Final Project: Fungi Biodyes

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Abstract

SECTION 1: ABSTRACT

The textile dyeing industry stands as one of the most chemically intensive sectors in global manufacturing, responsible for approximately 20% of industrial water contamination worldwide. The indiscriminate discharge of synthetics dyes compounds, acutely toxic to aquatic organisms, is associated with carcinogenic risk in humans. Has searched for sustainable dyeing alternatives not merely desirable, but scientifically imperative. Beyond environmental concern, fungal systems offer a compelling and largely unexplored aesthetic dimension: the organic, unpredictable growth patterns of fungi produce inherently unique colorations, transforming dyed textiles into one-of-a-kind pieces.

The project objective is to optimize the pigment-producing capacity of *Fusarium* as the basis for a biotechnological dyeing system that is simultaneously sustainable, scalable, and visually distinctive. We hypothesize that integrating controlled culture optimization and in silico-guided strain

improvement strategies can enhance fungal pigment production to levels that are reproducible, stable, and functionally competitive with conventional synthetic dyes.

The general aims of this work: (1) optimize *Fusarium* pigment production through growth condition tuning and computationally guided mutation of biosynthetic pathway enzymes; (2) validate the system for direct textile application, ensuring stable and consistent coloration; and (3) scale the process into bioreactor systems to achieve reproducibility at industrially relevant levels.

The methodology will focus on an in silico pipeline targeting key enzymes of the *Fusarium* bikaverin and fusarubin biosynthetic pathways. Protein sequences of rate-limiting enzymes will be retrieved from PDT and UniProt, and their three-dimensional structures predicted using AlphaFold2 or PyMOL. Evolutionary conservation and functional residues will be mapped using ESM-2, a protein language model, to identify candidate positions tolerant to beneficial mutations. Gibson Assembly, enabling seamless, scarless construction of modified expression cassettes. This approach will allow direct overexpression or functional replacement of key pigment pathway enzymes in Fusarium, with the ultimate goal of generating strains that produce significantly more intense and stable pigmentation on textile substrates.

PROJECT AIMS

Aims

1	The first aim of my final project is to enhance pigment production intensity and fungal growth rate in *Fusarium* by identifying and designing gain-of-function mutations in rate-limiting enzymes of the bikaverin and fusarubin biosynthetic pathways using an in silico pipeline (AlphaFold2, ESM-2, DeMaSk/EVmutation, FoldX), and constructing optimized mutant expression cassettes via Gibson Assembly for experimental validation.
2	The second aim is to experimentally validate the engineered mutations by transforming the Gibson-assembled constructs into *Fusarium* strains, culturing under optimized growth conditions (media composition, pH, temperature), and directly measuring pigment intensity and growth

	kinetics to identify the strain variants that produce the most vibrant coloration and fastest growth rate for textile applications.
3	The long-term vision is to scale optimized, high-pigment-producing *Fusarium* strains into bioreactor systems capable of generating intense, stable, and reproducible dyeing on textiles at an industrial scale, establishing fungal biodyes as a sustainable and commercially viable alternative to synthetic dyes while preserving the unique aesthetic patterns that make each piece one-of-a-kind.

