university of groningen

Cyanobacteria as Cell Factories for the Sustainable Production of Indigo from CO_2

Review Article

Thesis, BSc Life Sciences & Technology

Iris Gormezano Kasuto
Supervised by Marco W. Fraaije

University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands
Submitted 10th of July 2024

Abstract

Concrete evidence of the effects of climate change in recent years has encouraged a transition from the petrochemical paradigm to a circular economy model by developing more sustainable industrial processes. This review discusses the viability of using cyanobacteria as cell factories to replace the traditional indigo dye synthesis that is environmentally harmful. These biofactories could utilize carbon dioxide, mitigating the need for an organic source of carbon, that is in competition with other processes. Recent advancements in greener approaches are discussed, and a promising bifunctional enzyme that converts L-tryptophan to indigo is highlighted since it was the most productive. The review then delves into the potential of cyanobacteria as industrial workhorses, and the current state of genetic and metabolic engineering strategies for these organisms. Recent progress in developing molecular tools for cyanobacteria, including promoters, terminators, and gene regulation systems, is outlined. Finally, a theoretical route from carbon dioxide to indigo is proposed based on the gathered literature. This approach could potentially lead to carbon-negative indigo production, contributing to a circular economy model and demonstrating the broader applicability of cyanobacteria as prokaryotic photoautotrophs in the green production of other chemicals. The challenges and future directions for optimizing this process are mentioned, emphasizing the need for more studies.

Keywords: Climate change, Circular economy, Biobased Revolution, Cell Factory, Cyanobacteria, Indigo, L-Tryptophan

1. Climate Change & the Necessity for a Biobased Revolution:

While climate change is seen to occur throughout history, evidence of anthropogenically induced rise in temperature has been gathered since ~ the 1970s. As the demand for industrial production increases with the world population, the traditional linear economy model, (produce, consume, dispose) basing production on the fastest or cheapest petrochemical process, threatens global biosustainability. Solid waste accumulating, energy production through fossil fuels; and the emission of greenhouse gases (GHGs) like carbon dioxide or methane, lead to the absorption and trapping of heat in the atmosphere. Importantly, as marine, terrestrial, and freshwater environments are altered, the mitigation of the effects will be less possible, since biodiversity within ecosystems is essential for limiting climate variance and sequestering carbon. For example, the destruction of parts of the Amazon rainforest due to deforestation might cause the once vital sinks to become sources. Also, wetlands like salt marshes and mangrove swamps are at risk, with 85% already lost, even though they provide carbon sequestration, storage, water, and disaster regulation. This will eventually cause irretrievable deterioration of ecosystems, which can be predicted to cause the point of no return to arrive for the whole biome.[32,42] Some potential tipping points identified by the Intergovernmental Panel on Climate Change (IPCC) were: global average temperatures rising more than 1.5°C pre-industrial times; causing the thawing of permafrost, melting of the Arctic sea ice, loss of 50% of coral reefs by 2050, and collapse of the West Antarctic ice sheet by 2100.[8] Considering the current rate of temperature rise, the need to prevent further progress and learn to cope with current levels is urgent. In 2015, the internationally binding Paris Agreement was signed by 196 parties (countries) to collaborate in combating and adapting to climate change globally. Accordingly, the maximum amount of GHGs reached must be before 2025 latest, and decline 43% by 2030.[37] However, fuel combustion for the major gas, crude oil, coal, and petroleum industries, along with the current demands, keeps saturating the atmosphere and waters with carbon dioxide. The emissions owing to these reach 17.4 billion metric tons (2020 estimate) annually, with the growth rate projected to reach 1.9% each year in the present decade. The transition from the current petrochemical paradigm to a more sustainable circular economy model in the production of chemicals can be achieved through improving biotechnological applications. This is a complex but crucial shift.[17]

2. Petrochemical Synthesis of Indigo is not Sustainable

One of the industrial processes of prime concern is the petrochemical synthesis of indigo dye. With its characteristic blue color, indigo has found widespread industrial applications. These include use in inks, paints, cosmetics, food coloring, and currently research for organic semiconductors, and in the medical industry. Indigo that is produced for denim dying accounts for 95% of the chemically produced indigo globally, which reaches approximately 80,000 tonnes each year, with over 4 billion denim garments dyed.[42,39] The earliest use of indigo dates back to 4000 BC, and was naturally extracted from plants like Indigofera tinctoria (South Asia), *I. suffruticosa* (America). These plants contain indican, a derivative of tryptophan and the precursor of indigo, in their leaves. The fermentation of the leaf liberates indoxyl, which spontaneously dimerizes to indigo. Then, as

industrialization progressed, a more economic alternative for the mass production was searched for, and ~ 1890s onwards multiple procedures were developed (Figure 1) and implemented since it provided a cheaper, more efficient means.[30] However, in the dominating commercial method, the starting material is aniline; a cheap petrol based feedstock. Furthermore, strong reducing agents and metal catalysts like sodium amide are used, with the reaction requiring high temperatures.[7]

