

Bio-Based Production of Uroporphyrin and Protoporphyrin-IX in
Escherichia coli

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Porphyrins are tetrapyrrole compounds with diverse medical applications but challenging chemical and biological synthesis. This thesis reports a novel bioprocess approach for enhanced production of porphyrins including uroporphyrin (UP) and protoporphyrin IX (PP-IX) in *Escherichia coli* (*E. coli*) through the implementation of the C4/Shemin pathway. We first heterologously implemented the C4/Shemin pathway in an *E. coli* strain with an enlarged intracellular succinyl-CoA pool. Using plasmids with *trc* and *gracmax* promoters regulating the expression of synthesized gene operons, we characterized the effects of key pathway genes (*hemA*, *hemB*, *hemC*, *hemD*, *hemE*, and *hemF*, and *hemG*) on UP and PP-IX biosynthesis. By cultivating the resulting engineered *E. coli* strains in a glycerol-fed batch bioreactor under aerobic conditions, we produced up to 901.9 mg/L UP and 64.7 mg/L PP-IX. This thesis demonstrates the utility of synthetic biology and metabolic engineering strategies for large-scale bio-based production of UP and PP-IX.

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Dedication

I dedicate my dissertation work to my parents, lovely sisters, and Camelia.

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List of Abbreviations

UP	Uroporphyrin
TCA	Tricarboxylic acid
<i>hemA</i>	Gene encoding 5-aminolevulinate synthase
<i>hemB</i>	Gene encoding porphobilinogen synthase
<i>hemC</i>	Gene encoding porphobilinogen deaminase
<i>hemD</i>	Gene encoding uroporphyrinogen III synthase
<i>hemE</i>	Gene encoding uroporphyrinogen decarboxylase
<i>hemF</i>	Gene encoding coproporphyrinogen III decarboxylase
<i>hemG</i>	Gene encoding protoporphyrinogen III oxidase
5-ALA	5-aminolevulinic acid
PPD	Protoporphyrin-dependent
CPD	Coproporphyrin-dependent
SHD	Siroheme-dependent
CP	Coproporphyrin
CPG	Coproporphyrinogen
PDT	Photodynamic therapy
HMB	Hydroxymehtylbilane
UPG	Uroporphyrinogen
ATP	Adenosine triphosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
GSA	Glutamate-1-semialdehyde
PBG	Porphobilinogen

<i>sdhA</i>	Gene encoding succinate dehydrogenase
<i>iclR</i>	Gene encoding AceBAK operon repressor
<i>ldhA</i>	Gene encoding lactate dehydrogenase
DNA	Deoxyribonucleic acid
LB	Lysogeny broth
SB	Super broth
rpm	Revolutions per minute
IPTG	isopropyl β -D-1-thiogalactopyranoside
OD	Optical Density
HPLC	High-performance liquid chromatography
RID	Refractive index detector
PDA	Photodiode array
PP-IX	Protoporphyrin-IX
RBS	Ribosome binding site

List of Symbols

Δ	Gene deletion
$^{\circ}$	Degrees of temperature
$\pm$	Plus-minus (precision of approximation)
μ	Mu (indicating “micro”)
R	Resistance marker

Chapter 1: Introduction

Porphyrins are a class of pigmented tetrapyrrole compounds characterized by their unique interconnected ring structure (Cook, Brewer, & Wong-Ng, 2017). The word "porphyrin" is derived from the Greek word *porphura* meaning purple, reflecting the vivid pigmentation produced by these biomolecules that play indispensable roles in oxygen transport, photosynthesis, and electron transfer processes across living systems. In addition to their biological significance, porphyrin's versatile applications in various sectors, including therapeutics, molecular electronics, and polymers, have incited great interests in further exploring their properties and industrial production of these pigments (Shi, Zhang, & Linhardt, 2021; Zhou et al., 2017). While chemical synthesis methods for producing porphyrins exist, they typically result in low yields and lack of selectivity for specific isomer production (Hassner & Namboothiri, 2012; Kwon, De Boer, Petri, & Schmidt-Dannert, 2003). The intricate purification procedures needed to isolate the synthesized porphyrins also reduce the efficiency and economic viability of chemical porphyrin production, making such techniques less appealing for industrial use. Taking a bio-based approach for porphyrin production through metabolic engineering and synthetic biology could potentially overcome these limitations.

Heme biosynthesis involves conserved yet flexible pathways across diverse organisms, with the core steps centered on the generation of 5-aminolevulinic acid (5-ALA) and its conversion to the branch point intermediate uroporphyrinogen III (UPG-III) as illustrated in Figure 1. 5-ALA can be derived in two ways: a C4 pathway and a C5 pathway. Its subsequent transformation to UPG-III via porphobilinogen and hydroxymethylbilane is shared (Jiang et al., 2022) as illustrated in Fig. 1, green module. At this juncture, three distinct pathways emerge: the protoporphyrin-dependent (PPD; Fig. 1, red module) pathway prevalent in eukaryotes and some bacteria, the coproporphyrin-dependent (CPD; Fig. 1, yellow module)

pathway in gram-positive bacteria, and the siroheme-dependent (SHD; Fig. 1, blue module) pathway in certain archaea and bacteria (Q. Yang, Zhao, Zheng, Chen, & Wang, 2023). The PPD pathway involves oxidative decarboxylation of UPG-III to eventually generate protoporphyrin IX (PP-IX) and heme. In contrast, the CPD pathway diverges at coproporphyrinogen III (CPG-III) through alternate oxidation steps to produce heme. The SHD pathway uniquely uses SAM-dependent methylation during heme biosynthesis. While the specific sequences of transformations vary, the conserved initiation phase and divergence at the UPG-III branch point underscore the adaptable and essential nature of heme biosynthesis across living systems (Q. Yang et al., 2023; Zappa, Li, & Bauer, 2010; Zhang, Kang, Chen, & Du, 2015).

In the present study, we have implemented the C4/Shemin pathway into an *Escherichia coli* (*E. coli*) strain with an enlarged pool of succinyl-CoA through the heterologous expression of *hemA* from *Rhodobacter sphaeroides* (*R. sphaeroides*). Our investigation focused on elucidating the impacts of overexpressing various combinations of heme genes to facilitate the biosynthesis of UP and PP-IX. This approach enables a more comprehensive understanding of the most efficacious genes for enhanced production of UP and PP-IX. Glycerol was selected as the carbon source in this study, as it is a cost-effective and widely available substrate with a highly reduced chemical nature. Research has demonstrated its feasibility as a primary carbon source for engineered *E. coli* strains, eliminating the need for supplementary compounds (Lall et al., 2021b; D. Miscevic et al., 2021). The outcomes of this investigation are poised to offer valuable insights into the development of more efficient and economically viable strategies for the biotechnological production of UP and PP-IX.

Fig. 1: Heme biosynthesis pathways for porphyrin biosynthesis from C4/Shemin pathway in *E. coli*. **Green:** Denotes shared steps common to all three pathways. **Blue:** Identifies steps specific to the SHD pathway. **Red:** Highlights processes unique to the PPD pathway. **Yellow:** Indicates steps exclusive to the CPD pathway. Colored proteins: heterologous expression (in purple) and native expression (in blue). Enzyme abbreviations: HemA (ALAS), ALA synthase; CysG, siroheme synthase; HemB, PBG synthase, HemC, HMB synthase; HemD, UPG-III synthase; HemE, UPG decarboxylase; HemF, CPG-III decarboxylase ; HemG, PPG-IX oxidase or PPG-IX hydrogenase; HemH, ferrochelatase; YfeX, CPG oxidase; HemH*, CP ferrochelatase; HemN, CPG-III dehydrogenase; HemQ*, coproheme oxidative decarboxylase.

1.1. Hypotheses and objectives

The hypotheses of this research are as follows:

1. It is hypothesized that implementation of the C4/Shemin pathway for 5-ALA formation will significantly improve tetrapyrrole production, including UP and PP-IX, in *E. coli*.
2. It is further hypothesized that identification and removal of critical pathway bottlenecks in the tetrapyrrole biosynthesis pathway will lead to an increase in the product yield.

The objectives of this research are as follows:

1. To develop an efficient bio-based production method for tetrapyrroles including UP and PP-IX in *E. coli* by the implementation of the C4/Shemin pathway and optimization of the tetrapyrrole biosynthesis pathway.
2. To identify and address bottlenecks in the *E. coli* tetrapyrrole pathway to maximize UP and PP-IX yield.

Chapter 2: Literature review

2.1. Bio-based chemical production: A sustainable paradigm shift

In response to mounting environmental concerns, the last few decades have witnessed a significant surge in interest surrounding the production of bulk chemicals via bio-based routes, as opposed to conventional petrochemical processes. This paradigm shift is driven by a conscientious move towards sustainability and seeks to supplant finite fossil fuel resources with renewable biomass feedstocks such as sugars and plant oils. This shift is characterized by the conversion of biomass into targeted chemicals through microbial fermentation or enzymatic conversions, offering enhanced economic efficiency, sustainability, and environmental friendliness compared to conventional petrochemical procedures (Du Preez, 2016; Hermann, Blok, & Patel, 2007).

Extensive studies have underscored a series of interconnected advantages intrinsic to bio-based production. These advantages illuminate its superior standing over traditional petrochemical approaches in terms of sustainability, economic viability, energy utilization, emissions reduction, and product versatility (Ioannidou et al., 2020; Kirschning & Hahn, 2012; Weiss et al., 2012). Bio-based routes substantially reduce the reliance on fossil resources, capitalizing on renewable biomass feedstocks. Notably, this transition culminates in a significant reduction of the lifecycle environmental footprint; bio-based chemicals exhibit a remarkable decrease of over 100% in fossil energy consumption and greenhouse gas emissions relative to their petrochemical counterparts (Hermann et al., 2007). Furthermore, the viability of biomass feedstocks as a cost-effective alternative could potentially strengthen over the long term in light of escalating oil prices. Moreover, the bio-based production paradigm fosters a level of chemical tailoring previously unattainable, thus facilitating the engineering of chemicals endowed with desired characteristics (Kirschning & Hahn, 2012). These benefits in

terms of sustainability, energy efficiency, emissions reduction, economic viability, and flexibility make bio-based routes attractive alternatives to conventional petrochemical methods.

Nevertheless, bio-based production is not without its limitations. While single-pass yields in bio-based reactions often significantly outperform chemical reactions, the overall yields tend to be lower because a significant portion of the material consists of biomass, which is considered a byproduct in production of a specific substance. An additional challenge facing bio-based production is the potential alterations in land use associated with biomass agriculture (Philp, Ritchie, & Allan, 2013; Weiss et al., 2012). Mitigating these drawbacks requires the strategic deployment of synthetic biology and metabolic engineering to optimize metabolic pathways, augmenting product yields (Hara et al., 2014; Keasling, 2010). Additionally, using waste biomass as the essential carbon source has the potential to mitigate the adverse effects of land use change. A prominent illustration of this principle is the use of glycerol, a prevalent byproduct of the biofuel industry, which has demonstrated promise as a viable feedstock for fermentation processes (Westbrook et al., 2019; F. Yang, Hanna, & Sun, 2012). By utilizing glycerol as the primary carbon source and optimizing bio-based production derived from it, we can significantly reduce the demand for extensive agricultural biomass, thus decreasing our reliance on fields for feedstock production. To this end, the continuous evolution of industrial biotechnology is poised to unlock the full spectrum of interconnected advantages associated with bio-based production, substantiating its key role in diminishing reliance on fossil resources and mitigating the adverse impacts of climate change.

In summary, chemical production is marked by a paradigmatic shift towards bio-based methodologies. This transformation, motivated by environmental concerns and resource scarcity, capitalizes on renewable biomass feedstocks and manifests itself in processes characterized by heightened sustainability, energy efficiency, emissions reduction, and

economic viability. While certain challenges persist, advancements in synthetic biology and metabolic engineering hold the potential to surmount these limitations, further solidifying the standing of bio-based production as a transformative and sustainable avenue for chemical synthesis.

2.2. Challenges and potentials of using *E. coli* for bio-based production of chemicals

One of the most widely used organisms in industrial biotechnology for bio-based production of various natural products, biofuel, chemicals, and precursors for pharmaceuticals is *E. coli*. This bacterium has gained significant prominence as a heterologous host in the field of metabolic engineering, owing to its extensive repertoire of genetic tools (Keasling, 2010). In the realm of modern biotechnological methodologies encompassing synthetic biology, metabolic engineering, and genome manipulation, the construction of microbial cell factories has been considerably streamlined (Davy, Kildegaard, & Andersen, 2017; McArthur & Fong, 2010). Nonetheless, the complexity of bio-based production methods frequently presents challenges, primarily stemming from the intricate molecular and regulatory mechanisms inherent to engineered genetic and metabolic components.

Several specific challenges stand out as obstacles for advancing metabolic engineering and synthetic biology. One is the lack of characterization data for biological components like promoters and ribosome binding sites that limits rational design (García-Granados, Lerma-Escalera, & Morones-Ramírez, 2019). There is also a shortage of quantitative data on biological phenomena like transcription, translation, protein degradation rates, and enzyme kinetics that restricts predictive modeling capabilities (García-Granados et al., 2019). Additionally, constructing microbial cell factories is labor-intensive and time-consuming due to biological complexity and the need for iterative cycles of design, building, and testing (Y. Liu, Cao, Du, & Liu, 2020). Other key challenges include contending with genetic and non-genetic heterogeneity in engineered strains that reduces productivity over long production runs

(Y. Liu et al., 2020) and overcoming toxicity from intermediates that inhibit pathways (García-Granados et al., 2019). These challenges become particularly pronounced when dealing with complex metabolic pathways such as the tetrapyrrole biosynthesis pathway, which is essential for UP and PP-IX production in *E. coli*. Tackling these specific barriers will drive advances in metabolic engineering and synthetic biology.