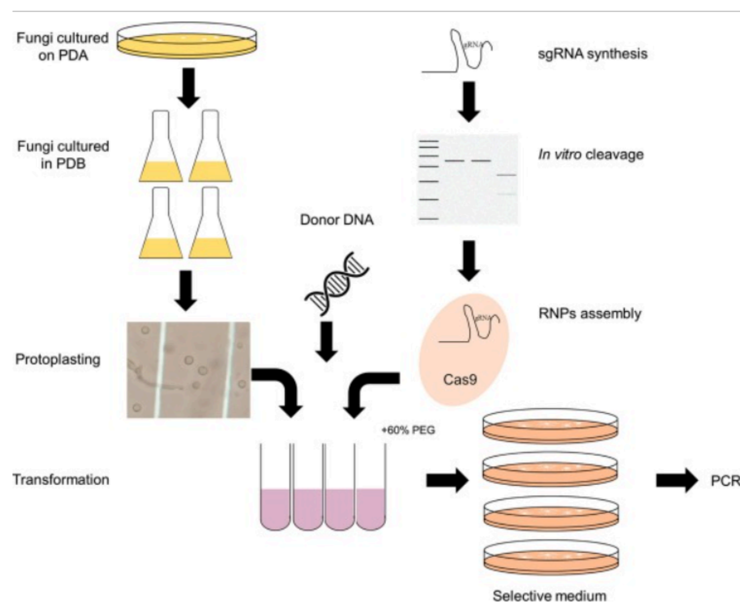
BACKGROUND

1. Literature context:

Fungi are extraordinary organisms with exceptional capabilities. These include not only their intrinsic chemical properties but also their wide range of applications, including architecture, programming, and biomaterials. They possess unique morphologies with distinctive shapes and the distinctive pigmentation they produce naturally. Each species of fungus can exhibit unique colors that emerge spontaneously during growth. These chromatic manifestations offer both a window into fungal metabolism and an untapped resource for sustainable color innovation. The article by Venil (2020) explores the application of dye-producing fungi in industry. The authors emphasized that fungal-derived pigments can achieve color fastness comparable to conventional dyes while simultaneously reducing environmental contamination, making them viable candidates for sustainable textile applications.

As a consequence, I began working with the fungus Fusarium oxysporum to dye textiles, but it did not grow sufficiently even after adjusting the environmental conditions. Consequently, I sought to change the species, which led me to a study on genetic editing using CRISPR/Cas9 in Fusarium oxysporum (Wang, 2018) developed an effective transformation system using a CRISPR/Cas9 ribonucleoprotein (RNP) specifically optimized for Fusarium oxysporum, a filamentous fungus capable of producing various secondary metabolites. The system utilized a fungus-optimized Cas9 protein fused to an endogenous nuclear localization sequence (NLSH2B), enabling direct protoplast transformation without plasmid integration and achieving a mutation efficiency of approximately 50% for target genes. Crucially, the researchers demonstrated the system's utility by inactivating the FoBIK1 gene, which encodes a polyketide synthase (PKS), confirming that this enzyme is responsible for the biosynthesis of bikaverin, a red pigment compound. This work establishes CRISPR/Cas9 as a viable molecular tool for manipulating secondary metabolite pathways in Fusarium species, directly supporting gain-of-function strategies for pigment enhancement.

Complementing this genetic approach, Westphal et al. (2018) examined the function of the transcription factor AurR1 in *Fusarium graminearum*, identifying it as a critical regulator of pigment biosynthesis. The study demonstrated that overexpression of AurR1 significantly increased pigment production, while gene disruption led to complete loss of pigmentation. In addition, related research in *Fusarium* species has explored genetic strategies to reduce pathogenicity and enhance fungal growth, aiming to improve their safety and efficiency for biotechnological applications. This work established that transcriptional regulation plays a fundamental role in controlling secondary metabolite pathways in fungi.



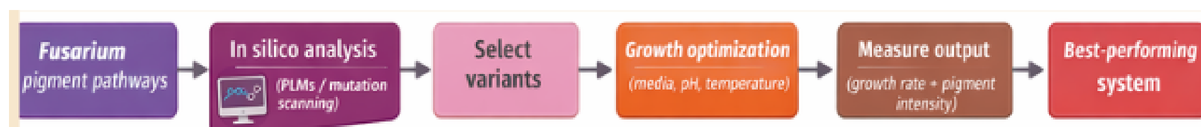
2. Project Novelty:

While both studies demonstrate powerful individual approaches—CRISPR-based gene editing and transcription factor overexpression—they remain largely independent strategies applied to either Fusarium oxysporum (gene disruption focus) or Fusarium graminearum (pigment enhancement focus). Our project innovates in three critical dimensions:

- This project is innovative because it applies a fully computational approach to optimizing the production of fungal pigments for textile applications, moving from trial-and-error experimentation to computational prediction in the early stages of bioprocess design. By integrating sequence analysis, protein structure prediction, and functional modeling, it proposes a new way to identify and optimize key targets, such as regulatory proteins and biosynthetic enzymes, without immediate intervention in the laboratory.

- Multi-enzyme optimization: Instead of single-gene disruption or global transcriptional upregulation, we focus specifically on critical enzymes (PKS, monooxygenases, dehydratases) using computational predictions of stability-enhancing substitutions. This precision approach differs from massive overexpression, offering adjustable control of the metabolic pathway flux without generating unwanted metabolic byproducts.

- Application to textile biotechnology: While previous work has characterized the chemistry of pigments in laboratory settings, we designed Fusarium strains specifically for direct application to textile fibers, combining enhanced pigment intensity with the preservation of the organic growth pattern: a unique aesthetic and functional dimension, absent in traditional synthetic dyes or in previous literature on fungal pigments. This establishes a link between synthetic biology and applied textile biotechnology. The focus is on ensuring that the strains are non-pathogenic, enhancing color, and promoting growth using an already established application methodology.