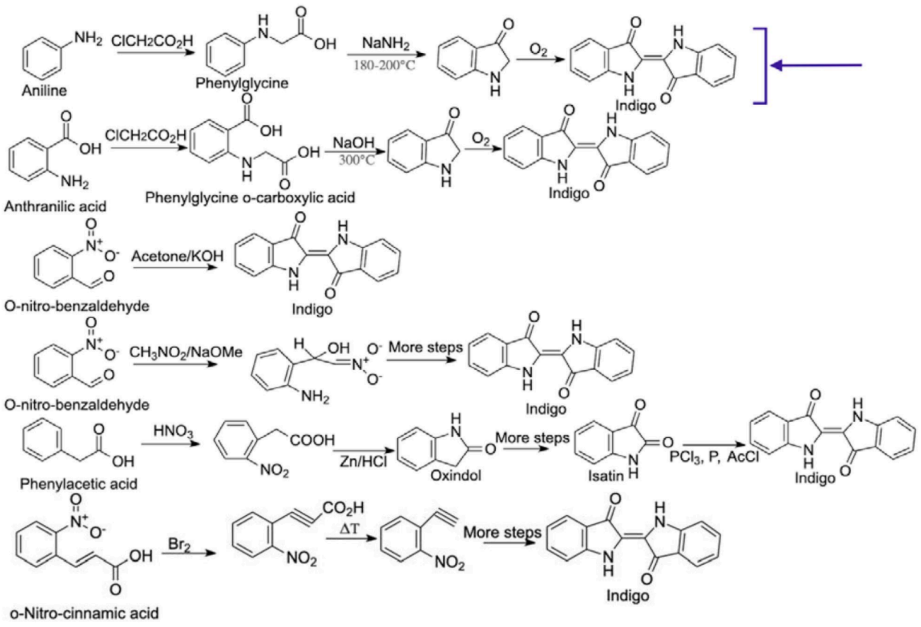

Figure 1 Chemical reactions utilized in producing synthetic indigo. The main route is depicted with a purple arrow. These reactions require harsh conditions: high temperatures like 180-200°C or 300°C, and strong chemicals like sodium hydroxide, nitric acid, and phosphorus trichloride. The starting materials aniline, anthranilic acid, o-nitrobenzaldehyde are non-renewable petroleum based sources. Chloroacetic acid, sodium hydride, nitric acid, acetyl chloride are toxic or hazardous agents, harmful to the environment and for human health. (Reproduced from Linke, Rayat and Ward, 2023)[29]

Therefore, the chemical process relies on non-renewable fossil fuels and involves energy-intensive processes, with harsh conditions; impacting the environment negatively.[6] In addition to the production, indigo being insoluble in water necessitates using harsh chemicals to obtain the reduced and soluble form; leucoindigo, to bind onto cloth fibers. Linking to this stage, human health risks and pollution of the water streams and soil have been outlined. In fact, one of the most significant causes of water pollution is the textile industry. Considering current jean production levels, Welner's team projected the reduction of carbon dioxide emissions by over three million tons, if the traditional method were substituted with a more sustainable alternative.[7] Thus, the urgency

of a green transition in this industry has spurred research into various approaches.

2.1 Greener Approaches to Indigo Production

Going back to using plants has been considered. This application would certainly be greener than the aniline-based production, but with its own drawbacks. One tonne of biomass is required to produce 10 kilograms of indigo, which would entail that the plantations for indigo would be competing with food and feed industries.[34] Biocatalyst-based processes have also been explored, and Fabara (2020) provided an overview of the microbial indigo-forming enzymes. These include; depending on their cofactors, non-heme iron oxygenases, heme-containing oxygenases, and flavin-dependent mono-oxygenases, which all oxidize indole; an aromatic pollutant in industrial and agricultural wastewater, to form indoxyl, which converts to indigo in the presence of oxygen. Oxygenases catalyze the integration of one (monooxygenase), or two oxygens (dioxygenase) using O2 and are chemo-, regio-, and stereoselective. Their intrinsic need for oxygen and reducing cofactors like NADH or NADPH renders using whole-cell factories compelling, and they have often been expressed in Escherichia coli to test efficiency on a small scale.[13]

2.1.1 A fusion Enzyme can Convert L-Tryptophan to Indigo

It has been stated that of the studied biocatalysts, flavin dependent monooxygenases (FMOs) are distinctively productive, making them an appropriate candidate for the biobased manufacture of the dye. Of the natural and engineered enzymes that have been reported, a fusion of tryptophanase (TRP) and flavin dependent monooxygenase (FMO) exhibited the highest amount of indigo obtained from whole cells. This bifunctional enzyme first converts L-tryptophan intro pyruvate, ammonia and indole via TRP, and then indigo is formed using indole as a substrate for FMO. (Figure 2) The regeneration of NADPH required by the enzyme

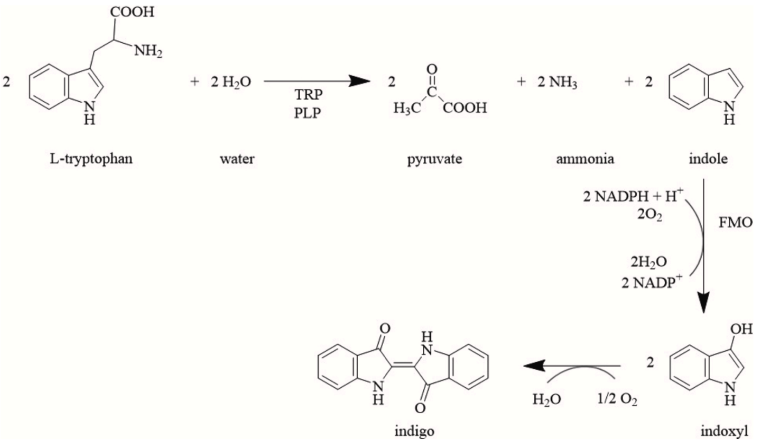

Figure 2 The reactions of the bifunctional enzyme from L-tryptophan to indigo. The TRP domain catalyzes the conversion to pyruvate, ammonia and indole. Pyruvate is used to regenerate the reducing cofactor and indole is oxidized to indigo by FMO. (Reproduced from Fabara, Fraaije. 2020)

is driven by pyruvate used in intracellular metabolism. Ideal tryptophan concentrations were tested and mutations were introduced to improve the turnover (kcat) and thermostability. In this study, they were able to achieve the yield of 1.7 g of indigo per liter of culture, starting from 2 g of L-tryptophan per liter of culture. This way, without the need of a cosubstrate, a realistic non hazardous starting material for indigo has been established.[14] In terms of a larger scale, even though it has not been practiced, Linke et al. (2023) reviewed the factors in translating it to an industrial scale. Based on available data and common bioprocess facilities, they concluded that the annual output would be capable of supplying the mass production of denim. Optimization efforts are required but the large-scale biotechnological application of indigo

production is attractive for business exploitation. As of yet, substrates like indole, indican, or tryptophan have been used as the input.[30]