2.3. Glycerol: A Sustainable alternative feedstock for enhanced biorefinery applications in *E. coli*

Glycerol's emergence as a versatile biorefinery feedstock presents a compelling alternative to conventional sugars like glucose, primarily due to its unique properties, cost-effectiveness, and abundance. The two latter features stem from its status as a common byproduct in industries such as biodiesel and bioethanol production. It offers an enticing option for sustainable resource utilization (Blankschien et al., 2010). Its pronounced redox potential, with a redox ratio of 4.67 as opposed to glucose's 4.0, renders it particularly suitable for the synthesis of highly reduced compounds, such as succinate (Blankschien et al., 2010). Comparatively, the theoretical maximum yield of succinate derived from glycerol is notably higher, reaching 1.6 mol/mol, in contrast to glucose's potential yield of 1.3 mol/mol. This numerical advantage underscores glycerol's efficacy as a precursor for succinate production (Murarka et al., 2008). In *E. coli*, glycerol degradation follows two distinct pathways (Fig. 2): respiratory and fermentative. Under respiratory conditions, glycerol is enzymatically converted into glycerol-3-phosphate (G3P) via glycerol kinase. Subsequently, G3P is transformed into dihydroxyacetone phosphate (DHAP) by aerobic or anaerobic G3P dehydrogenase. Alternatively, the fermentative pathway involves the oxidation of glycerol into dihydroxyacetone (DHA) by NAD⁺-linked glycerol dehydrogenase, followed by the phosphorylation of DHA into DHAP through dihydroxyacetone kinase. The fermentative route

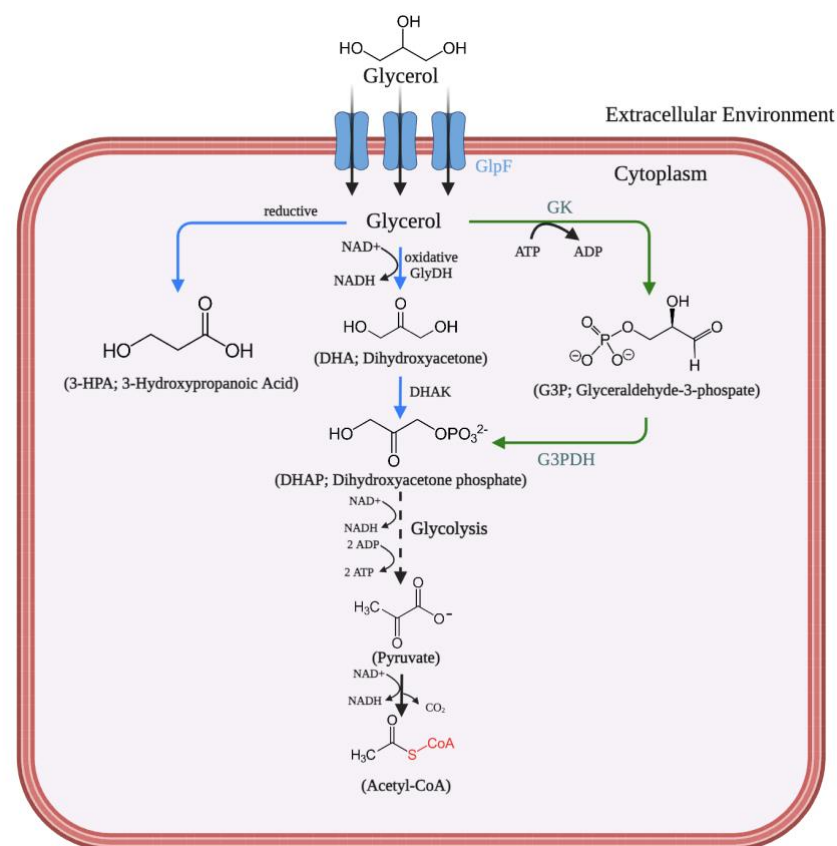


Fig. 2: Schematic representation of glycerol dissimilation pathways in *E. coli*. **Blue** arrows show the fermentative branch reactions; **green** arrows show the respiratory branch reactions; **solid** lines show one-step bioconversion; **dashed** line show multi-steps bioconversion. Enzyme abbreviation: DHAK; dihydroxyacetone kinase, G3PDH; glycerol-3-phosphate dehydrogenase, GK; glycerol kinase, GlyDH; glycerol dehydrogenase.

displays greater efficiency, particularly in anaerobic environments. DHAP then integrates into glycolysis, offering diverse metabolic possibilities, including succinate production (Durnin et al., 2009).

2.4. Enhanced succinate production through TCA cycle

The tricarboxylic acid (TCA) cycle is a vital metabolic pathway in bacteria such as *E. coli* that generates ATP as well as precursors for biosynthesis. A key intermediate in this cycle is succinic acid, a C₄ dicarboxylic acid that can serve as a precursor for many biosynthetic pathways including tetrapyrrole biosynthesis (X. Liu et al., 2022; Shemin & Kumin, 1952). Succinic acid production pathways branch off from the TCA cycle. Under anaerobic conditions, the reductive TCA branch coupled to phosphoenolpyruvate (PEP) carboxylase converts PEP and CO₂ into succinic acid (Fig. 3). Alternatively, under aerobic conditions, the oxidative TCA branch or glyoxylate shunt pathway produce succinic acid from acetyl-CoA and oxaloacetate (X. Liu et al., 2022) as illustrated in Fig. 3. Wild-type *E. coli* can only produce minor amounts of succinic acid under anaerobic conditions, whereas under aerobic conditions succinic acid is formed only as an intermediate of the TCA cycle unless the glyoxylate bypass is operating (Thakker, Martínez, San, & Bennett, 2012). Metabolic engineering strategies can redirect carbon flux to substantially increase succinic acid biosynthesis under both aerobic and anaerobic states. For example, deletion of subunit A of lactate dehydrogenase (*ldhA*) and subunit A of succinate dehydrogenase (*sdhA*) shifts carbon flux towards anaerobic succinic acid production. Additional gene disruptions such as *iclR* can further optimize succinic acid yields under aerobic conditions (Dragan Miscevic, Mao, Moo-Young, & Chou, 2020). These genetic modifications reconfigure the organism's core metabolism to favor succinic acid generation via the TCA cycle. Accordingly, the accumulated succinate can be converted to succinyl-coenzyme A (succinyl-CoA) by succinyl-CoA synthetase to serve as a precursor for downstream biosynthetic pathways like the C₄/Shemin or C₅.

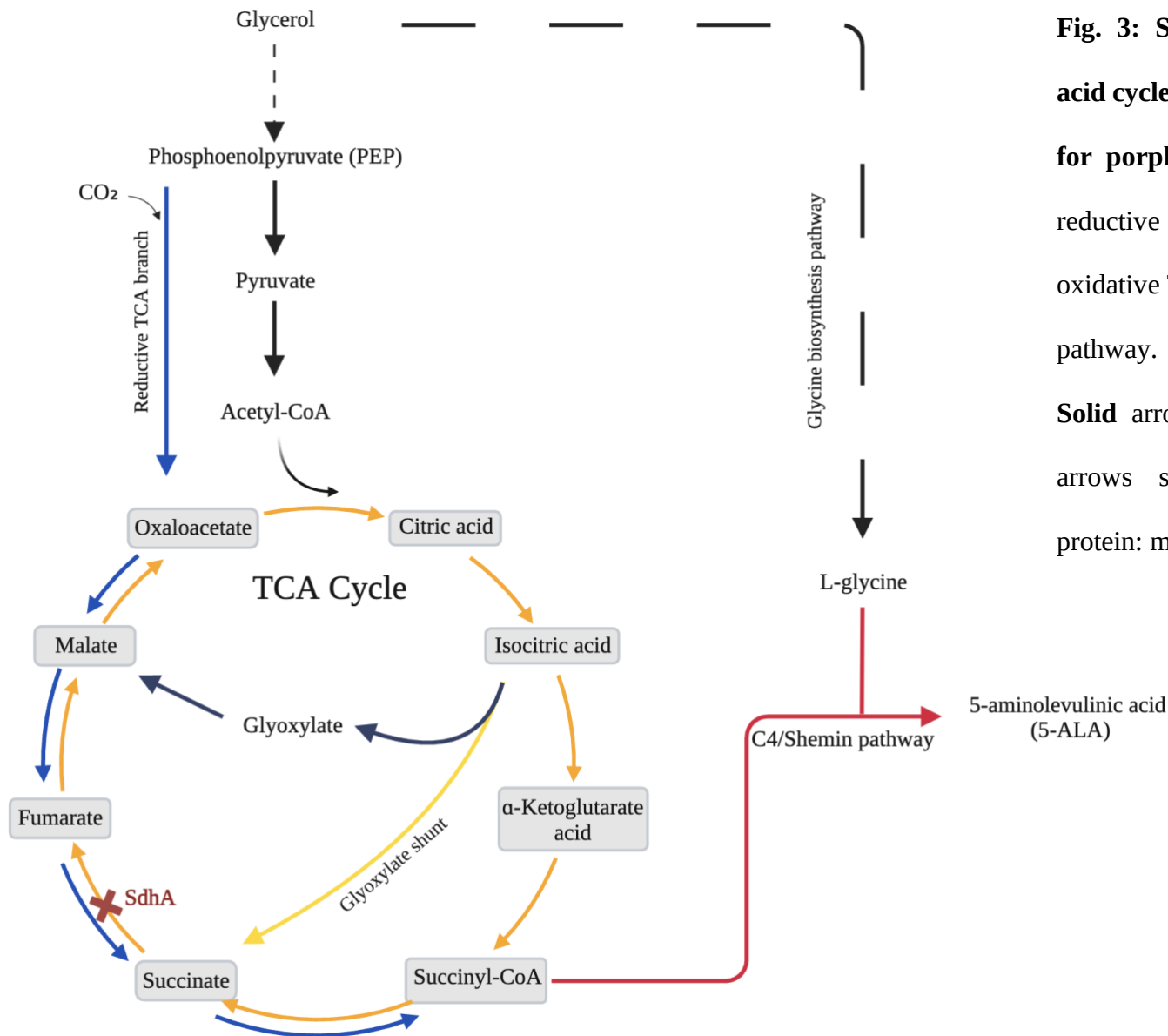


Fig. 3: Schematic representation of tricarboxylic acid cycle and the implemented C4/Shemin pathway for porphyrin biosynthesis. Blue arrows show the reductive TCA branch. Orange arrows show the oxidative TCA cycle. Red arrow shows the C4/Shemin pathway. Yellow arrow shows the glyoxylate shunt. Solid arrows show one-step bioconversion. Dashed arrows show multi-steps bioconversion. Colored protein: mutation (in red).

2.5. Heme Biosynthesis Pathways Across Biological Kingdoms

Heme biosynthesis, despite variations in specific pathways and enzyme arrangements, is a conserved and essential process that occurs across virtually all living organisms (Kořený, Oborník, Horáková, Waller, & Lukeš, 2022). These pathways are not only essential for the production of heme, which serves as a prosthetic group in hemoglobin and various enzymes, but also for the synthesis of other important tetrapyrrole compounds such as uroporphyrin (UP) and protoporphyrin IX (PP-IX). While the exact enzymatic steps and pathways may vary between different organisms, the core process of heme biosynthesis involves the assembly of a tetrapyrrole ring structure from simpler precursor molecules. This process begins with the production of 5-aminolevulinic acid (5-ALA), which acts as the immediate precursor for heme synthesis. One of the pathways leading to the production of 5-ALA is the C4/shemin pathway (Fig. 4A). In this pathway, the condensation of succinyl-CoA and glycine takes place, catalyzed by the enzyme δ -aminolevulinic acid synthase (ALAS). This step is a bottleneck and regulatory step in heme biosynthesis, making ALAS a crucial enzyme in the regulation of heme production. The C4/Shemin pathway is prevalent in many organisms, including animals and some bacteria. Another pathway leading to the production of 5-ALA is the C5 pathway, which operates in certain bacteria including *E. coli*, archaea, and plants. This pathway involves the conversion of glutamate to 5-ALA through a series of enzymatic reactions (Fig. 4B). The key enzymes involved in this pathway include glutamyl-tRNA synthetase (GltX), glutamyl-tRNA reductase (HemA), and glutamate-1-semialdehyde aminotransferase (HemL)(Jiang et al., 2022). However, the native C5 pathway has certain limitations that can impose metabolic and energetic constraints on its efficiency. This pathway demands the expression of several enzymes in a tightly regulated manner. Additionally, the C5 pathway involves the utilization of ATP and NADPH as limiting cofactors, which could potentially divert these energy-rich molecules from other essential cellular processes. Another notable disadvantage of the C5

pathway is the formation of unstable glutamate-1-semialdehyde (GSA) intermediate during the enzymatic conversion of glutamate. The inherent instability of GSA can lead to unwanted side reactions or degradation, reducing the overall yield of 5-ALA (D. Miscevic et al., 2021; Yi, Shih, Yu, Lee, & Ng, 2021). To overcome these constraints, the current study capitalizes on the C4/Shemin pathway to achieve heterologous biosynthesis of 5-ALA by overexpression of *hemA* from *R. sphaeroides*.

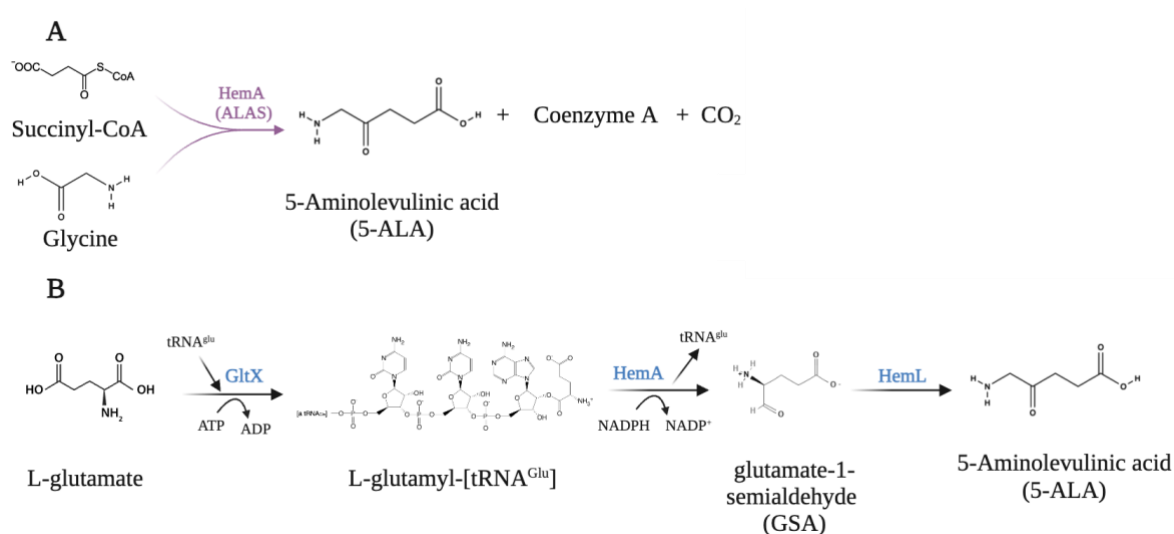


Fig. 4: Schematic representation of 5-ALA production pathways. A: C4/Shemin pathway, **B:** C5 pathway. Enzyme abbreviations: ALAS (HemA); 5-ALA synthase, HemA (in B); glutamyl-tRNA reductase, HemL; glutamate-1-semialdehyde 2,1-aminomutase, GltX; Glutamate-tRNA ligase.