3. Impact:

The textile dyeing industry discharges toxic synthetic dyes that persist in aquatic ecosystems while textile workers and waterway-dependent

communities in developing nations bear disproportionate exposure to chemical pollution. The industry consumes 79 trillion liters of water annually for dyeing, with 85% contaminated and discharged untreated into rivers and oceans. No comprehensive, scalable alternative to synthetic dyes currently exists that combines environmental safety, cost-effectiveness, and the color intensity required for commercial viability—this project directly addresses that critical barrier.

If successful, establishing fungal-based biodyes as commercially viable would enable a paradigm shift: reducing water consumption by 50-80%, eliminating toxic chemical mordants that pose occupational health risks, creating economic opportunities in communities through localized fermentation-based production, and producing wearable textiles with distinctive organic pigmentation patterns that command premium value. Beyond fashion, fungal pigments could be adapted for food coloring, cosmetics, and industrial coatings.

Comparison of what synthetic textile dyes do in water.

4. Ethical implications:
This project involves ethical considerations related to biosafety and the use of potentially pathogenic organisms, such as Fusarium species. Although this is an in silico study, it proposes the optimization of biological systems that, in real-world applications, could pose a risk if pathogenic traits are not properly addressed. Therefore, the principle of non-maleficence (avoiding harm) is fundamental, as the project aims to reduce or eliminate pathogenicity while enhancing beneficial traits, such as pigment production and fungal growth to improve the areas stained by the fungus. At the same time, the principle of beneficence is reflected in the goal of developing safer and more sustainable modifications; responsibility is also important, as computational predictions must be used cautiously to guide future experimental work without creating unintended risks.

To ensure ethical conduct, I propose:

(1) Transparent computational development and risk assessment of all proposed genetic modifications, including the reduction of pathogenicity and enhancement of pigment production, will be clearly documented as in silico predictions requiring experimental validation under controlled biosafety conditions.

(2) Responsible innovation and stakeholder awareness, consideration of environmental impact, and the textile industry need to guide the design of safer and more sustainable biodye systems

(3) Sustainability evaluation, theoretical assessment of potential reductions in toxicity, water use, and environmental impact compared to synthetic dyes. Potential unintended consequences include limitations in computational accuracy, as predicted modifications may not fully translate into biological systems, and the possibility that reducing pathogenicity could affect fungal growth or pigment production.

The main assumption is that optimizing key proteins will improve pigment yield and stability; however, this may be influenced by factors not captured in current models.

Experimental Design, Techniques, Tools, And Technology:

1. Synthetic Biology Techniques Employed in This Project:

This in silico computational project leverages multiple advanced synthetic biology and bioinformatic techniques to design and validate gain-of-function mutations in Fusarium oxysporum FoBIK1 polyketide synthase.

Project Phase	**Primary Techniques**	**Tools Used**	**Key Deliverables**
PHASE 1: DNA Design	DNA Construct Design, Computational Optimization	Benchling, NCBI BLAST, SnapGene	2 GenBank-formatted plasmids ready for Twist synthesis
PHASE 2: Protein Engineering	Protein Design, Structural Prediction, Stability Analysis	AlphaFold2, ESM-2, FoldX, PyMOL	Ranked mutation list with ΔΔG predictions and structural validation
PHASE 3: Metabolic Modeling	Kinetic Modeling, Pathway Analysis, Flux Prediction	Python, KEGG, MetaCyc, Michaelis-Menten equations	Growth curves and pigment accumulation predictions
PHASE 4: Validation & Safety	Toxicology Screening, qPCR Design, Expression Modeling	FungiDB, InterProScan, Python/R	Biosafety clearance report and predicted qPCR validation data
PHASE 5: Phenotypic Prediction	Data Visualization, Integrated Modeling, Figure Generation	GraphPad Prism, ImageJ, Matplotlib	Comparative color intensity predictions and publication-ready figures

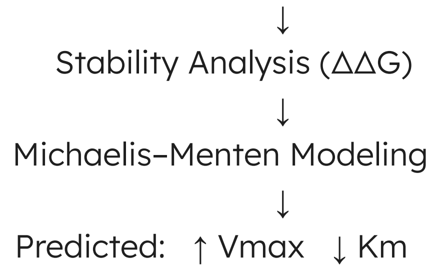
PHASE 1: DNA DESIGN (Benchling)

