixed acid fermentations, a natural metabolic pathway in *E. coli* that can produce hydrogen, ethanol, succinate, formate, and lactate due to anaerobic metabolism

(Ciani *et al.* 2013). We will keep it in anaerobic conditions to make side products to keep an income of money to help us buy more expensive growing supplies starting out. To prevent acetate production and its inhibition of cell growth (Tripathi *et al.* 2009), a dissolved oxygen level of 30% may be sufficient (Phue and Shiloach 2005). This also improves the carbon efficiency for the yield of PPIX, due to having higher oxygen levels to convert it to anaerobic fermentation creating fewer organic side products (Phue and Shiloach 2005).

The medium will be iron limiting to minimize the conversion of PPIX to heme. Our medium to grow PPIX will be modeled after Citric Acid production in *Aspergillus niger* for the approximate iron levels of iron we will use for the medium (Najafpour 2007). This is because citric acid production also contains a limit on iron being used in the medium (Najafpour 2007). All this can change though depending on fermenting conditions that arise during scale-up.

To avoid contamination, proper sterilization techniques will be used to ensure that the fermenter will only contain *E.coli*. From Marisch *et al.* mentioned strains of *E. coli* that were efficient at producing proteins in fed-batch conditions (2013). The strains we recommend are BL21(DE3) due to its ability to produce more proteins; however, this strain can lose its plasmid to maintain growth if under stressful conditions (Marisch *et al.* 2013).

In lab-scale production, the main concern is guaranteeing a sufficient carbon-nitrogen ratio to optimize growth; therefore, as reported by Wang *et al.,* we suggest using a modified NBS medium II for fed-batch culturing (2019). The NBS medium II contains, per litre, 7 g KH_2PO_4, 10 g K_2HPO_4, 7 g $(NH_4)_2HPO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 30 mg $CaCl_2$, 1 mg thiamine, 0.1% (v/v) trace element solution, 5 g yeast extract, and 50 g glucose (Wang *et al.* 2019).

This nutritional medium will allow the bacteria to grow and release the PPIX through the drug efflux pumps into the supernatant, which will then be retrieved via centrifugation (Turlin *et al.*

2014).With increase in PPIX production from our *E. coli*, our production will increase 10-fold until we reach 10 L, ending the lab-scale production.

When moving to the pilot scale, the fermentation tanks used will range from 10 L (from the end of the bench-scale) to 100 L. At the start of the pilot, we will use the aforementioned medium; however, as the fermentation size increases, the media will be changed to be more cost-effective. To reduce production costs, we suggest substituting the NBS medium for waste products easily obtained. There will be a slow shift starting supplementing the NBS medium with at 20 L to using the waste products from other facilities to start moving to the industrial scale.

The carbon source for a more extensive operation could be Dried Distiller Grains with Solubles (DDGS), due to its protein, fat, and carbohydrate composition (Li *et al.* 2019) and, as nitrogen source, we could use Brewer's yeast extract. This will be attainable and, as DDGS is the waste products of brewery companies, would make PPIX production more cost effective.

In industrial-scale, we will use 1000 L tanks will be used, and the size of the end tank will be determined based on annual sales estimatives. The media used will be DDGS and Brewer's yeast. All the same controls and additives that were at the beginning of lab scale may have changed by this stage in development, which will depend on how APIS is best produced moving up in scale.

The University of Calgary report did not have an accurate upstream processing. The medium they were planning on using was RPMI 1640 medium, which is specific for mammalian cells rather than bacterial cultures. Hence, we decided to use glucose and yeast extract in our lab-scale instead. They also intended to continue using pure glucose until the end of industrial scale. However, this would be extremely expensive - for example, Sigma Aldrich sells glucose for 338 CAD for only 25 kg (2019). There was also no mention in the report on the inoculum train culture conditions to get to higher commercial volumes when moving from lab-scale to pilot and eventually industrial.

Moreover, the UofC report did not mention the type of reactor operation they would use (fed-batch, batch). We recommend using a fed-batch operation mode. The report also did not define reactor sizes when transitioning from the various steps in production scale-up, what style of fermentation they would be using, controls and proper growth conditions. Their plan is to keep the cell in aerobic growth.

Downstream Processing (DSP)

As PPIX is excreted through drug efflux pumps, it can be collected in the supernatant by separation (Turlin *et al.* 2014). Moreover PPIX is not soluble in the supernatant; therefore, more expensive or challenging DSP is not needed. However, there will be added expenses, such as cleaning out antifoam and purifying the PPIX chemical that will be produced.

It is challenging to determine the other steps of DSP at the current project development stage. The simplest purification method is to turn the PPIX phase of the fermentation system into a dry chemical. The beekeeper would then dilute it in sugar water to administer the drug to the hive. Dry products typically have longer shelf-lives, which would make the storage and distribution of the final PPIX product easier.

According to Sigma Aldrich, the storage temperature for PPIX needs to be between 2-8 degrees celsius; therefore, beekeepers of our product will need to keep it in the fridge at all times before use. As mentioned above, we wanted our product to be a dry chemical and based on DrugBanks literature, PPIX is in a solid state (2019).

The by-products of Mixed Acid Fermentation are hydrogen, ethanol, succinate, formate, and lactate. These can be sold off to help keep our profitability (Ciani *et al.* 2013).

The UofC report suggested using high-pressure homogenization to disrupt the cell; however, this would not be needed since PPIX is excreted from the cell and is in the supernatant. This would be a

needless, unnecessary, and expensive step. The report also mentions using an high-performance liquid chromatography (HPLC) column to collect the PPIX from the supernatant, but this also would not be needed since PPIX has a solubility of 169 mg/L at 25°C and can be easily collected by centrifugation (Yalkowsky & Dannenfelser 1992).

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The University of Alberta iGEM 2019 Team thanks the University of Calgary team members for your contribution to last year's project. The above study was based on the information we have received from your scale-up report and it is our hope to continue improving the APIS project as well as to our knowledge of industrial biotechnology principles

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