The heme biosynthesis pathway encompasses three distinct pathways – the protoporphyrin-dependent (PPD), coproporphyrin-dependent (CPD), and siroheme-dependent (SHD) pathways (**FIG**). Despite their divergent paths, the initial stages of these pathways are characterized by common steps, all originating from 5-ALA and progressing to uroporphyrinogen III (UPG III), a crucial intermediate in heme biosynthesis (Q. Yang et al., 2023).

First, two 5-ALA molecules are enzymatically converted into porphobilinogen (PBG) through a reaction catalyzed by porphobilinogen synthase (PBGS or HemB). Subsequently, four molecules of PBG are condensed to form a linear tetrapyrrole known as hydroxymethylbilane (HMB) through the action of hydroxymethylbilane synthase (HMBS or HemC). This linear tetrapyrrole is then enzymatically cyclized into the cyclic form known as uroporphyrinogen III (UPG-III) by uroporphyrinogen III synthase (UROS or HemD). It is important to note that an alternative route exists for the linear HMB derived from PBG. In the non-classical pathway, HMB can spontaneously undergo oxidation, leading to the formation of uroporphyrinogen I (UPG-I; Fig.1). The formation of UPG III marks a critical juncture, as it serves as a precursor for further downstream branching into the distinctive PPD, CPD, and SHD pathways (Fig. 1). These pathways, each with unique enzymatic transformations and functional significance, subsequently diverge to synthesize heme through their respective mechanisms (Q. Yang et al., 2023; Zappa et al., 2010; Zhang et al., 2015).

The PPD pathway primarily operates in eukaryotes and certain gram-negative bacteria (Fig. 1, red module). It commences with the decarboxylation of uroporphyrinogen III (UPG-III) by uroporphyrinogen decarboxylase (UROD or HemE), yielding coproporphyrinogen III (CPG-III). The subsequent oxidative steps involve enzymes coproporphyrinogen III decarboxylase (CgdC or HemF) and coproporphyrinogen III dehydrogenase (CgdH or HemN), leading to the formation of protoporphyrinogen IX (PPG-IX). Oxidation of PPG-IX produces protoporphyrin IX (PP-IX) via protoporphyrinogen IX oxidase (PgoX or HemG) or protoporphyrinogen IX dehydrogenases (also called HemG) culminating in the insertion of a ferrous ion catalyzed by ferrochelatase (HemH) to produce heme (Q. Yang et al., 2023; Zhang et al., 2015).

Conversely, the CPD pathway, prevalent among gram-positive bacteria, shares the precursor CPG-III with the PPD pathway (Fig. 1, yellow module). Oxidation of CPG-III into

coproporphyrin III (CP-III) is driven by coproporphyrinogen oxidase (YfeX). Subsequent insertion of ferrous ions to generate Fe-coproheme III is facilitated by coproporphyrin ferrochelatase (HemH), while coproheme III oxidative decarboxylase (HemQ) catalyzes oxidative decarboxylation to produce heme. The CPD pathway, considered transitional between the SHD and PPD pathways, serves as an important evolutionary link (Q. Yang et al., 2023; Zhang et al., 2015).

In contrast, the SHD pathway, prevalent in specific archaea and sulfate-reducing bacteria, synthesizes siroheme, later transformed into heme (Fig. 1, blue module). Notably, this pathway involves S-adenosyl-L-methionine (SAM)-dependent methylation of the tetrapyrrole backbone, distinguishing it from classical routes (Q. Yang et al., 2023).

2.6. Literature review summary

In the preceding sections, we have explored the paradigm shift towards bio-based chemical production as a sustainable alternative to conventional petrochemical processes. This shift capitalizes on renewable biomass feedstocks, offering enhanced sustainability, economic viability, and environmental friendliness. However, challenges in metabolic engineering and synthetic biology, particularly in organisms like *E. coli*, have been highlighted as obstacles to optimizing bio-based production.

The methodologies delineated in the next section provide a comprehensive overview of the scientific and procedural foundation for this M.A.Sc. research. These described techniques and experimental procedures, which were designed to provide robust and reliable results for this study, were frequently applied and optimized. Building upon the insights gained from the preceding sections on bio-based chemical production, succinate enhancement, glycerol utilization, and heme biosynthesis pathways, this methodology section aims to outline the practical approaches and strategies employed to enhance the bio-based production of UP and PP-IX.

Chapter 3: Materials and methods

3.1. Bacterial strains and plasmids

The bacterial strains and plasmids utilized in this research are described in Table 1, whereas the primer sequences are provided in Table S1. Genomic DNA from bacterial cells was extracted with the Qiagen Blood & Tissue DNA Isolation Kit (Hilden, Germany). Taq DNA polymerase was obtained from New England Biolabs (Ipswich, MA, USA). All host cells in this study were derived from CPC-Sbm by introducing mutation(s) in the *sdhA* and/or *iclR* genes (Dragan Miscevic et al., 2020). Note that CPC-Sbm was derived from BW25113 by inactivating the *ldhA* gene (Srirangan et al., 2014). *E. coli* HI-Control 10G (Lucigen, Middleton, WI, USA) was used for molecular cloning. Plasmids were purified using the Qiagen Miniprep kit. DNA sequencing was performed by The Centre for Applied Genomics (TCAG) (Toronto, ON, Canada). Gibson assembly was employed for plasmid construction. All oligonucleotides were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA).

The initial construction of the pK-hemABCD plasmid involved several steps, where specific genes and backbone components were amplified and assembled using various primers. This process aimed to establish pK-hemABCD as a template for the subsequent construction of derivative plasmids, each carrying a distinct combination of heme genes. The following paragraphs detail the construction of these plasmids.

To begin, the *hemA* gene was obtained by polymerase chain reaction (PCR) amplification from the genomic DNA of *R. sphaeroides* DSM 158. This amplification was achieved using the primer pair P001/P002. Similarly, the *hemB*, *hemC*, and *hemD* genes were obtained by PCR amplification from the genomic DNA of *E. coli* MG1655, employing the primer pairs P003/P004, P005/P006, and P007/P008, respectively.

The backbone of the plasmid, which consisted of the p15A origin of replication (ori) and the *trc* promoter region, along with a kanamycin resistance marker, was extracted from a laboratory-created plasmid. This backbone was amplified using the primer pair P009/P010.

Subsequently, these five distinct fragments, namely *hemA*, *hemB*, *hemC*, *hemD*, and the plasmid backbone, were assembled through Gibson assembly methodology. This assembly process resulted in the construction of the pK-hemABCD plasmid.

The subsequent construction of derivative plasmids from pK-hemABCD followed a similar protocol. To create pK-hemA, primers P011 and P012, which were initially phosphorylated, were utilized to amplify a single fragment from the pK-hemABCD plasmid. This amplified fragment was then self-ligated to generate pK-hemA.

In a similar fashion, a single fragment was amplified from pK-hemABCD using primers P011 (phosphorylated) and P013, and this fragment was subsequently self-ligated to create pK-hemAB.

The pK-hemABC plasmid was constructed by employing two primer sets: P014/P002 and P003/P015. The first set of primers facilitated the amplification of both the plasmid backbone and the *hemA* gene in one fragment, while the second set amplified *hemB* and *hemC* in another fragment. These two fragments were then assembled using the Gibson assembly method to form pK-hemABC.

For the construction of pK-hemABC^B, *hemA* and *hemB* were initially amplified as a single fragment from pK-hemABCD using primers P001/P004. Simultaneously, *hemC* was amplified from the genomic DNA of *Bacillus subtilis* 168 with primers P016/P017. The plasmid backbone was also amplified using primers P018/P019 from pK-hemABCD. These three fragments were subsequently assembled using Gibson assembly, resulting in the formation of pK-hemABCB.

The pK-hemABD plasmid was created by amplifying *hemA* and *hemB* using primers P001/P004 and simultaneously amplifying *hemD* along with the plasmid backbone using primers P020/P021. The pK-hemABCD plasmid served as the template for these amplifications. The resulting two fragments were assembled through Gibson assembly to generate the pK-hemABD plasmid.

For the pK-hemABD-EFG plasmid, two sets of primers, P022/P023 and P024/P025, were employed. P022/P023 were utilized to amplify the *hemA*, *hemB*, *hemD*, and *gracmax* promoter regions from the pK-hemABD-E plasmid. Simultaneously, P024/P025 were used to amplify the *hemE*, *hemF*, *hemG*, and backbone from the pK-hemABCD-EFG plasmid. These two sets of fragments were subsequently assembled using the Gibson assembly method to create the pK-hemABD-EFG plasmid.

Finally, for the construction of the pK-hemAB-EFG plasmid, a different set of primers was utilized. P026/P027 were employed to amplify *hemA* and *hemB* from the pK-hemABD-EFG plasmid. Additionally, P028/P029 were used to amplify *hemE*, *hemF*, and *hemG* from the same pK-hemABD-EFG plasmid. Finally, P030/P031 were used to amplify the backbone from the pK-hemABD-EFG plasmid. These three sets of fragments, corresponding to *hemA* and *hemB*, *hemE*, *hemF*, *hemG*, and the backbone, were assembled through the Gibson assembly method, resulting in the construction of the pK-hemAB-EFG plasmid.

3.2. Media and bacterial cell cultivation

All medium components were obtained from Sigma-Aldrich Co. (St. Louis), except for yeast extract and tryptone, which were obtained from BD Diagnostic Systems (Franklin Lakes). *E. coli* strains were preserved as glycerol stocks at -80 °C and streaked on lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) agar plates, followed by incubation at 37 °C for 14-16 h.

For bioreactor cultivation, individual single colonies were picked from LB plates to inoculate 12 mL of super broth (SB) medium (32 g/L tryptone, 20 g/L yeast extract, and 5 g/L NaCl) in a 125 mL conical flask. The culture was incubated at 37 °C and 280 revolutions per min (rpm) using a rotary shaker (New Brunswick Scientific) for 4-6 h and subsequently used as a seed culture to inoculate 220 mL of SB medium at a 2% (vol/vol) concentration in a 1 L conical flask. This second seed culture was incubated at 37 °C and 280 rpm for 14-16 h. Cells were harvested by centrifugation at 4,500 g and 20 °C for 8 min and resuspended in 30 mL of fresh SB medium. The resuspended culture was used to inoculate a stirred tank bioreactor (CelliGen 115, Eppendorf AG) with a working volume of 0.8 L at 37 °C and 430 rpm. The semi-defined production medium in the batch bioreactor contained 30 g/L glycerol, 0.23 g/L K₂HPO₄, 0.51 g/L NH₄Cl, 49.8 mg/L MgCl₂, 48.1 mg/L K₂SO₄, 1.52 mg/L FeSO₄, 0.055 mg/L CaCl₂, 2.93 g/L NaCl, 0.72 g/L tricine, 10 g/L yeast extract, 10 mM NaHCO₃, and 1 mL/L trace elements (2.86 g/L H₃BO₃, 1.81 g/L MnCl₂·4H₂O, 0.222 g/L ZnSO₄·7H₂O, 0.39 g/L Na₂MoO₄·2H₂O, 79 µg/L CuSO₄·5H₂O, 49.4 µg/L Co(NO₃)₂·6H₂O); (Neidhardt, Bloch, & Smith, 1974), supplemented with 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). To avoid glycine limiting during the cultivation (as one mole porphyrin requires 8 moles of L-glycine), 2 g/L of L-glycine was supplemented into the bioreactor ~30 h after the inoculation.

Aerobic conditions were maintained by continuously purging the air into the bulk culture at 1 volume of air per volume of liquid per min (vvm). Microaerobic conditions were maintained by purging the air into the headspace at 0.1 vvm. Finally, anaerobic conditions were maintained by purging nitrogen (N₂) into the headspace. The pH of the bioreactor culture was maintained at 7.0 ± 0.1 using 3 M NH₄OH and 3 M H₃PO₄.

Table 1: Bacterial strains and plasmids used in this study.