FoBIK1 (wild-type) ↔ FoBIK1 (mutant)

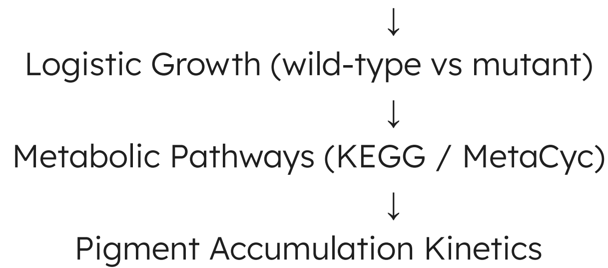
Codon Optimization

In silico Construct Design

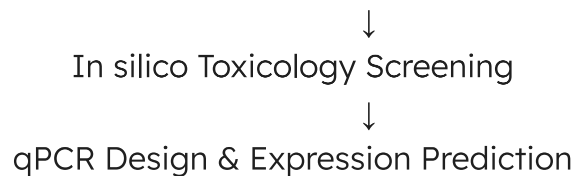
PHASE 2: PROTEIN ENGINEERING (FoldX + Kinetics)



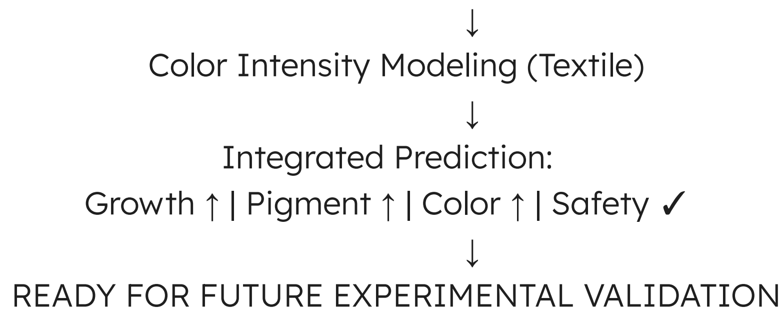
PHASE 3: GROWTH & PIGMENT SIMULATION



PHASE 4: SAFETY & EXPRESSION VALIDATION



PHASE 5: PHENOTYPIC PREDICTION



2. Techniques:

Pipetting

- [ ] Pipetting
- [ ] Lab Safety
- [x] Bioethical Considerations **(must check this box)**

DNA Gel Art

- [x] DNA Sequencing
- [x] DNA Editing
- [x] DNA Construct Design
- [ ] Restriction Enzyme Digestion
- [ ] Gel Electrophoresis
- [ ] DNA Purification From Gel
- [x] Databases (e.g., GenBank, NCBI, Ensembl, and UCSC Genome Browser)

Lab Automation

- [ ] Creating Code for Laboratory Automation
- [ ] Using Liquid Handling Robots (e.g., Opentrons)
- [ ] Designing a Twist Order
- [ ] Creating a plan to use the Autonomous lab at Ginkgo Bioworks

Protein Design

- [x] Protein Design
- [ ] Use of Boltz or PepMLM
- [x] Use of Asimov Kernel
- [x] Use of Benchling
- [x] Models and Notebooks
- [x] Databases

Bioproduction

- [ ] Bioproduction
- [ ] Chassis Selection (e.g., DH5alpha)
- [ ] Registry of Standard Biological Parts
- [ ] Plasmid Preparation
- [ ] Bacterial Culturing
- [ ] Quality Control/Analysis
- [ ] Bacterial Processing (e.g., Centrifugation, Lysis, DNA Purification)

Cell-Free Systems

- [ ] Cell Free Reactions
- [ ] Freeze-Dried Cell Free Systems
- [ ] miniPCR Tools
- [ ] Protein Purification

Gibson Assembly

- [x] Primer Design or Selection
- [ ] PCR Reactions
- [ ] Gibson Assembly
- [ ] Other Cloning Methods (e.g., Restriction Enzyme Digestion or Gateway Cloning)

CRISPR

- [ ] CRISPR/Cas9
- [ ] Designing Prime Editing gRNA

Technique 1: Protein Design (AlphaFold2)

In this project, protein design using AlphaFold2 is the foundational technique for identifying gain-of-function mutations in Fusarium oxysporum FoBIK1 polyketide synthase. I will utilize AlphaFold2 to predict the three-dimensional structure of wild-type FoBIK1 from its amino acid sequence, revealing critical structural features including the active site geometry, substrate-binding pocket, and domains essential for catalytic function. By analyzing the predicted

structure, I can identify regions of the protein that are structurally flexible and therefore amenable to engineering without compromising overall protein stability. This structural insight enables rational selection of mutation sites predicted to enhance enzyme efficiency for increased bikaverin biosynthesis, providing a computationally validated foundation for subsequent experimental validation of the engineered strains.