While these advancements in indigo biosynthesis are promising, they still rely on tryptophan as a starting material, which is derived from biomass. This approach, although greener than traditional methods, still competes with other industries for resources and arable land[10], and so is inadequate in achieving a circular economy. To further mitigate the environmental impact of indigo production, there is a growing interest in utilizing waste products or harmful substances as input materials. An attractive course of action for research would be making use of biofactories that can natively use greenhouse gases; like cyanobacteria; that can synthesize tryptophan autonomously, requiring only carbon dioxide, light, and a nitrogen source. This way, a system that not only makes indigo production more sustainable, but also sequesters carbon can be potentially established. If the bifunctional fusion enzyme presented by Fabara et al. were to be heterologously expressed in cyanobacteria, a route from carbon dioxide to tryptophan to indigo can theoretically be employed.

3. Cyanobacteria is a promising candidate for valorization

3.1 Biobased Revolution of the Industry: Exploitation of Photoautotrophs

The goal of the circular economy model is to utilize waste as resources, attending to the problems of loss of value and waste accumulation. This model should be implemented since, for instance, it was shown that the majority of plastic packaging is used just once, resulting in a loss of 95% of the utility, implying up to 120 billion US dollars.[38] If biochemical recycling technologies are used to their potential, it would enable efficient recovery of carbon and heterogeneous solid waste to employ in the industry. While bio-based manufacturing using heterotrophic bacteria has gained traction, it competes with the food industry for organic biomass. On the contrary, autotrophic microorganisms, particularly photoautotrophs, offer an attractive alternative as they simply require light to fix carbon dioxide and produce metabolites. By exploiting synthetic biology tools and manipulating metabolism, photoautotrophs can be used for gas fermentation to produce commodity chemicals, biopolymers, and biofuels without generating toxic byproducts.[5] Companies like LanzaTech have successfully used chemoautotrophs like *Clostridium autoethanogenum* for large-scale carbon-negative production of platform chemicals.[1] However, these processes require anaerobic conditions that are complex to maintain in an industrial setting, and require sugar as feedstock; implying competition with arable land for the food industry. Oxygenic photoautotrophs utilizing the Calvin-Benson-Bassham cycle offer a more diverse product range and operate under aerobic conditions, bypassing the problems arising with chemoautotrophs. With comprehensive genetic, metabolic, and biochemical engineering, they could sustainably generate high-value compounds like pigments, vitamins, and bioplastics, using free and abundant sunlight for energy. While engineering strategies for photoautotrophs are less explored compared to other microorganisms, they present a promising method of large scale carbon-negative production, addressing the greenhouse emission goals and resilience of the industry.[9]

3.2 Why Cyanobacteria?

Information in this section on the metabolism and energetics, and engineering strategies of cyanobacteria, and the model strains used was mainly adapted from "Cyanobacteria in Biotechnology, Applications and Quantitative Perspectives" by P. Lindberg et al.[5]

3.2.1 Cyanobacterial Metabolism and Energetics

The first oxygenic photoautotrophs, known to be responsible for the oxygenation of the atmosphere: cyanobacteria, are the only prokaryotes able to perform oxygenic photosynthesis, and are responsible for 20-30% of the global recycling of carbon dioxide.[37] They provide a large microbial consortia with organic carbon by just using light, water and carbon dioxide. This attribute makes them enticing host organisms for biotechnology. Various chemicals have been produced from cyanobacteria, but growth being light dependent causes lower cell densities and yields, with the photobioreactor setups non-optimal as well. Moreover, especially on a regulatory level, research is not sufficient enough to rationally design process specific manipulations to amplify output. The fundamentals of the relevant pathways, strategies for engineering cyanobacteria and the existing toolbox, potential bottlenecks that would be relevant for cyanobacteria becoming workhorses for the indigo industry, will be discussed.

The primary carbon fixation pathway in cyanobacteria is the Calvin-Benson-Bassham (CBB) cycle. It consists of the steps: carboxylation, reduction, and regeneration of ribulose 1,5 bisphosphate (RuBP). The first step, which involves producing 3-phosphoglyceric acid (3PGA) from RuBP and CO2, is catalyzed by Ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCo). The enzyme RuBisCo is notoriously inefficient, with a low turnover rate and poor substrate specificity due to photorespiration activity. This might have been due to it having evolved in ancient times in a low-oxygen, high-carbon-dioxide environment. To enhance its carboxylase activity, microcompartments encapsulating RuBisCo and carbonic anhydrase (CA) have evolved in cyanobacteria, called carboxysomes. The necessary energy: in the form of adenosine triphosphate (ATP), and reducing power: in the form of NADH and NADPH is provided by the light reactions of photosynthesis. Photosystem II (PS II) directs the light energy to generate the very powerful oxidant; elemental oxygen by splitting water molecules, and the electrons are passed through the photosynthetic electron transport chain (PETC). Operating later in the light-dependent reactions, Photosystem I (PS I) serves as the central redox energy hub that couples the light reactions to the central cyanobacterial metabolism. The electrons from the PETC are further energized, and the high energy electrons are used in reducing NADP+ to NADPH; to use in converting inorganic substrates like CO2 and NO3 to sugar and amino acids. A proton gradient is then generated from the protons pumped by PS II and cytochrome b6f complex, with the indirect contribution of PSI through the electron flow. The protons accumulated in the thylakoid lumen lead to the release of energy in the form of ATP when they flow down the gradient through the ATP synthase.

3.2.2 Cyanobacteria as Industrial Workhorses

Initially, as they can exploit the abundantly available solar energy, cyanobacteria was found to be suitable for the biocatalysis of biofuels. In the past 20 years, their potential in becoming cell factories for the manufacture of a wide range of sustainable chemicals have increasingly gained attention. While native products were mostly applied in nutrition and as coloring agents[31], engineering efforts demonstrated non-native products can be produced too. [22,35,28,23] Commercial cultivation of cyanobacteria for the industry has been going on for the past half-century.