Name	Description or relevant genotype	Source
Cell strains		
HI-Control 10G	<i>mcrA</i> , $\Delta(mrr\text{-}hsdRMS\text{-}mcrBC)$, <i>endA1</i> , <i>recA1</i> , $\phi80dlacZ\Delta M15$, $\Delta lacX74$, <i>araD139</i> , $\Delta(ara\ leu)7697$, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Str ^R), <i>nupG</i> , λ^- , <i>tonA</i> , Mini-F <i>lacI</i> ^{q1} (Gent ^R)	Lucigen
MG1655	K-12; F ⁻ , λ^- , <i>rph-1</i>	Lab stock
<i>Bacillus Subtilis</i> 168	Wild type	Lab stock
CPC-Sbm $\Delta iclR\Delta sdhA$	F ⁻ , $\Delta(araD\text{-}araB)567$, $\Delta lacZ4787(::rrnB\text{-}3)$, λ^- , <i>rph-1</i> , $\Delta(rhaD\text{-}rhaB)568$, <i>hsdR514</i> , $\Delta ldhA$, P _{trc} :: <i>sbm</i> (i.e., with the FRT-P _{trc} cassette replacing the 204-bp upstream of the Sbm operon), $\Delta iclR$, $\Delta sdhA$	(Dragan Miscevic et al., 2020)
CPC-Sbm $\Delta sdhA$	F ⁻ , $\Delta(araD\text{-}araB)567$, $\Delta lacZ4787(::rrnB\text{-}3)$, λ^- , <i>rph-1</i> , $\Delta(rhaD\text{-}rhaB)568$, <i>hsdR514</i> , $\Delta ldhA$, P _{trc} :: <i>sbm</i> (i.e., with the FRT-P _{trc} cassette replacing the 204-bp upstream of the Sbm operon), $\Delta sdhA$	(Dragan Miscevic et al., 2020)
DMA	CPC-Sbm $\Delta iclR\Delta sdhA$ /pK-hemA	This study
DMB	CPC-Sbm $\Delta iclR\Delta sdhA$ /pK-hemAB	This study
DMC	CPC-Sbm $\Delta iclR\Delta sdhA$ /pK-hemABC	This study
DMC ^B	CPC-Sbm $\Delta iclR\Delta sdhA$ /pK-hemABC ^B	This study
DMD	CPC-Sbm $\Delta iclR\Delta sdhA$ /pK-hemABD	This study

SMD	CPC-Sbm Δ <i>sdhA</i> /pK-hemABD	This study
DMG	CPC-Sbm Δ <i>iclR</i> Δ <i>sdhA</i> /pK-hemAB-EFG	This study
DMG ^D	CPC-Sbm Δ <i>iclR</i> Δ <i>sdhA</i> /pK-hemABD-EFG	This study
Plasmids		
pK184	p15A ori, Km ^R , P _{lac} :: <i>lacZ</i> '	(Jobling & Holmes, 1990)
pK-hemABCD	p15A ori, Km ^R , P _{trc} :: <i>hemABCD</i>	This study
pK-hemA	p15A ori, Km ^R , P _{trc} :: <i>hemA</i>	This study
pK-hemAB	p15A ori, Km ^R , P _{trc} :: <i>hemAB</i>	This study
pK-hemABC	p15A ori, Km ^R , P _{trc} :: <i>hemABC</i>	This study
pK-hemABC ^B	p15A ori, Km ^R , P _{trc} :: <i>hemABC</i> - <i>hemC</i> from <i>B. subtilis</i> 168-	This study
pK-hemABD	p15A ori, Km ^R , P _{trc} :: <i>hemABD</i>	This study
pK-hemAB-EFG	p15A ori, Km ^R , P _{trc} :: <i>hemAB</i> , P _{gracmax} :: <i>hemEFG</i>	This study
pK-hemABD-EFG	p15A ori, Km ^R , P _{trc} :: <i>hemABD</i> , P _{gracmax} :: <i>hemEFG</i>	This study
pK-hemABCD-EFG	p15A ori, Km ^R , P _{trc} :: <i>hemABCD</i> , P _{gracmax} :: <i>hemEFG</i>	Lab Stock
pK-hemABD-E	p15A ori, Km ^R , P _{trc} :: <i>hemABD</i> , P _{gracmax} :: <i>hemE</i>	Lab Stock

3.3. Analysis

Culture samples were appropriately diluted using a 0.15 M saline solution to measure cell density in OD₆₀₀ with a spectrophotometer (DU520, Beckman Coulter). All culture samples were washed one time with 0.15 M saline solution prior to OD₆₀₀ absorbance measurement. To prepare cell-free medium, culture samples were centrifuged at 17,000 g for 1 min and then filter sterilized using a 0.2 µm syringe filter. Extracellular metabolites and glycerol were analyzed by high-performance liquid chromatography (HPLC; LC-10AT; Shimadzu) with a refractive index detector (RID; RID-10A; Shimadzu) and a chromatographic column (Aminex HPX-87H; Bio-Rad Laboratories). The HPLC column temperature was maintained at 35 °C and the mobile phase was 5 mM H₂SO₄ (pH 2) running at 0.6 ml/min. The RID signal was analyzed by a data processing software (Clarity Lite, DataApex, Prague, Czech Republic). The concentrations of 5-ALA and PBG in the cell-free medium were determined using an assay with a modified Ehrlich's reagent (Mauzerall & Granick, 1956). Porphyrins were analyzed using an HPLC (2690 separation module, Waters™) with a photodiode array (PDA; 2996 PDA detector, Waters™) detector and a chromatographic column (Chromolith® HighResolution RP-18 endcapped, Supelco). The UV absorbance was measured at 400 nm and the signal was analyzed using a data processing software (Empower 3, Waters™, Milford, USA). A previously described mobile-phase system was employed (Kwon et al., 2003) with minor modifications. Briefly, the mobile phase consisted of two solvent mixtures: solvent A (7.7 g/L ammonium acetate, 125 mL/L acetonitrile, adjusted to pH 5.17 with glacial acetic acid) and solvent B (a methanol-glacial acetic acid mixture with a volume ratio at 10:1) (Kwon et al., 2003). The flow rate of the mobile phase was 1 mL/min. An isocratic elution step was used with 100% solvent A for 5 min, followed by a linear gradient from 0 to 100% solvent B over 30 min, and further followed by 100% solvent B for 5 min. The column temperature was maintained at 45 °C. The percentage yield of UP was calculated as the mole ratio of the

produced UP to the theoretical maximum UP production based on the consumed glycerol, assuming a one-to-sixteen molar ratio (i.e., one mole UP produced per sixteen moles of glycerol consumed). Note that eight moles of succinyl-CoA (derived from 16 moles of glycerol) and eight moles of L-glycine are needed to generate one mole UP. All measurements were performed in duplicate and calculations and data visualizations were performed in Excel.

Chapter 4: Results

4.1. Redirection of carbon flux from the TCA cycle to the C4/Shemin pathway

We implemented the C4/Shemin pathway with heterologous expression of *hemA* from *R. sphaeroides* in the CPC-Sbm Δ *iclR* Δ *sdhA* strain, resulting in the DMA strain. Note that to the deletion of *iclR* and *sdhA* genes, under aerobic conditions, redirects carbon flux towards succinyl-CoA, the precursor of the C4/Shemin pathway. Additionally, we supplemented all the cultures with 2 g/L L-glycine, another precursor of the C4/Shemin pathway, to ensure that the pathway flux was not limited by glycine availability. Both the control strain and DMA were cultivated under aerobic conditions. Unless otherwise noted, all titers reported here are for the final cultivation sample, and the percentage yields are calculated as the ratio of the produced metabolite to the theoretical maximum based on the glycerol consumption.

In contrast to the control strain, which exhibited an uninhibited cell growth characterized by fast glycerol consumption, DMA had an inhibited and slow cell growth accompanied with significant acetogenesis and a decreased glycerol dissimilation rate (Fig. 5A and Fig. 6A). Moreover, DMA exhibited notable production of 5-ALA and PBG, reaching their peak levels at 71 h, with 5-ALA reaching 215 mg/L (22.6 mg/OD₆₀₀) and PBG reaching 130 mg/L (15 mg/OD₆₀₀) (Fig. 6B). Additionally, this production was accompanied by significant pigmentation, resulting in the generation of 55.3 mg/L (0.32% yield) of UP-I and 41.9 mg/L (0.25% yield) of UP-III (Fig. 6C and 6D). These findings provide clear evidence of the successful integration of the C4/Shemin pathway within the cell, achieved by heterologous expression of *hemA*. They also indicate that the carbon flux has been redirected from the TCA cycle toward the heme biosynthesis pathway.

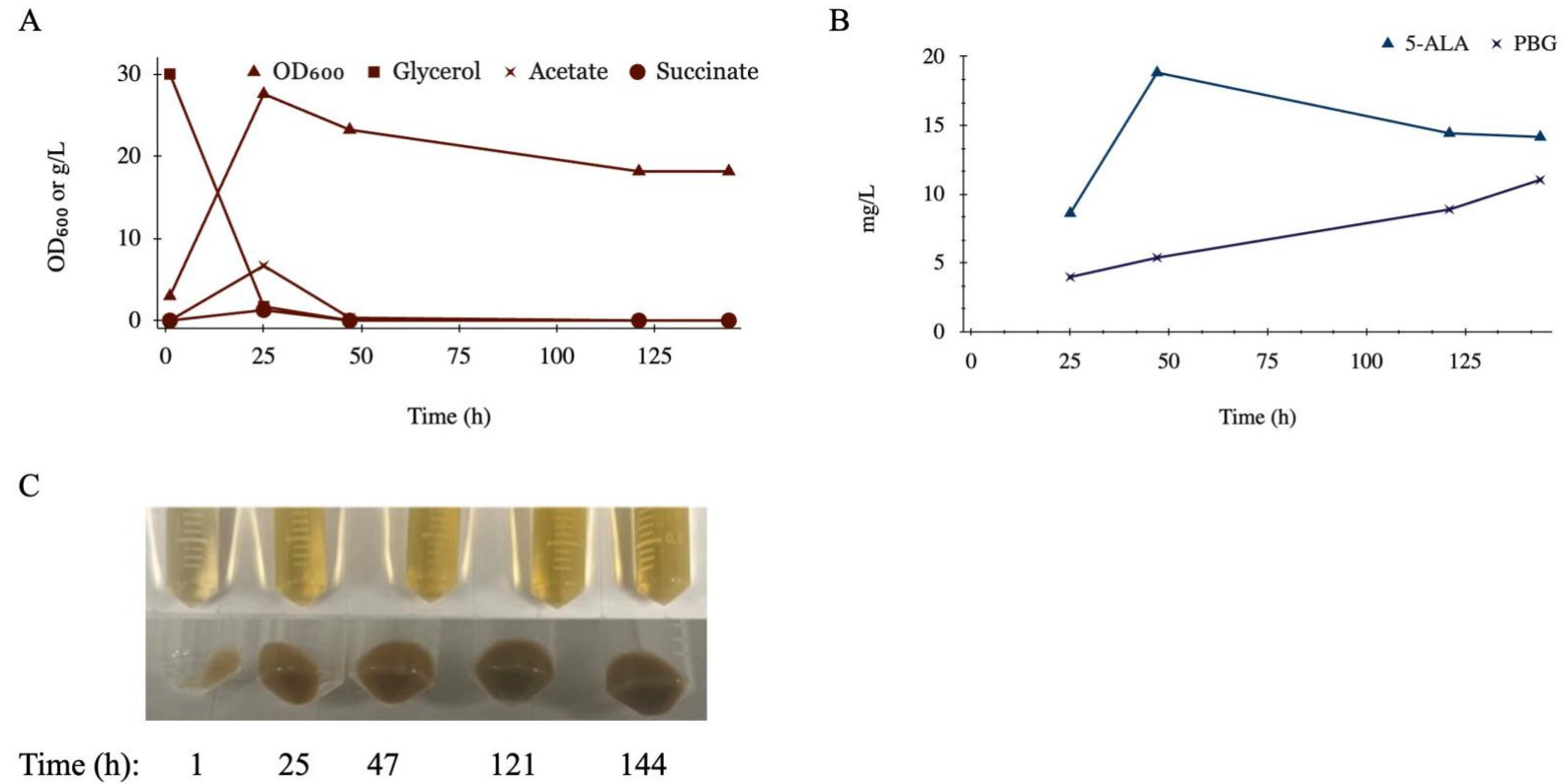


Fig. 5: Aerobic bioreactor cultivation of the control strain. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate production, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points. All values are reported as means (n=2).

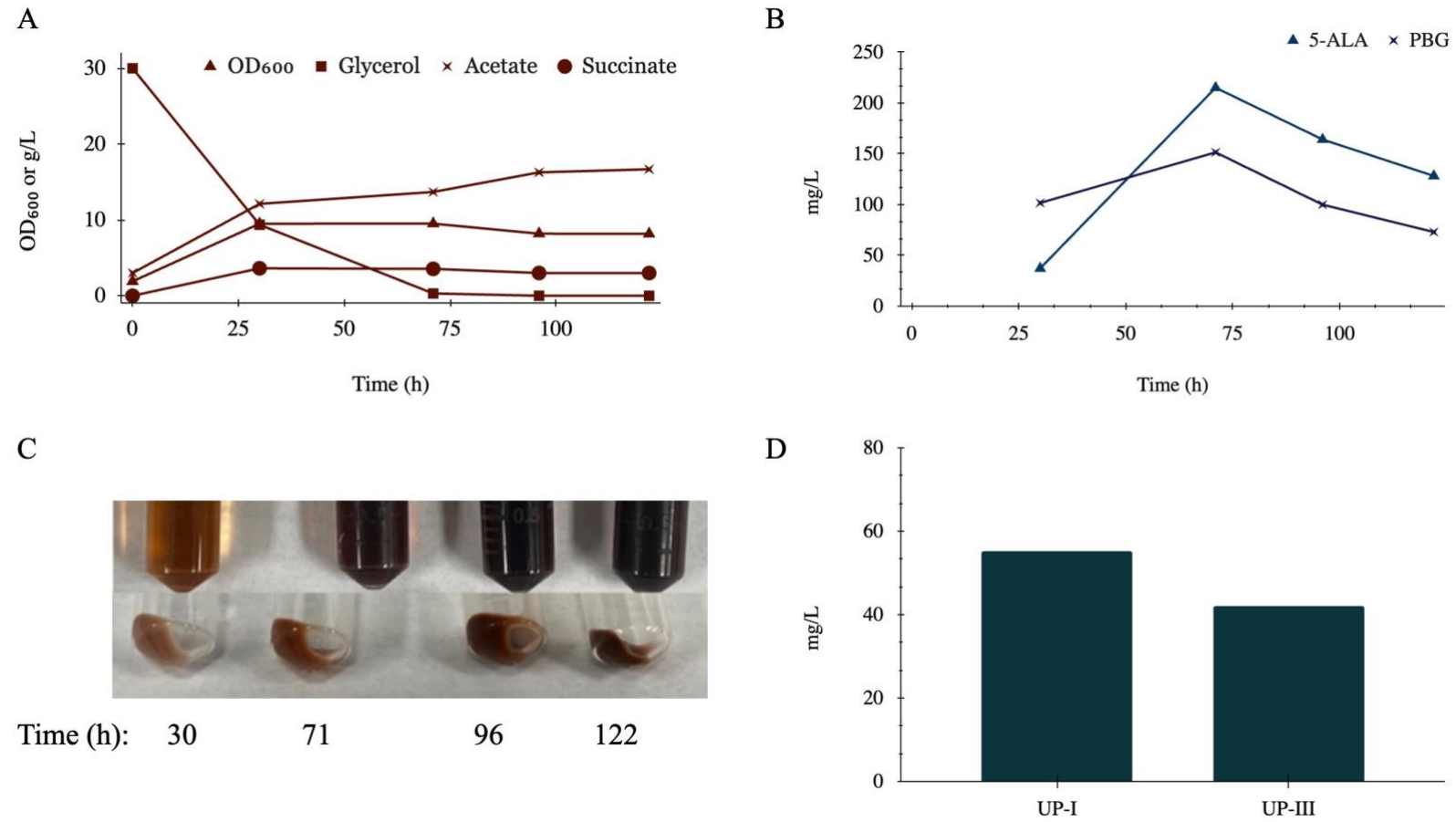


Fig. 6: Aerobic bioreactor cultivation of DMA for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points. (D) Porphyrin biosynthesis. All values are reported as means (n=2).

4.2. Effects of the native HemB overexpression on UP production

Next, to explore the effects of the native HemB on UP production, we generated another control strain, DMB. This strain was constructed by coexpressing *hemB* from *E. coli* and *hemA* from *R. sphaeroides* using a plasmid, pK-HemAB, with single operon regulated by a robust common promoter, *trc*, and an individual strong ribosome binding site (RBS) for each gene. In contrast to DMA, aerobic cultivation of DMB demonstrated a slightly improved biomass yield while exhibiting a reduced glycerol consumption rate, along with a comparable level of acetogenesis (Fig. 7A). However, DMB exhibited significantly elevated production of 5-ALA and PBG, reaching peak levels of 304.3 mg/L (20.9 mg/OD₆₀₀/L) at 144 h and 1995 mg/L (193 mg/OD₆₀₀/L) at 47 h, respectively (Fig. 7B). Furthermore, the DMB strain also appeared to generate significantly higher porphyrin levels as evidenced by intensified pigmentation, with production reaching 555.3 mg/L (3.7% yield) and 346.6 mg/L (2.3% yield) for UP-I and UP-III, respectively (Fig. 7C and 7D). These findings imply that HemB activity could potentially be another bottleneck within the pathway.

4.3. Effects of HemC overexpression on UP production

Subsequently, to investigate the impact of *hemC* overexpression on UP production, two distinct strains were engineered, DMC and DMC^B. In DMC, the native *hemC* gene from *E. coli* was co-overexpressed in conjunction with the native *hemB* from *E. coli* and *hemA* from *R. sphaeroides*. Conversely, in DMC^B, the analogous *hemC* gene from *Bacillus subtilis* 168 (*B. subtilis*) was overexpressed alongside the same set of genes, the native *hemB* from *E. coli* and *hemA* from *R. sphaeroides*. Similar to pK-HemAB, we organized all three genes in a single operon controlled by *trc* promoter with each gene having its own distinct RBS. Surprisingly, the DMC aerobic fermentation displayed behavior highly reminiscent of the control strain, without any pigmentation. In contrast to DMA and DMB, the DMC strain exhibited uninhibited

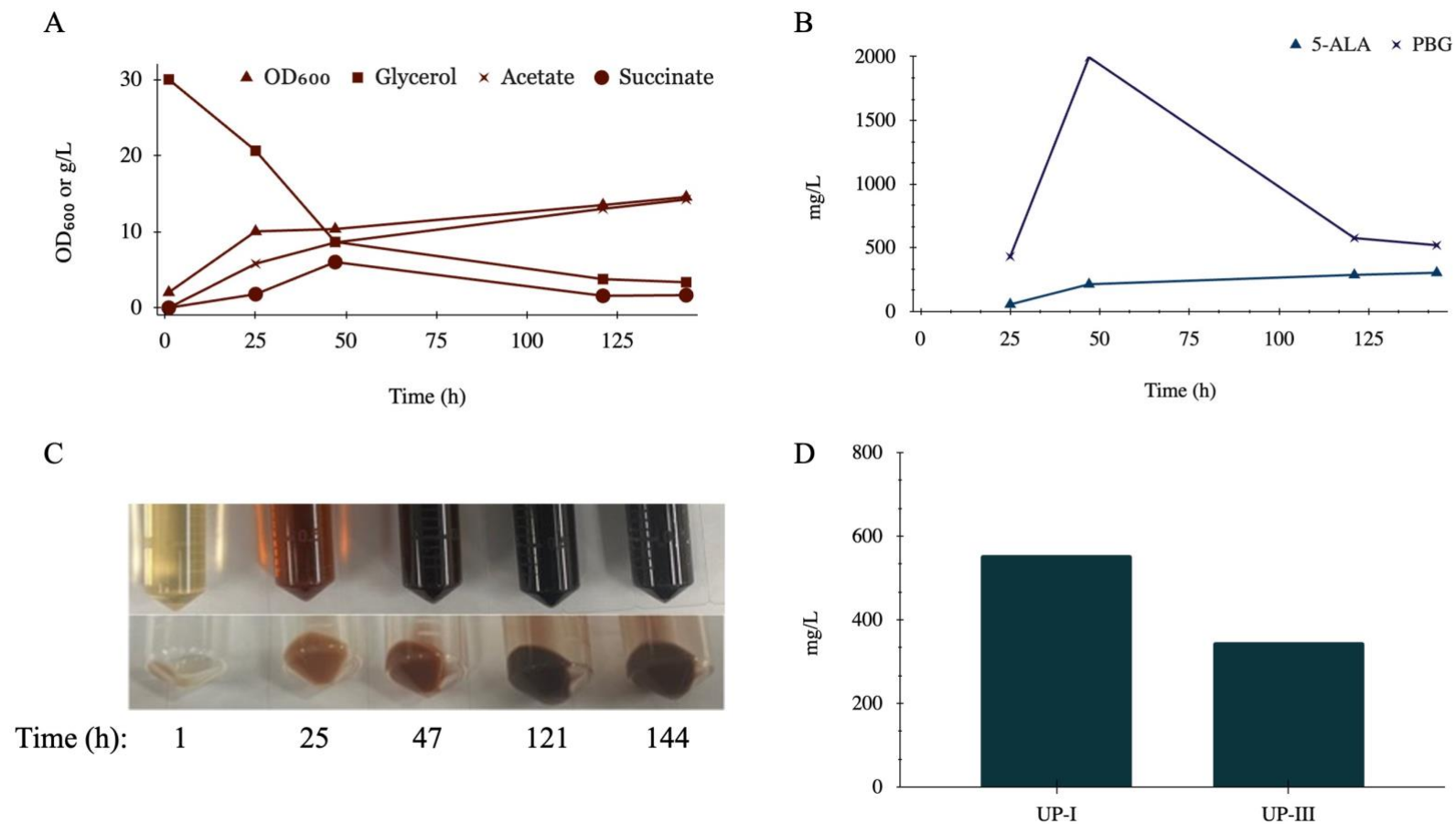


Fig. 7: Aerobic bioreactor cultivation of DMB for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points, (D) Porphyrin biosynthesis. All values are reported as means (n=2).

cell growth, characterized by a fast glycerol consumption rate and only modest levels of acetogenesis. (Fig. 8A). The 5-ALA and PBG production levels in DMC were also similar to those of the control strain and notably lower than the quantities observed in the DMA and DMB strains. DMC only achieved a maximum levels of 14 mg/L (0.84 mg/OD₆₀₀/L) of 5-ALA and 11 mg/L (0.67 mg/OD₆₀₀/L) of PBG at 12 h, a pattern that closely mirrored the levels exhibited by the control strain (Fig. 8B). These findings indicate a disruption in the C4/Shemin pathway, possibly attributable to the overexpression of the native *hemC*.

On the other hand, the aerobic fermentation of DMC^B behaved more similarly to DMB, with significant pigmentation and limited cell growth while producing minimal amount of acetate (Fig. 9A and 9C). This strain also produced 81.2 mg/L (5.25 mg/OD₆₀₀/L) of 5-ALA and 108.3 mg/L (7 mg/OD₆₀₀/L) of PBG at 142 h (Fig. 9B). It should be noted that the significant PBG accumulation observed in DMB was not present in DMC^B, showing the overexpression of *hemC* from *B. subtilis* resulted in the efficient conversion of PBG to HMB (Fig. 7B and 9B). Additionally, DMC^B yielded UP-I and UP-III at notably reduced levels compared to DMB, peaking at 200.4 mg/L (1.18% yield) and 135 mg/L (0.8% yield), respectively (Fig. 9D). These findings further support the idea that *hemC* overexpression has detrimental effects on UP biosynthesis.

4.4. Effects of the native HemD and different aeration conditions on UP production

To further explore the impact of HemD on UP biosynthesis under varying oxygen levels, we employed the pK-HemABD plasmid, which shares the identical construct as pK-HemABC. This plasmid was used for the transformation of both CPC-SbmΔ*iclR*Δ*sdhA* and CPC-SbmΔ*sdhA* strains, resulting in the generation of DMD and SMD strains, respectively. We did not include *hemC* as our results showed that its overexpression has a negative effect on the C4/Shemin pathway. During aerobic cultivation, DMD exhibited a growth pattern and glycerol

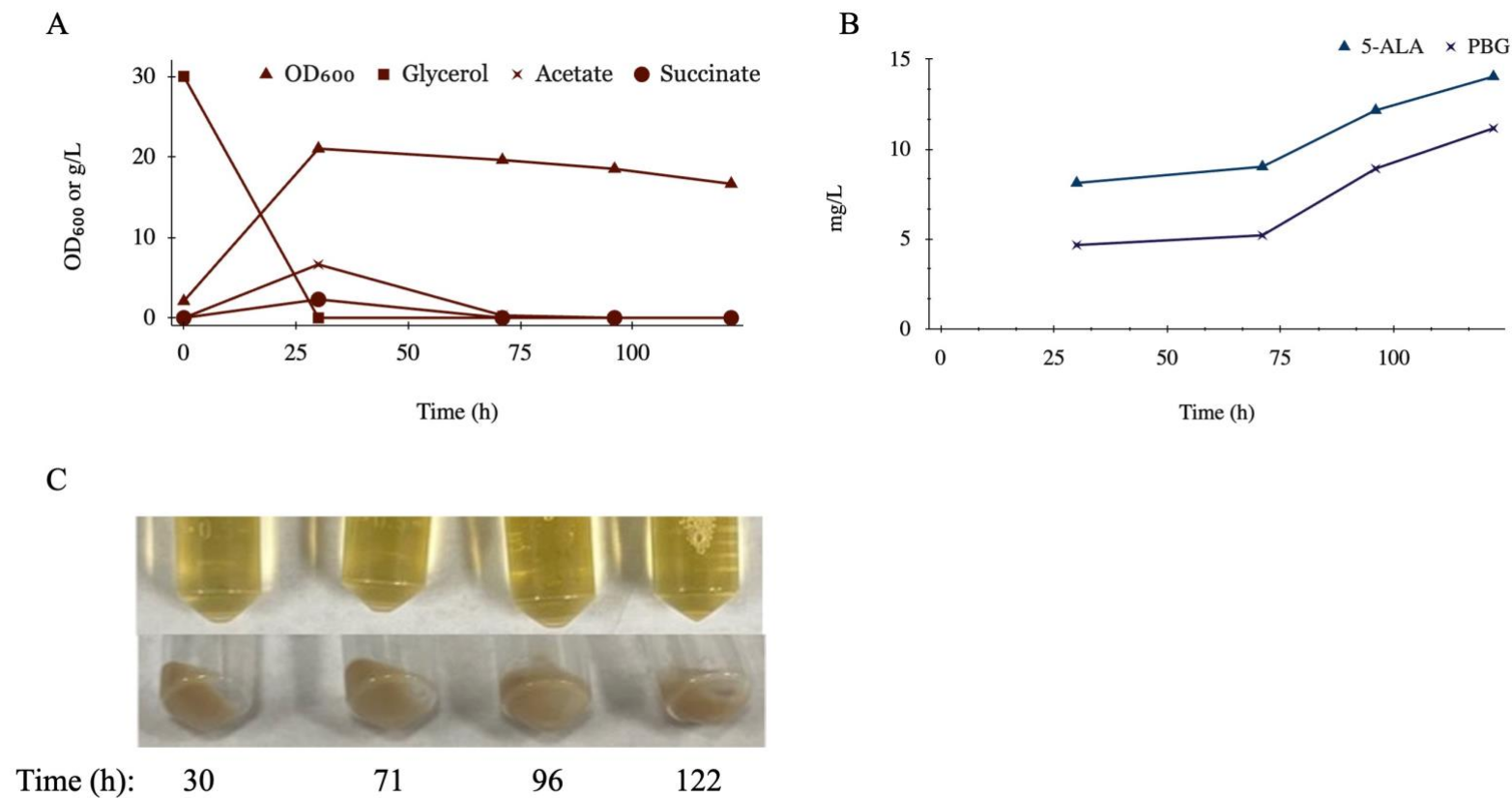


Fig. 8: Aerobic bioreactor cultivation of DMC for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points. All values are reported as means (n=2).