Cyanobacteria are diverse, due to the adaptations to various ecosystems. Model strains with different attributes are preferred depending on the research or application. The *Synechococcus* genus is systemically understood, with well annotated genome sequences and molecular tools for genetic engineering. *Synechococcus* sp. PCC 7002 (sp. 7002) and sp. PCC 7942 can survive high light intensities and temperatures up to 40°C, while the marine strain sp. 7002 can also tolerate high salt concentrations. UTEX 2973, PCC 11901, PCC 11801 are among the strains with the highest growth rate. Elements like vectors or promoters for the control of gene expression have been developed for *Synechococcus elongatus* PCC 7942, commonly used in circadian clock studies. *Synechocystis* sp. PCC 6803 (sp. 6803), the first photosynthetic organism to be sequenced (Kaneko et al. 1996), meets many of the limitations of becoming a chassis strain. Although only 30% of the coding sequence has its function identified, this strain serves as a model that can utilize different carbon metabolisms (phototrophic, mixotrophic, heterotrophic) and facilitates handling, especially for higher plants that might trigger stress responses. The CyanoBase, the genome database for cyanobacteria, contains 290 draft and 84 full genomes (2023).[5] This accumulation catalyzes the progress in building genome-scale models (GSMs) that will facilitate proposing and predicting outcomes of engineering strategies significantly.

3.2.3 Engineering Strategies for Cyanobacteria

The useful approaches to improving the productivity of a desired metabolite in cyanobacterial cell factories have been identified. The pull strategy is where a sink for cellular resources is created so that the cell will counteract and upregulate the upstream processes. For *S. elongatus* PCC 7942, implementing a sucrose production pathway, or an oxygenase consuming NADH enhanced the electron flux into the PETC, increasing the quantum yield of PS II. [40] Conversely, the flux of precursors could be increased by overexpressing relevant enzymes, or knocking out competing pathways. Namely, the push strategy.[50] To enhance the availability of carbon precursors, carbon fixation can be enhanced by addressing the rate limiting step of RuBisCo. This can be done by expressing a more efficient RuBisCo heterologously, overexpressing carbon transporters, or the overexpression of CBB enzymes like transketolase.[28] Additionally, the photosynthetic inefficiency of cyanobacteria can be tackled. The conversion yield of light energy to chemical energy is in the range of 1-3%. This is due to the fact that only the wavelengths within the photosynthetically active radiation (PAR, 400-700 nm) can be utilized, and the rest of the energy is lost as heat. The biotechnological application of different pigments like chlorophyll (Chl) d (absorbs at 700-750 nm), or chlorophyll f (~706 nm) might expand the exploitable spectrum. Alternatively, the growth of

cyanobacteria can be tuned by exposure to a restricted range of light. The Spirulina platensis growth rate was increased by 37.5% compared to white light, when red light corresponding to the absorption peak of Chl a and phycocyanobilin was radiated. Also, in sp. 6803, it was found that blue light might be causing an imbalance between the two photosystems, while orange-red light (525-660 nm) amplified growth and oxygen concentrations. A compromise for these strategies is that optimizing growth is not always correlated with higher amounts of the target product forming. Engineering the cofactor supply might favor metabolite formation over growth. NADPH-dependent enzymes and/or trans-hydrogenases interconverting NADH with NADPH, may increase the affiliated enzyme activity and thus the overall carbon flux toward the desired metabolic pathways. For example, the intracellular NADPH concentrations were increased in the strain PCC 6803 by overexpressing glucose-6-phosphate dehydrogenase, and the ethanol titers were improved from 440 to 690 mg/L.[6] Another bottleneck that can be addressed is the low cell densities in the photobioreactors. Investigating different reactor geometries[20], or exploring biofilm formation methods (PCC 6803) has been done by Hoschek et al. for higher cell densities, though the latter has only been done on a laboratory scale.[5]

3.2.4 Molecular Toolbox for Building Cyanobacterial Cell Factories

A hindrance in reaching an ample toolbox for engineering cyanobacteria is: the toolset that has been developed for E.coli often can not be translated directly. This is often because of the differences in the initiation and regulation of transcription. Nevertheless, the development of tools for the control of the flux into the desired product has been progressing for the different model strains. A range of promoters have been developed, including adaptations of heterologous inducible promoters.[3,26,27,31] For example, the orthogonality, heightened expression, transcription rates and versatility of the T7 RNA polymerase systems has been demonstrated to be useful in the model strains.[45,25] Transcription terminators that are efficient have been identified for multiple species too.[31,26,] Ribosome binding sites were characterized[51] with in-silico manipulation techniques for their design. Regulatory RNAs, including small RNAs (sRNA), and riboswitches were used for tight control of transcription [19,], often implemented in parallel with promoters, as well as Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi) systems. CRISPRi systems facilitate reversible knocking down of genes to deal with competitive pathways.[19,20,12,32,53] Heterologous proteases to degrade proteins as a post translational control mechanism has further expanded the toolset.[21] Additionally, CyanoGate: a modular cloning suite, uniting cyanobacteria with plant and algal systems[16], has facilitated accommodating standardized synthetic biology tools in cyanobacteria. Overall, these advancements (Figure 3) will enable adjusting the metabolic pathway in a targeted approach, and help construct the genetic circuits for optimized production.[21] The progress in this area has already led to increased fatty acid[46] and valencene yield[12].