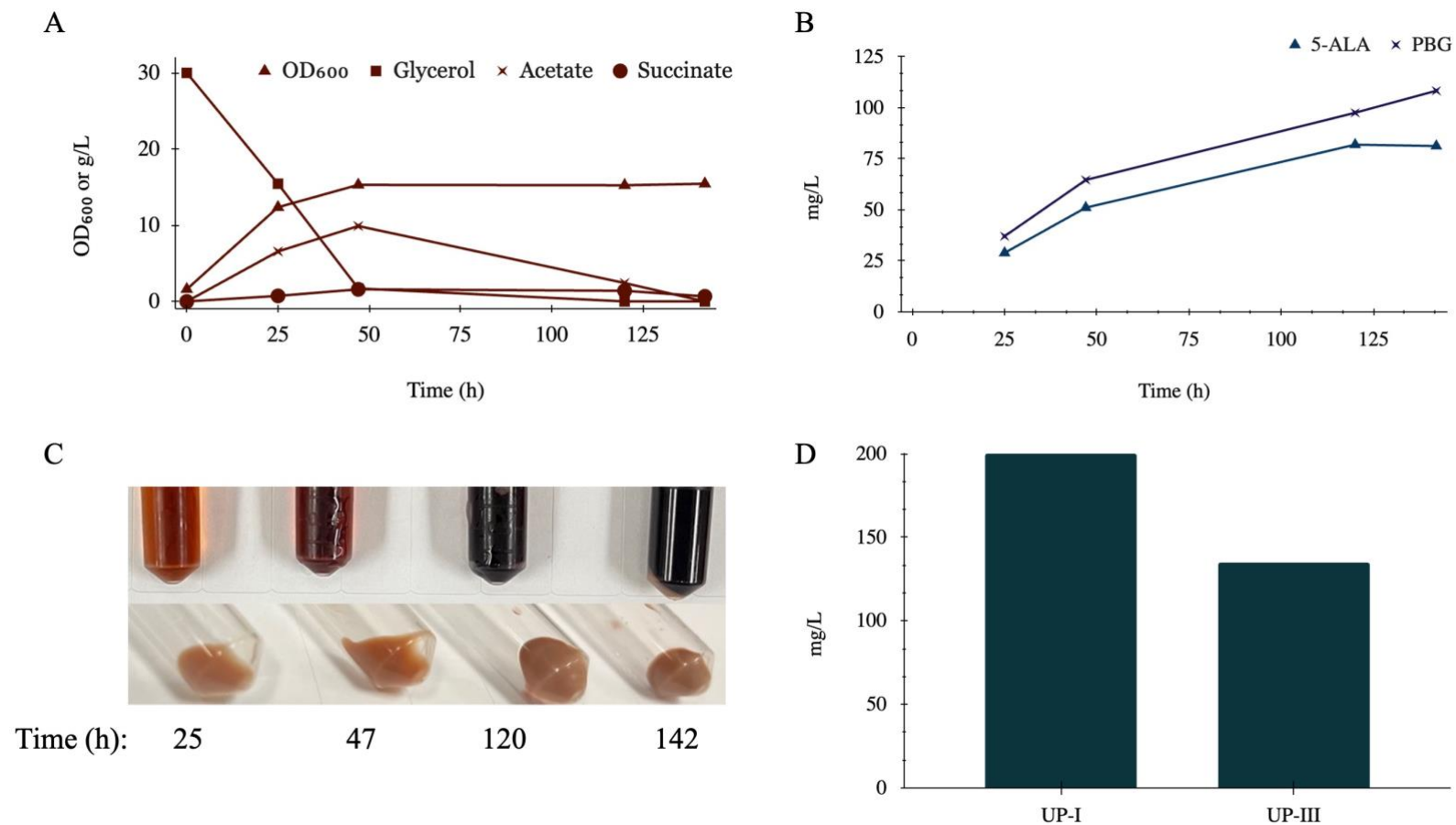


Fig. 9: Aerobic bioreactor cultivation of DMC^B for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points. (D) Porphyrin biosynthesis. All values are reported as means (n=2).

consumption rate closely resembling that of DMB, accompanied by notable acetogenesis (Fig. 10A). Remarkably, DMD achieved substantial production of 5-ALA and PBG, with peak levels of 257.6 mg/L (19.6 mg/OD₆₀₀/L) at the end of the fermentation and 1472 mg/L (112 mg/OD₆₀₀/L) at 57 h, respectively (Fig. 10B). Similar to its pigmented counterpart, DMB, DMD generated a total of 793.7 mg/L of UP, comprising 430.7 mg/L UP-I and 363 mg/L UP-III, with respective yields of 2.54% and 2.14% (Fig. 10C and 10D). Interestingly, the similarity in the distribution of UP-I and UP-III between DMD and DMB suggests that the overexpression of *hemD* did not result in a substantial improvement in the metabolic flux towards the classical pathway (Fig. 15B). These results imply that, despite the overexpression of the *hemD* gene, the efficiency of the autoxidation reaction in converting HMB seemed to surpass that of the enzymatic reaction catalyzed by HemD.

In the next step, to explore the impact of oxygen availability on both autoxidation rate and porphyrin distribution, we cultivated the SMD strain under aerobic conditions for the first 30 h to support cell growth during the early stages of the fermentation. It's worth highlighting that our findings indicated that the majority of porphyrin biosynthesis occurred following the addition of L-glycine at the 30 h (Data not shown). Subsequently, in one experiment, we switched to microaerobic conditions, while in the other, we transitioned to anaerobic conditions. In our SMD fermentation experiments, we observed a similar culture performance during the initial 30 h in comparison to DMD aerobic cultivation (Fig. 11A and 12A). Nevertheless, it is important to note that limiting the oxygen availability had a discernible impact on the metabolic activity associated with UP production. In contrast to DMD, SMD produced only 314.9 mg/L UP-I (yield 1.86%), 171.7 mg/L UP-III (yield 1.01%), and 47.28 mg/L (0.35% yield) CP-III under microaerobic conditions, and 37.1 mg/L UP-I (yield 0.22%), 4.78 mg/L UP-III (yield 0.02%), and 19.6 mg/L CP-III (yield 0.14%) under anaerobic conditions, along with considerably lower levels of 5-ALA and PBG (Figs. 11B-D and 12B-D).

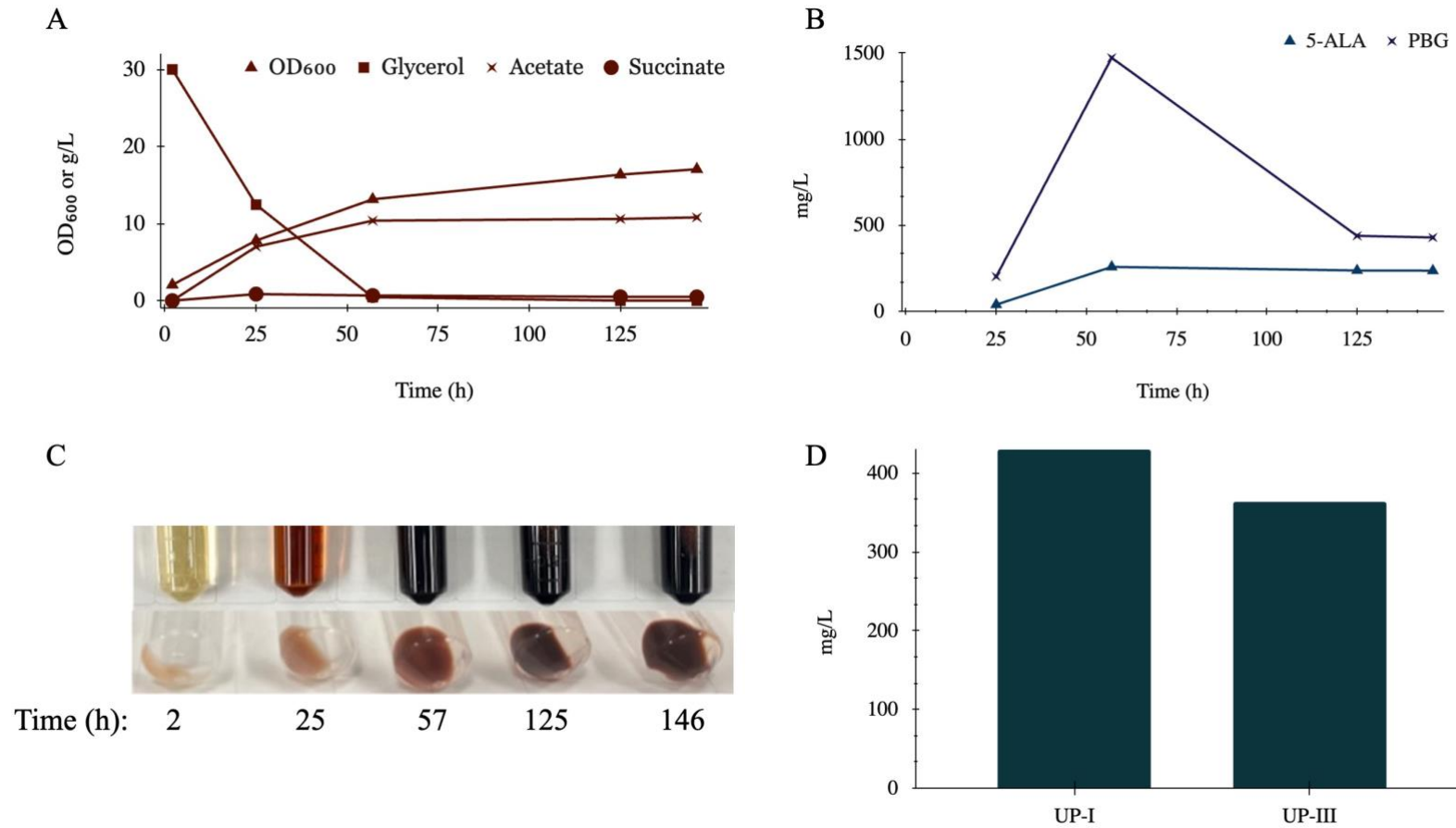


Fig. 10: Aerobic bioreactor cultivation of DMD for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points, (D) Porphyrin biosynthesis. All values are reported as means (n=2).

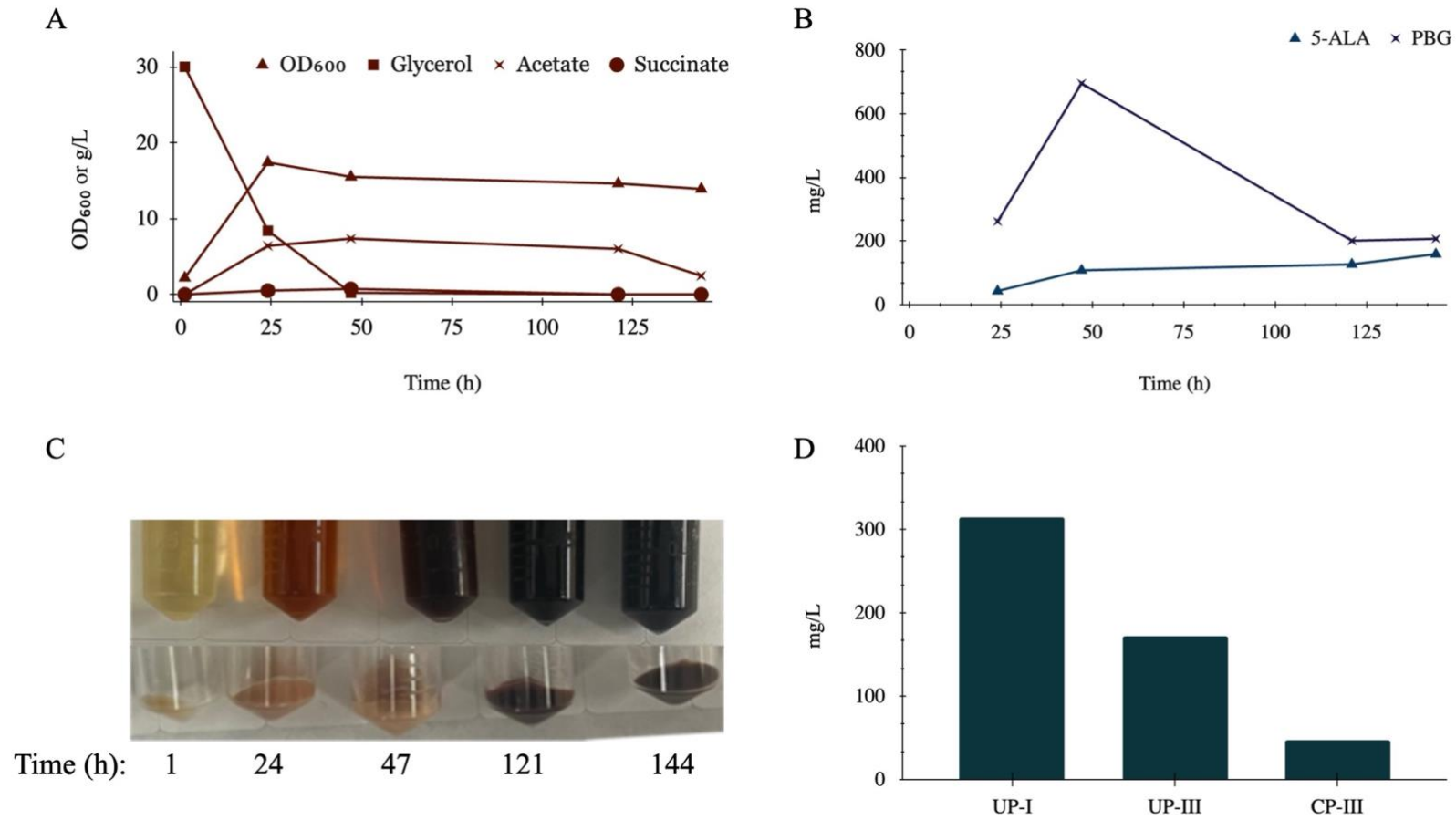


Fig. 11: Aerobic bioreactor cultivation of SMD for porphyrin biosynthesis under microaerobic conditions. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points, (D) Porphyrin biosynthesis. All values are reported as means (n=2).

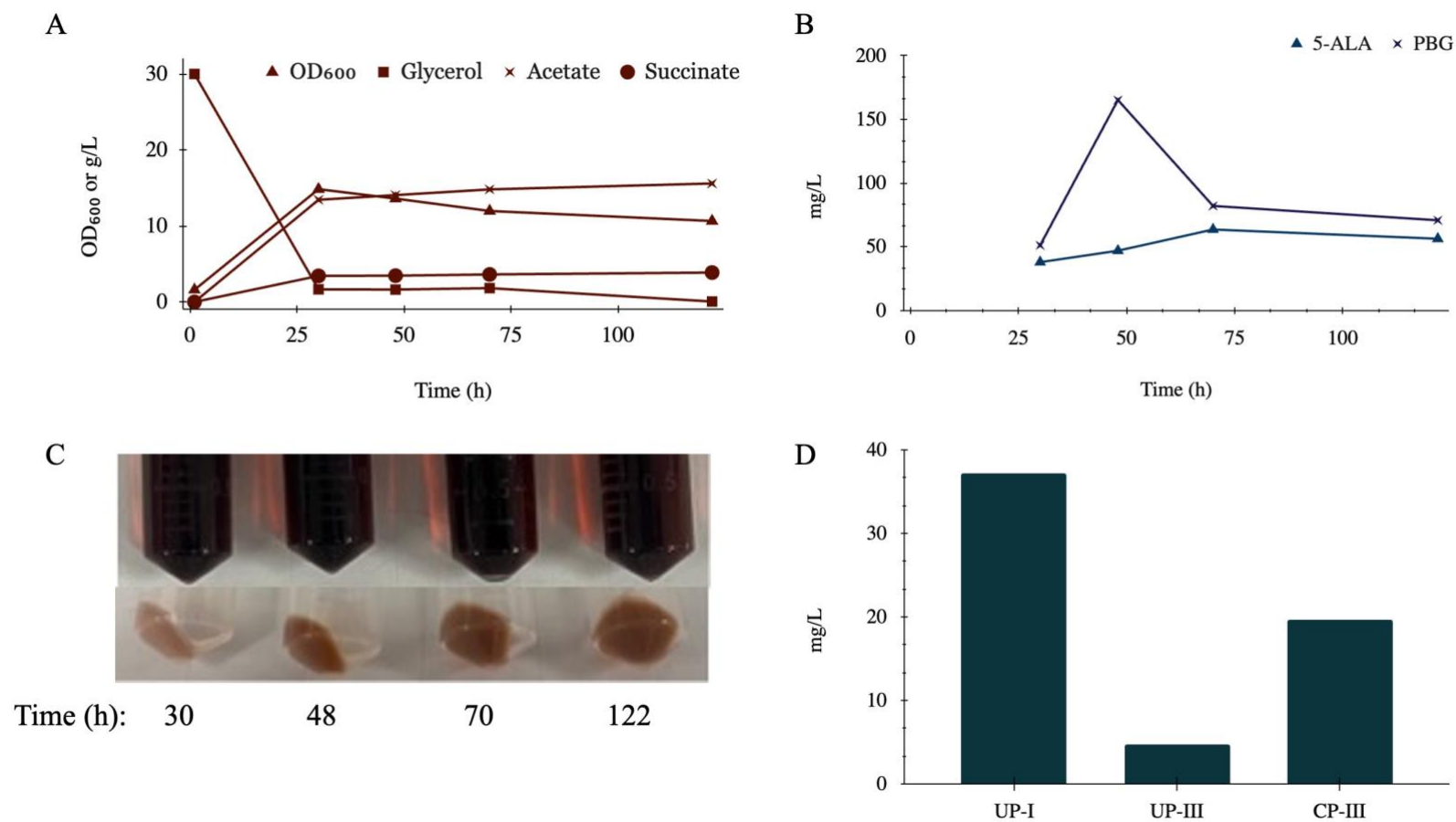


Fig. 12: Aerobic bioreactor cultivation of SMD for porphyrin biosynthesis under anaerobic conditions. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points, (D) Porphyrin biosynthesis. All values are reported as means (n=2).

These findings suggest that the decrease in oxygen levels did not alter the distribution of UP and had a detrimental impact on the overall process of UP biosynthesis.

4.5. Redirection of carbon flux to PP-IX biosynthesis

The outcomes of the fermentation involving the DMD strain indicated a preference for the conversion of UPG-I to UP-I and UPG-III to UP-III, rather than their conversion to CPG-I and CP-III, respectively. This observation raised the possibility that the activity of HemE could represent an additional limitation within the heme biosynthesis pathway. This hypothesis was subsequently confirmed by the results obtained from the DME strain, which involved the overexpression of *hemA*, *hemB*, *hemD*, and *hemE*, revealing higher titers of CP-I and CP-III compared to UP-I and UP-III, respectively (Data not shown). Similarly, the results from the DME strain fermentation (not shown) also hinted at HemF (Coproporphyrinogen III oxidase) as another potential bottleneck within the pathway. Hence, we derived another plasmid, pK-HemAB-EFG, for co-overexpression of *hemA* from *R. sphaeroides* along with the native *hemB*, *hemE*, *hemF*, and *hemG* in DMG strain to explore the PP-IX production performance in this strain. Note that to ensure efficient expression of the genes, *hemA* and *hemB* were aligned to form a *hemAB* operon under the control of the strong promoter, *trc*, and *hemE*, *hemF*, and *hemG* were aligned to form a *hemEFG* operon controlled by another strong promoter, *gracmax*. It should also be noted that we did not include *hemD* as our results hinted that its overexpression does not substantially change the carbon flux towards the classical pathway.

The aerobic cultivation of DMG displayed robust growth following a diauxic pattern, with glycerol as the primary carbon source and acetate as the secondary (Fig. 13A). However, the DMG culture exhibited distinctive traits, including diminished pigmentation and reduced production of 5-ALA and PBG in comparison to the DMB strain. Specifically, DMG achieved peak concentrations of 57.2 mg/L (2.73 mg/OD₆₀₀/L) for 5-ALA and 201.1 mg/L (9.61 mg/OD₆₀₀/L) for PBG (Fig. 13B). The reduced pigmentation observed in the DMG strain could

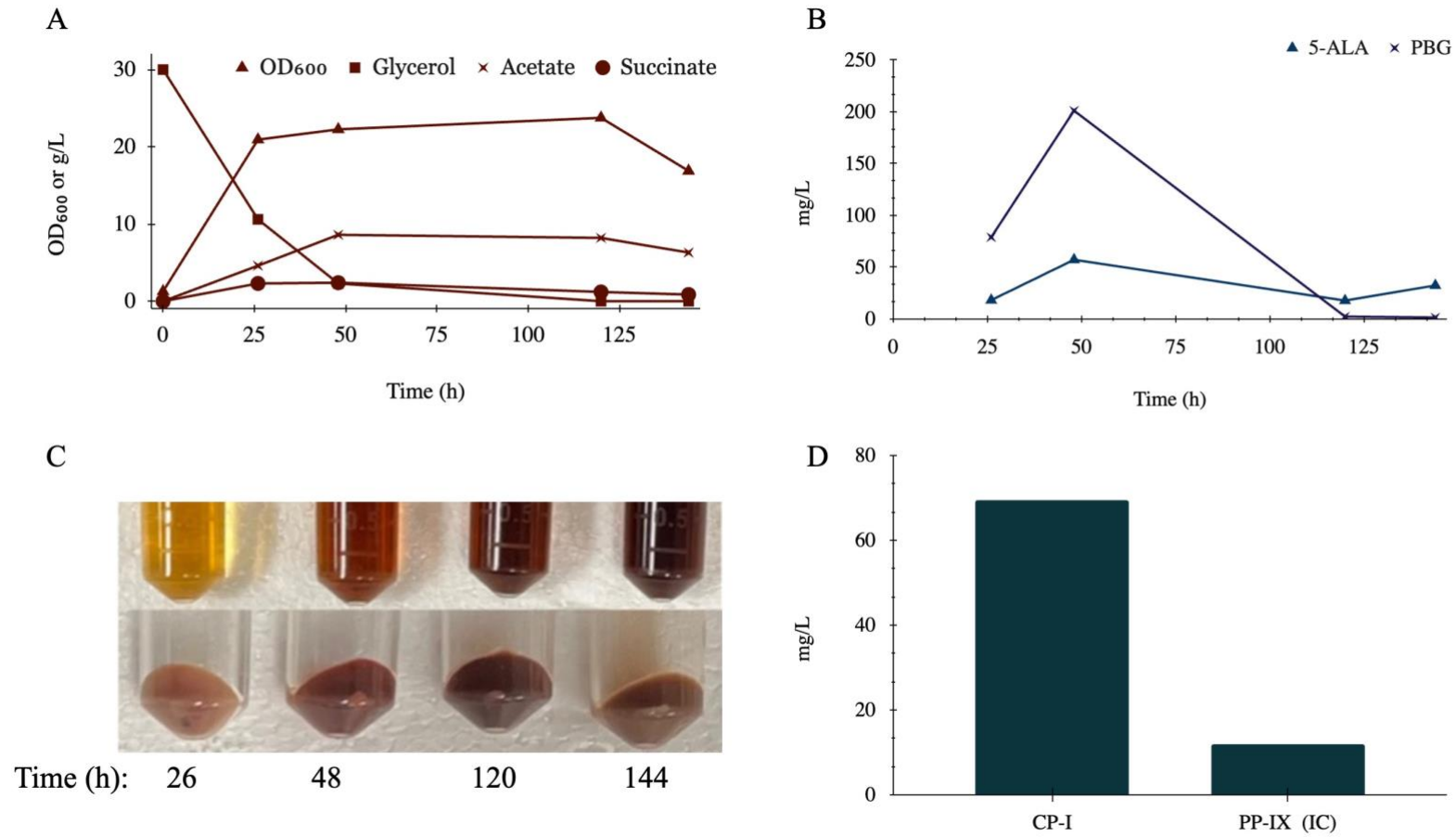


Fig. 13: Aerobic bioreactor cultivation of DMG for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points, (D) Porphyrin biosynthesis. Abbreviation: IC; Intracellular. All values are reported as means (n=2).

be attributed to its limited porphyrin output, which was only 69.4 mg/L (0.6% yield) of CP-I and 11.8 mg/L (0.13% yield) of PP-IX (Fig. 13C and D). Notably, while DMG predominantly secreted CP-I, the majority of PP-IX was detected within the intracellular environment (Fig. 13D). This finding implies that *E. coli* does not efficiently secrete PP-IX. Furthermore, our results unveiled a substantial contrast in carbon flux between the classical and non-classical pathways (by comparing CP-I and PP-IX titers), indicating a preference for the non-classical pathway within the DMG strain over the classical pathway (Fig. 15B).

4.6. Effect of the native HemD overexpression on PP-IX production

Next, to explore the effects of HemD overexpression on PP-IX production, we integrated the native *hemD* gene into the *hemAB* operon, resulting in pK-HemABDEFG plasmid, to engineer another PP-IX producing strain, DMG^D. While aerobic cultivation of DMG^D resembled DMG culture in terms of cell growth, and 5-ALA and PBG production (Fig. 14A and B), there was a marked improvement in pigmentation (Fig. 14C). DMG^D demonstrated a substantial enhancement in PP-IX production, reaching a yield of 64.8 mg/L (0.63% yield) as illustrated in Fig. 14D. Nonetheless, it is noteworthy that 54.7 mg/L of the total PP-IX produced was retained intracellularly (Fig. 14D), indicating an ineffective secretion for PP-IX in *E. coli*. This observation aligns with the findings observed in the DMG fermentation. Additionally, the DMG^D strain exhibited a porphyrin production of 76.4 mg/L through the classical pathway, while the non-classical pathway yielded 33.6 mg/L (Fig 15). This porphyrin distribution, in contrast to the opposite distribution observed with the DMG strain, highlights the classical pathway's dominance upon introduction of the *hemD* gene in DMG^D.

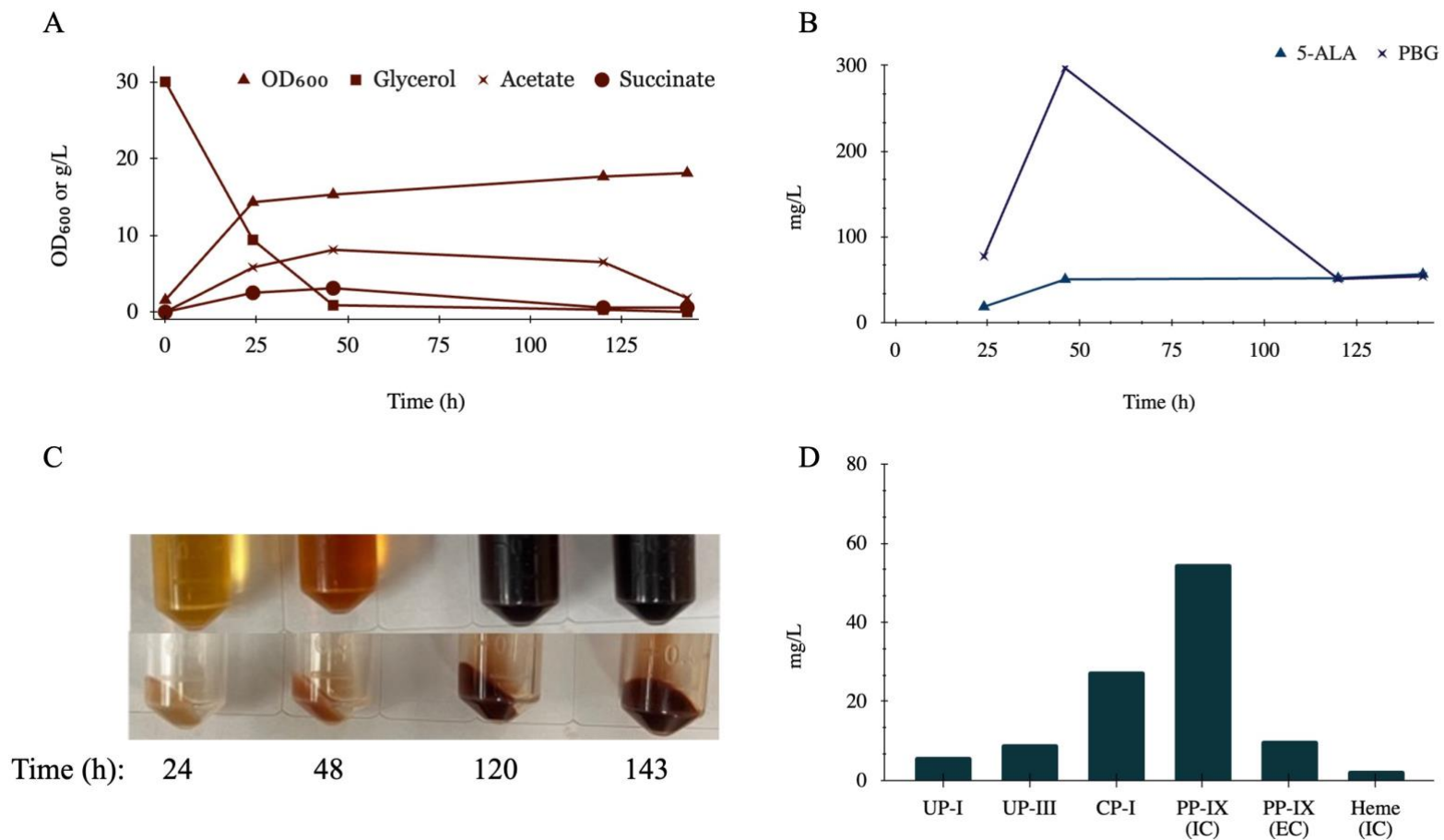


Fig. 14: Aerobic bioreactor cultivation of DMG^D for porphyrin biosynthesis. Time profiles of (A) cell density (OD₆₀₀), glycerol consumption, and acetate/succinate formation, (B) 5-ALA and PBG biosynthesis, and (C) Image of CFM and cell paste samples at different time points, (D) Porphyrin biosynthesis. Abbreviation: IC; Intracellular, EC; Extracellular. All values are reported as means (n=2).