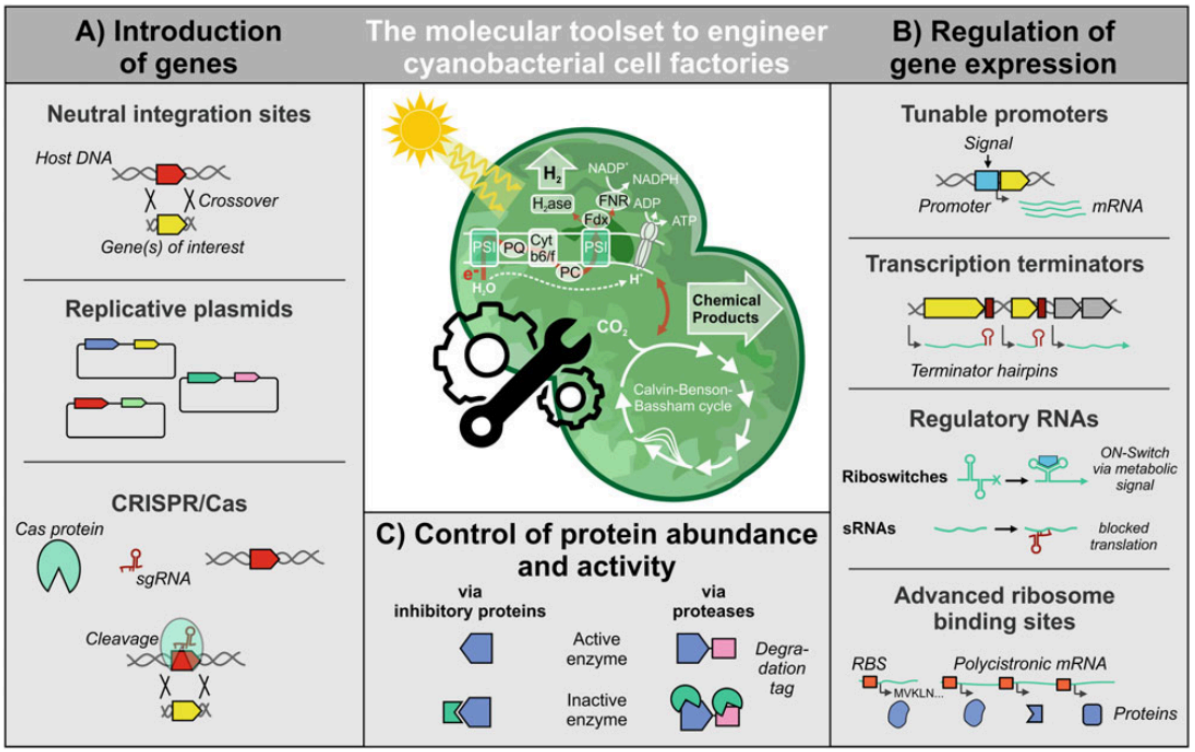

Figure 3 The synthetic biology toolbox for the genomic and metabolic engineering of cyanobacteria. Several tools for introduction of genes, regulation of gene expression, and control of protein abundance and activity have been developed. Including: Neutral integration sites, replicative plasmids, CRISPR/Cas systems, tunable promoters, transcription terminators, regulatory RNAs, advanced ribosome binding sites. (Reproduced from Bühler and Lindberg, 2023)

3.2.5 Metabolic Engineering of Cyanobacteria for the Production of Tryptophan

Aromatic amino acids (AAA) and their derivatives may be produced from Cyanobacteria to find use in nutrition or in the chemical industry. This only requires light, a carbon source, and a nitrogen source[1]. While cumulative efforts have been made in engineering Cyanobacteria for AAA biosynthesis, better yields need to be obtained for the transition to larger scale.[4]

The relevant pathways from carbon dioxide to the aromatic acids can be outlined as: First, the Calvin cycle fixes carbon dioxide into 3PGA. Then, through passageways in glycolysis and the pentose-phosphate-pathway,phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) enter the Shikimate-Pathway to produce 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP), catalyzed by DAHPS. Through shikimate as an intermediate, chorismate is formed, which is the common precursor for the biosynthesis of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp). Chorismate mutase (CM) converts chorismate to phenylalanine and tyrosine, while anthranilate synthase (AS) converts it to tryptophan. Figure 3 shows the feedback inhibition points within this biosynthetic route. The process of engineering aromatic amino acid overproduction has mainly been focused on enzymes subject to feedback inhibition by their end products: DAHPS, AS,

[1] Cyanobacteria cultures were grown in BG11 media (with nitrogen sources) for the studies mentioned here.

CM.[5] Furthermore, targeting transcriptional regulators of the Shikimate pathway in E.coli has been attempted[31]. It has been demonstrated that heterologous expression of feedback inhibition resistant genes from *E.coli*, can increase the titers significantly for phenylalanine and tyrosine from CO2 in *Synechocystis sp.* PCC 6803.[4] This suggests that this same orthogonality can be applied to producing tryptophan. Applying this, Deshpande showed that expressing feedback-resistant DAHPS helped achieve a titer of 211.6±23.2 mg/L. Additionally, mitigating competing pathways was effective; introducing the mutation CMV52F in chorismate mutase redirected flux to the tryptophan branch. It has been suggested that shikimate feeding the cyanobacteria, coupled with metabolite profiling could help pinpoint bottlenecks upstream and downstream of it. Moreover, overexpressing AroG^{fbr} and trpE^{fbr} (see Figure 4) was effective. Overall, combining random mutagenesis with metabolic engineering was deemed more effective than either of the methods implemented exclusively. [10,11] These small scale studies have been carried out in the chassis strain *Synechocystis sp*. PCC 6803. However, more autotrophically productive strains that can grow faster could be better for industrial applications. For example, *Synechococcus elongatus* UTEX 2973 seems to be poised for greater anabolic flux capacity.[29] If the mechanistic and systematic means of this higher capacity of the strain is realized, a critical step in engineering future steps would be taken. Moreover, after removing the allosteric feedback inhibitions, the next step should be to identify the rate limiting steps to improve flux, and the respective enzymes should be overexpressed.