Chapter 5: Discussion

In this study, we enhanced the biosynthesis of UP and PP-IX in *E. coli*. First, we implemented the C4/Shemin pathway in CPC-Sbm Δ *iclR* Δ *sdhA* with a redirected carbon flux from the TCA cycle. The introduction of *hemA* through heterologous expression in DMA led to a substantial increase in the levels of 5-ALA and PBG relative to that produced by the control strain. This enhancement was accompanied by pronounced culture pigmentation and significant UP formation. These findings collectively imply that within the genetic context of DMA, the bottleneck in UP biosynthesis might lie downstream of the 5-ALA formation step. The implementation of the C4/Shemin pathway also resulted in significant acetogenesis, which reduced the available carbon for UP biosynthesis and impacted cell growth/physiology, similar to previous observations (Lall et al., 2021a; D. Miscevic et al., 2021). Additionally, DMA produced a higher level of UP-I than UP-III, indicating that the HMB autoxidation conversion (under the aerobic conditions) was favored over the enzymatic conversion by HemD.

Second, we overexpressed the native *hemB* along with *hemA* to further increase the carbon flux within the pathway. Compared to DMA, the DMB strain showed a significant increase in maximum PBG titer accompanied with intensified pigmentation and UP formation (Fig. 15A). This suggests that in addition to HemA, HemB is another bottleneck in the heme biosynthesis pathway. Additionally, the enhanced pigmentation and UP production observed in DMB compared to DMA suggests that the genomic *hemC* was actively expressed in this strain, helping to maintain flux through the biosynthetic pathway. This aligns with previous findings by Davinder et al., where repression of *hemC* expression was required to enable accumulation of the intermediate PBG (Lall et al., 2021b). In addition, similar to the DMA strain, the higher production of UP-I compared to UP-III in DMB indicates that the autoxidation of HMB was preferred over the enzymatic

conversion catalyzed by HemD (Fig. 15B). This again suggests the autoxidation reaction was more active than the HemD-catalyzed step under the aerobic conditions tested.

In addition, our research findings indicate that *hemC* exhibits active expression from the *E. coli* genome. However, it is noteworthy that an unexpected outcome was observed when the native *hemC* gene was overexpressed. Specifically, a complete disruption was noted in the C4/Shemin pathway. It should be noted that this disruption resulted not only in the absence of pigmentation but also in the production of significantly reduced levels of 5-ALA and PBG, which aligns the observation from the control strain. This intriguing observation strongly suggests that the overexpression of *hemC* likely played an important role in deactivating the C4/Shemin pathway. This outcome could potentially be attributed to the suppressive impact of elevated *hemC* expression on the metabolic processes or gene transcription associated with the C4/Shemin pathway. Further research is required to reveal the underlying cause of the *hemC* overexpression effects on the C4/Shemin pathway.

Moreover, we found that non-enzymatic autoxidation of HMB to UPG-I was more efficient compared to the enzymatic HemD-mediated conversion to UPG-III. This indicates that autoxidation is likely the primary biosynthetic route for HMB conversion under the conditions tested in this study. We further showed that modulating *hemD* overexpression and aeration did not improve total UP yields or the UP-III/UP-I ratio (Fig. 15B). This suggests that neither the overexpression of *hemD* nor limitations in oxygen availability resulted in a redirection of carbon flux towards the classical pathway. Nevertheless, the overexpression of the native *hemE* gene resulted in a notable increase in the activity of the classical pathway compared to the non-classical pathway (data not shown). This observation raises the possibility of a potential repression of *hemD* activity coinciding with the accumulation of UP/UPG. Further research should investigate if

HemD activity is somehow altered with UP/UPG accumulation. Furthermore, the conversion of UPG-III was favored to CPG-III over UP-III (evidenced by higher CP-III titer than UP-III) under limited oxygen availability (Fig. 15B). This raises the possibility that oxygen availability impacts either the activity of the HemE enzyme, the autoxidation of UPG-III, or both. Further studies should explore if low oxygen level directly impacts HemE enzyme activity or UPG-III autoxidation rate, or if both factors are at play.

While we discovered that *hemD* overexpression did not significantly change the preferential conversion of HMB for UP production, we did observe a notable shift when examining PP-IX biosynthesis under these conditions. Our findings revealed that in the absence of *hemD* overexpression, a minimal quantity of PP-IX was generated, with the majority of carbon flux directed towards the non-classical pathway, leading to the formation of CP-I (Fig. 15A and 15B). In contrast, the concurrent overexpression of *hemD*, alongside other essential genes for PP-IX production, yielded a significantly enhanced PP-IX titer. This provides further support to the hypothesis suggesting the repression of HemD activity by UP/UPG accumulation. Additionally, most of the produced PP-IX was accumulated inside the cell suggesting an ineffective efflux mechanism for PP-IX in *E. coli*. Furthermore, the PP-IX producing strain, DMG^D, yielded considerably lower porphyrin titer than the UP-producing strain which can be attributed to the downregulation of the pathway by PP-IX and heme (Choby & Skaar, 2016; Zhang et al., 2015). Interestingly, overexpression of the MacAB-TolC, the complex that has been attributed to PP-IX efflux, facilitated PP-IX efflux and increased its production by more than 10 times in shake flask (Data not shown). However, this strain showed a completely different behavior in bioreactor and did not produce any porphyrin. Further research should reveal the underlying reasons.

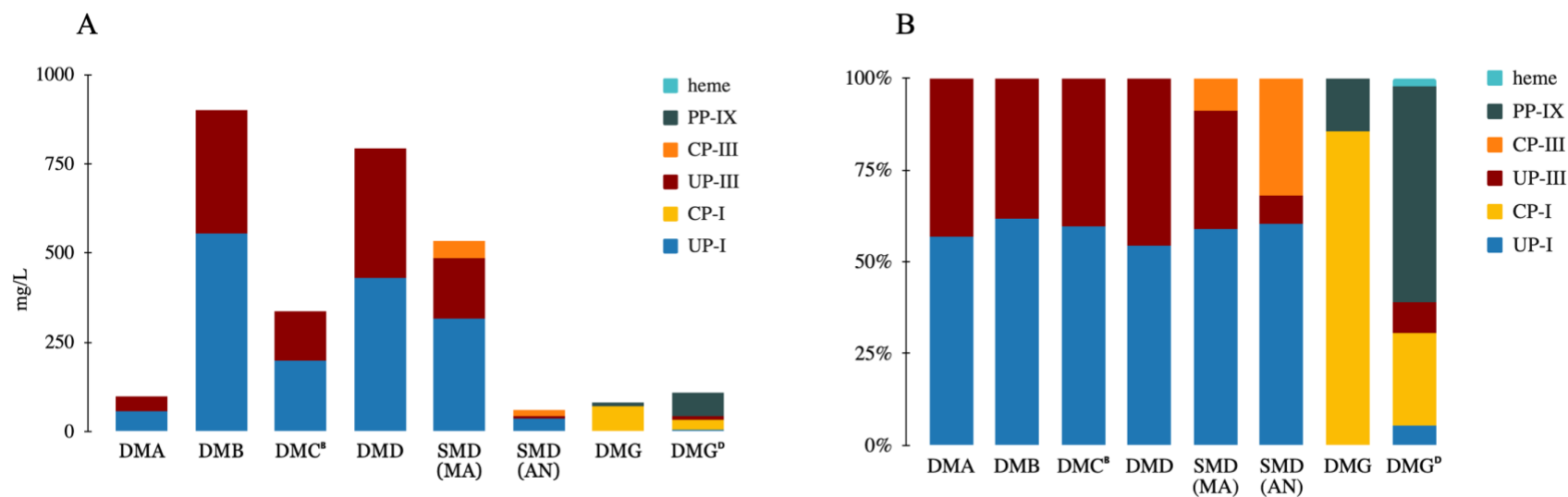


Fig. 15: Porphyrin production profiles of the engineered strains. A: provides a breakdown of the total porphyrin production within each strain. B: provides a comparative view, showing the relative proportion of each porphyrin produced in each strain. Abbreviations: MA: microaerobic, AN: anaerobic.

Chapter 6: Conclusion

In conclusion, this study demonstrates several important insights into UP and PP-IX bio-based production enhancement in *E. coli*. Building upon our findings, we have sequentially identified constraints on the tetrapyrrole biosynthesis pathway to reveal the next bottleneck and, ultimately, improve UP and PP-IX production. Implementing the C4/Shemin pathway with *hemA* overexpression enabled carbon flux redirection to the tetrapyrrole biosynthesis pathway. Additionally, *hemB* co-overexpression further enhanced flux through the pathway resulted in much higher UP titer, indicating *hemB* as another bottleneck. However, *hemC* overexpression unexpectedly disrupted the C4/Shemin pathway, suggesting possible repression or inhibition.

Interestingly, for UP production, the non-enzymatic route via HMB autoxidation was preferred over the HemD-catalyzed reaction, even with *hemD* overexpression. However, for PP-IX biosynthesis, *hemD* overexpression was essential to redirect flux through the classical pathway. Furthermore, the PP-IX-producing strain exhibited a markedly lower porphyrin yield compared to the UP-producing strain, which can be attributed to the downregulation of the pathway resulting from the accumulation of PP-IX and heme within the cell. This deduction finds support in the observed increase in porphyrin production in the PP-IX-producing strain when PP-IX efflux was enhanced.

In summary, this study provides valuable insights into metabolic engineering strategies for improving UP and PP-IX production in *E. coli*. Key bottlenecks and pathway regulation mechanisms were identified. Future work should further investigate the apparent inhibition of the C4/Shemin pathway by *hemC* overexpression and repression of HemD activity by UP/UPG accumulation. Also, further metabolic and bioprocess engineering approaches should be

implemented to address the pathway downregulation by porphyrin. Overcoming these limitations could further enhance UP and PP-IX production in *E. coli*.

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Appendix

Table S1: Oligomers used in this study.

Primers/oligo name	Primer/oligo sequence (5' → 3')
P001	AAGGAGGAATAGAAAATGGACTACAATCTGGCACTCG
P002	TTTCGTACCTCCTTTGCTAGCTCAGGCAACGACCTCGGC
P003	CTAGCAAAGGAGGTACGAAATATGACAGACTTAATCCAACGC
P004	ATGTAAATTTCTCCTTTGGTACCTTAACGCAGAATCTTCTTCTCAG
P005	CCAAAGGAGGAAATTTACATGTTAGACAATGTTTTAAGAATTGCCAC
P006	TAATAATCCTCCTTTGCATGCTCATGCCGGGGCGTCTCC
P007	CATGCAAAGGAGGATTATTATATGAGTATCCTTGTCACCC
P008	CTGCGGATCCTTATTGTAATGCCCGTAAAAGC
P009	ATTACAATAAGGATCCGCAGCCCGCCTAATG
P010	TCCATTTTCTATTCTCCTTTAATTGTTATCCGCTC
P011	GGATCCGCAGCCCGCCTAATG
P012	TCAGGCAACGACCTCGGCG
P013	TTAACGCAGAATCTTCTTCTCAGCC
P014	CCCGGCATGAGGATCCGCAGCCCGCCTAATGAGC

P015	CTGCGGATCCTCATGCCGGGGCGTCTCCGTTATAG
P016	CCAAAGGAGGAAATTTACATGATGAGAACGATTAAAGTAGG
P017	CTGCGGATCCTCATTTTCCATCCTCGTCAAGC
P018	TGGAATGAGGATCCGCAGCCCGCCTAATGAGC
P019	GTCCATTTTCTATTCTCCTTTAATTGTTATCCGC
P020	TACCAAAGGAGGAAATTTACATGAGTATCCTTGTCACCCG
P021	GTCCATTTTCTATTCTCCTTTAATTGTTATCCG
P022	AAGGAGGAATAGAAAATGGACTACAATCTGGCACTCG
P023	TTAAGTTCGGTCATTATTGATTCCTCCTTTAATTGG
P024	GGAATCAATAATGACCGAACTTAAAAACGATCG
P025	GTCCATTTTCTATTCTCCTTTAATTGTTATCCG
P026	GGAAGCGGAAGAGCGCCCAATACGC
P027	CATTCTTTTCGGATGTGGTACCTTAACGCAGAATCTTCTCTCAGCC
P028	TCTGCGTTAAGGTACCACATCCGAAAAGAATGATGGATCAC
P029	CCAGTGCGATCAGGAAGGACA
P030	TCCTTCCTGATCGCACTGGCGCAG
P031	ATTGGGCGCTCTTCCGCTTCC