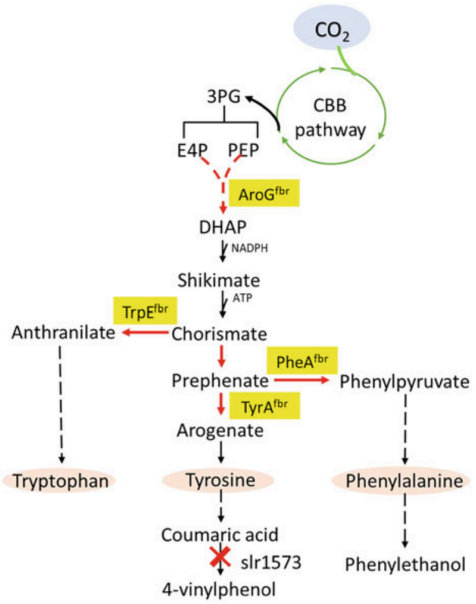

Figure 4 Strategy for increasing carbon flux toward tryptophan. Overexpressing the genes AroG^{fbr} coding for DAHPS and TrpE^{fbr} for AS is effective since these points of the pathway are inhibited by their products through feedback inhibition. Fbr; feedback resistant. (Reproduced from Bühler and Lindberg, 2023)

4. Cyanobacteria as Cell Factories for the Sustainable Production of Indigo

Currently, cyanobacteria is not valorized to its potential as a cell factory, even though it has the ability to fix carbon dioxide to make a wide range of useful products. As this review discussed, the indigo industry is in need of a transition to a greener alternative, and a number of biocatalysts; oxygenases, have been identified to generate indigo from indican or tryptophan. The bifunctional fusion enzyme engineered by Fabara et al. renders using L-tryptophan as a substrate a viable option, with the highest yields reached to date. However, amino acids are derived from biomass and this implies a competition with industries such as agriculture. In recent years, efforts in optimizing the productivity of the biosynthesis of aromatic amino acids from carbon dioxide in

cyanobacteria has gathered attention. Based on the literature collected, this review presents a route from carbon dioxide to indigo using cyanobacteria as a biofactory and L-tryptophan as an intermediate by heterologously expressing the TRP-FMO fusion enzyme in the strains most productive for L-tryptophan, indigo can theoretically be produced from merely CO2, media containing a nitrogen source like sodium nitrate, and light. The potential bottlenecks and how they could be tackled for each of the two concepts combined here were presented and could be of importance for consideration during the paradigm shift of the industry.

As the cyanobacterial toolbox expands, and genome-scale metabolic models and photobioreactor designs are improved, the viability of this process will increase. If this process were to become applicable, it would contribute to the circular economy model; reducing the carbon footprint of the indigo industry to be negative, show the potential of optimizing genetic and regulatory networks through synthetic biology for industrial processes, and offer a scalable platform to generate other high-value compounds using cyanobacteria as a cell factory.

References

1. "About." *LanzaTech*, lanzatech.com/about/. Accessed 10 July 2024.
2. Angermayr, S. Andreas, et al. "Metabolic Engineering of cyanobacteria for the synthesis of commodity products." *Trends in Biotechnology*, vol. 33, no. 6, June 2015, pp. 352–361, https://doi.org/10.1016/j.tibtech.2015.03.009.
3. Behle, Anna, et al. "Comparative dose–response analysis of inducible promoters in cyanobacteria." *ACS Synthetic Biology*, vol. 9, no. 4, 5 Mar. 2020, pp. 843–855, https://doi.org/10.1021/acssynbio.9b00505.
4. Brey, Laura Furelos, et al. "Metabolic engineering of Synechocystis SP.. PCC 6803 for the production of aromatic amino acids and derived phenylpropanoids." Metabolic Engineering, vol. 57, Jan. 2020, pp. 129–139, https://doi.org/10.1016/j.ymben.2019.11.002.
5. Bühler, Katja, and Pia Lindberg. Cyanobacteria in Biotechnology: Applications and Quantitative Perspectives. Springer, 2023.
6. Choi, Kwon-Young. "A review of recent progress in the synthesis of bio-indigoids and their biologically assisted end-use applications." *Dyes and Pigments*, vol. 181, Oct. 2020, p. 108570, https://doi.org/10.1016/j.dyepig.2020.108570.
7. Bidart, Gonzalo Nahuel, et al. "Chemoenzymatic Indican for light-driven denim dyeing." *Nature Communications*, vol. 15, no. 1, 27 Feb. 2024, https://doi.org/10.1038/s41467-024-45749-3.
8. "Climate Change 2021: The Physical Science Basis." *IPCC Â" Intergovernmental Panel on Climate Change*, www.ipcc.ch/report/ar6/wg1/ Accessed 10 July 2024.

9. Delrue, Florian, et al. “Optimization of arthrospira platensis (spirulina) growth: From laboratory scale to pilot scale.” *Fermentation*, vol. 3, no. 4, 7 Nov. 2017, p. 59, https://doi.org/10.3390/fermentation3040059.
10. Deshpande, Arnav. Sustainable Production of Amino Acids By Engineered Cyanobacteria. 2022. Purdue University, Doctoral Dissertation. Https://Www.Proquest.Com/Openview/927f40cb5e4fd3f684e45bb8056abc4d/1?Pq-origsite=Gscholar&Cbl=18750&Diss=Y.
11. Deshpande, Arnav, et al. “Combining random mutagenesis and metabolic engineering for enhanced tryptophan production in *synechocystis* SP.. strain PCC 6803.” *Applied and Environmental Microbiology*, vol. 86, no. 9, 17 Apr. 2020, https://doi.org/10.1128/aem.02816-19.
12. Dietsch, Maximilian, et al. “Metabolic engineering of Synechocystis SP.. PCC 6803 for the photoproduction of the Sesquiterpene Valencene.” *Metabolic Engineering Communications*, vol. 13, Dec. 2021, https://doi.org/10.1016/j.mec.2021.e00178.
13. Fabara, Andrea N., and Marco W. Fraaije. “An overview of microbial indigo-forming enzymes.” *Applied Microbiology and Biotechnology*, vol. 104, no. 3, 13 Dec. 2019, pp. 925–933, https://doi.org/10.1007/s00253-019-10292-5.
14. Fabara, Andrea N., and Marco W. Fraaije. “Production of indigo through the use of a dual-function substrate and a bifunctional fusion enzyme.” *Enzyme and Microbial Technology*, vol. 142, Dec. 2020, p. 109692, https://doi.org/10.1016/j.enzmictec.2020.109692.
15. Ferrari, Olivia. “Jeans Are Bad for the Environment-but a New Discovery May Help.” *Environment*, 27 Feb. 2024, www.nationalgeographic.com/environment/article/blue-jeans-indigo-indican-enzyme.
16. Gale, Grant A., et al. “Genetic modification of cyanobacteria by conjugation using the CyanoGate Modular Cloning Toolkit.” *Journal of Visualized Experiments*, no. 152, 31 Oct. 2019, https://doi.org/10.3791/60451.
17. Geissdoerfer, Martin, et al. “The circular economy – a new sustainability paradigm?” *Journal of Cleaner Production*, vol. 143, Feb. 2017, pp. 757–768, https://doi.org/10.1016/j.jclepro.2016.12.048
18. Ghiffary, Mohammad Rifqi, et al. “High-level production of the natural blue pigment indigoidine from metabolically engineered *corynebacterium glutamicum* for sustainable fabric dyes.” *ACS Sustainable Chemistry & Engineering*, vol. 9, no. 19, 19 Apr. 2021, pp. 6613–6622, https://doi.org/10.1021/acssuschemeng.0c09341.
19. Higo, Akiyoshi, et al. “Application of CRISPR interference for metabolic engineering of the heterocyst-forming multicellular cyanobacterium anabaena sp.. PCC 7120.” *Plant and Cell Physiology*, vol. 59, no. 1, 3 Nov. 2017, pp. 119–127, https://doi.org/10.1093/pcp/pcx166.
20. Huang, Chun-Hung, et al. “CRISPR interference (CRISPRI) for gene regulation and succinate production in Cyanobacterium S. elongatus PCC 7942.” *Microbial Cell Factories*, vol. 15, no. 1, 15 Nov. 2016, https://doi.org/10.1186/s12934-016-0595-3.
21. Immethun, Cheryl M., and Tae Seok Moon. “Synthetic gene regulation in cyanobacteria.” *Synthetic Biology of Cyanobacteria*, 2018, pp. 317–355, https://doi.org/10.1007/978-981-13-0854-3_13.
22. Iwai, Masako, et al. “Improved genetic transformation of the thermophilic cyanobacterium, Thermosynechococcus elongatus BP-1.” *Plant and Cell Physiology*, vol. 45, no. 2, 15 Feb. 2004, pp. 171–175, https://doi.org/10.1093/pcp/pch015.

23. Jaiswal, Damini, Annesha Sengupta, et al. “Genome features and biochemical characteristics of a robust, fast growing and naturally transformable cyanobacterium Synechococcus elongatus PCC 11801 isolated from India.” *Scientific Reports*, vol. 8, no. 1, 9 Nov. 2018, https://doi.org/10.1038/s41598-018-34872-z.
24. Jaiswal, Damini, Deepti Sahasrabuddhe, et al. “Cyanobacteria as cell factories: The roles of host and pathway engineering and translational research.” *Current Opinion in Biotechnology*, vol. 73, Feb. 2022, pp. 314–322, https://doi.org/10.1016/j.copbio.2021.09.010.
25. Jin, Haojie, et al. “Building an inducible T7 RNA polymerase/T7 promoter circuit in *synechocystis* SP.. PCC6803.” *ACS Synthetic Biology*, vol. 8, no. 4, 2 Apr. 2019, pp. 655–660, https://doi.org/10.1021/acssynbio.8b00515.
26. Kelly, Ciarán L., George M. Taylor, Aistė Šatkutė, et al. “Transcriptional terminators allow leak-free chromosomal integration of genetic constructs in cyanobacteria.” *Microorganisms*, vol. 7, no. 8, 16 Aug. 2019, p. 263, https://doi.org/10.3390/microorganisms7080263.
27. Kelly, Ciarán L., George M. Taylor, Andrew Hitchcock, et al. “A rhamnose-inducible system for precise and temporal control of gene expression in cyanobacteria.” *ACS Synthetic Biology*, vol. 7, no. 4, 15 Mar. 2018, pp. 1056–1066, https://doi.org/10.1021/acssynbio.7b00435.
28. Liang, Yuanmei, et al. “Thermosynechococcus as a thermophilic photosynthetic microbial cell factory for CO2 Utilisation.” *Bioresource Technology*, vol. 278, Apr. 2019, pp. 255–265, https://doi.org/10.1016/j.biortech.2019.01.089.
29. Lin, Po-Cheng, et al. “Enhanced production of sucrose in the fast-growing cyanobacterium Synechococcus elongatus UTEX 2973.” *Scientific Reports*, vol. 10, no. 1, 15 Jan. 2020, https://doi.org/10.1038/s41598-019-57319-5.
30. Linke, Julia A., et al. “Production of indigo by recombinant bacteria.” *Bioresources and Bioprocessing*, vol. 10, no. 1, 13 Mar. 2023, https://doi.org/10.1186/s40643-023-00626-7.
31. Liu, Deng, Michelle Liberton, et al. “Engineering biology approaches for food and nutrient production by cyanobacteria.” *Current Opinion in Biotechnology*, vol. 67, Feb. 2021, pp. 1–6, https://doi.org/10.1016/j.copbio.2020.09.011.
32. Liu, Deng, Virginia M. Johnson, et al. “A reversibly induced CRISPRI system targeting photosystem II in the cyanobacterium *synechocystis* SP.. PCC 6803.” *ACS Synthetic Biology*, vol. 9, no. 6, 7 May 2020, pp. 1441–1449, https://doi.org/10.1021/acssynbio.0c00106.
33. Mcleod, Elizabeth, et al. “A blueprint for Blue Carbon: Toward an improved understanding of the role of vegetated coastal habitats in sequestering co2.” *Frontiers in Ecology and the Environment*, vol. 9, no. 10, 20 Jan. 2011, pp. 552–560, https://doi.org/10.1890/110004.
34. Mills, Lauren A., et al. “Current knowledge and recent advances in understanding metabolism of the model cyanobacterium *synechocystis* SP.. PCC 6803.” *Bioscience Reports*, vol. 40, no. 4, Apr. 2020, https://doi.org/10.1042/bsr20193325.
35. Onai, K., et al. “Natural transformation of the thermophilic cyanobacterium Thermosynechococcus elongatus BP-1: A simple and efficient method for Gene Transfer.” *Molecular Genetics and Genomics*, vol. 271, no. 1, 25 Nov. 2003, pp. 50–59, https://doi.org/10.1007/s00438-003-0953-9.

36. Pattanaik, Lopa, et al. “Valorization of waste Indigofera tinctoria L. biomass generated from indigo dye extraction process—potential towards biofuels and compost.” *Biomass Conversion and Biorefinery*, vol. 9, no. 2, 4 Dec. 2018, pp. 445–457, https://doi.org/10.1007/s13399-018-0354-2.
37. Pavan, Marilene, et al. “Advances in systems metabolic engineering of autotrophic carbon oxide-fixing biocatalysts towards a circular economy.” Metabolic Engineering, vol. 71, May 2022, pp. 117–141, https://doi.org/10.1016/j.ymben.2022.01.015.
38. *Plastics and the Circular Economy*, www.ellenmacarthurfoundation.org/plastics-and-the-circular-economy-deep-dive. Accessed 10 July 2024.
39. Paul, Roshan, et al. “Indigo and Indigo Colorants.” *Ullmann’s Encyclopedia of Industrial Chemistry*, 31 Mar. 2021, pp. 1–16, https://doi.org/10.1002/14356007.a14_149.pub3.
40. Savakis, Philipp, and Klaas J Hellingwerf. “Engineering cyanobacteria for direct biofuel production from CO2.” *Current Opinion in Biotechnology*, vol. 33, June 2015, pp. 8–14, https://doi.org/10.1016/j.copbio.2014.09.007.
41. Savakis, Philipp, and Klaas J Hellingwerf. “Engineering cyanobacteria for direct biofuel production from CO2.” *Current Opinion in Biotechnology*, vol. 33, June 2015, pp. 8–14, https://doi.org/10.1016/j.copbio.2014.09.007.
42. Schimper, Christian B., et al. “Surface activation of dyed fabric for cellulase treatment.” *Biotechnology Journal*, vol. 6, no. 10, 13 Apr. 2011, pp. 1280–1285, https://doi.org/10.1002/biot.201100002.
43. Steffen, Will, et al. “Trajectories of the earth system in the anthropocene.” *Proceedings of the National Academy of Sciences*, vol. 115, no. 33, 6 Aug. 2018, pp. 8252–8259, https://doi.org/10.1073/pnas.1810141115
44. Sheldon, Roger A., and Dean Brady. “Green Chemistry, biocatalysis, and the chemical industry of the future.” *ChemSusChem*, vol. 15, no. 9, 9 Feb. 2022, https://doi.org/10.1002/cssc.202102628.
45. Shono, Chika, et al. “A green light-regulated T7 RNA polymerase gene expression system for cyanobacteria.” *Marine Biotechnology*, vol. 23, no. 1, 26 Sept. 2020, pp. 31–38, https://doi.org/10.1007/s10126-020-09997-w.
46. Sun, Tao, et al. “Re-direction of carbon flux to key precursor malonyl-COA via artificial small RNAS in photosynthetic synechocystis sp.. PCC 6803.” *Biotechnology for Biofuels*, vol. 11, no. 1, 5 Feb. 2018, https://doi.org/10.1186/s13068-018-1032-0.
47. Till, Petra, et al. “Regulatory systems for gene expression control in cyanobacteria.” *Applied Microbiology and Biotechnology*, vol. 104, no. 5, 22 Jan. 2020, pp. 1977–1991, https://doi.org/10.1007/s00253-019-10344-w.
48. Treece, Tanner R., et al. “Synthetic Biology Approaches for improving chemical production in cyanobacteria.” *Frontiers in Bioengineering and Biotechnology*, vol. 10, 11 Mar. 2022, https://doi.org/10.3389/fbioe.2022.869195.
49. Vasudevan, Ravendran, et al. “CyanoGate: A modular cloning suite for engineering cyanobacteria based on the plant MoClo syntax.” *Plant Physiology*, vol. 180, no. 1, 28 Feb. 2019, pp. 39–55, https://doi.org/10.1104/pp.18.01401.

50. Wang, Weihua, et al. “Engineering cyanobacteria to improve photosynthetic production of Alka(E)nes.” Biotechnology for Biofuels, vol. 6, no. 1, 6 May 2013, https://doi.org/10.1186/1754-6834-6-69.
51. Wang, Bo, et al. “A genetic toolbox for modulating the expression of heterologous genes in the cyanobacterium synechocystis SP.. PCC 6803.” ACS Synthetic Biology, vol. 7, no. 1, 22 Dec. 2017, pp. 276–286, https://doi.org/10.1021/acssynbio.7b00297.
52. Włodarczyk, Artur, et al. “Newly discovered Synechococcus sp.. PCC 11901 is a robust cyanobacterial strain for high biomass production.” *Communications Biology*, vol. 3, no. 1, 7 May 2020, https://doi.org/10.1038/s42003-020-0910-8.
53. Yao, Lun, et al. “Multiple gene repression in cyanobacteria using CRISPRI.” *ACS Synthetic Biology*, vol. 5, no. 3, 28 Dec. 2015, pp. 207–212, https://doi.org/10.1021/acssynbio.5b00264.
54. Yu, Jingjie, et al. “Synechococcus elongatus UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO2.” *Scientific Reports*, vol. 5, no. 1, 30 Jan. 2015, https://doi.org/10.1038/srep08132.