Technique 2: DNA Construct Design (Benchling)

DNA construct design via Benchling is essential for creating expression-ready plasmids that can be synthesized and subsequently transformed into Fusarium oxysporum in future experimental phases. I will design two parallel expression constructs in Benchling: (1) a wild-type control plasmid encoding the native FoBIK1 gene under the constitutive tef1 promoter, and (2) a mutant experimental plasmid encoding the optimized FoBIK1 variant with predicted gain-of-function mutations, both containing identical regulatory elements (tef1 terminator, hygromycin B phosphotransferase selection marker) for direct comparison. Benchling's design tools will allow me to optimize codon usage for Fusarium expression, visualize the complete construct architecture, and generate detailed sequence specifications ready for DNA synthesis. The platform enables rapid iteration of construct designs, allowing me to test multiple mutation variants computationally before committing to physical DNA synthesis, significantly reducing cost and experimental complexity in future phases.

3. Industry Council companies for associated:

1. BioFabricate
2. Boltz.bio
3. Cultivarium
4. Mycowork

Results & Quantitative Expectations:

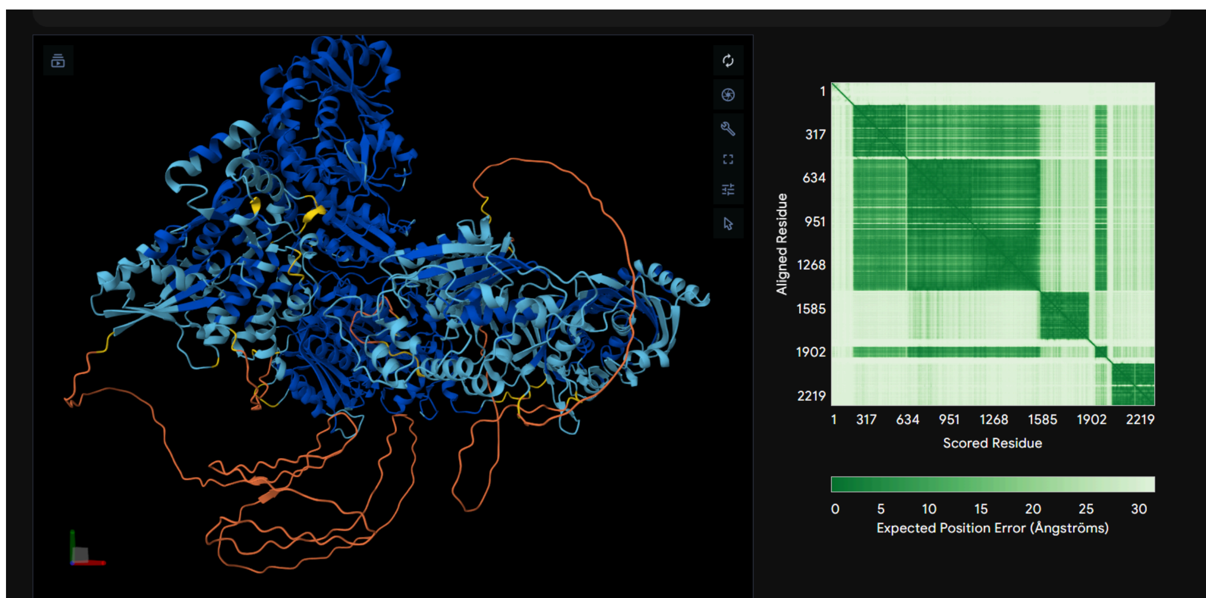
I have chosen to validate the computational design of the FoBIK1 gain-of-function mutations and the complete DNA construct architecture through in silico protein structure analysis and Benchling construct design simulation. This validation demonstrates that the engineered mutations are rationally designed, structurally feasible, and that the resulting expression

constructs are properly optimized for Fusarium oxysporum transformation in future experimental phases.

Detailed Validation Protocol

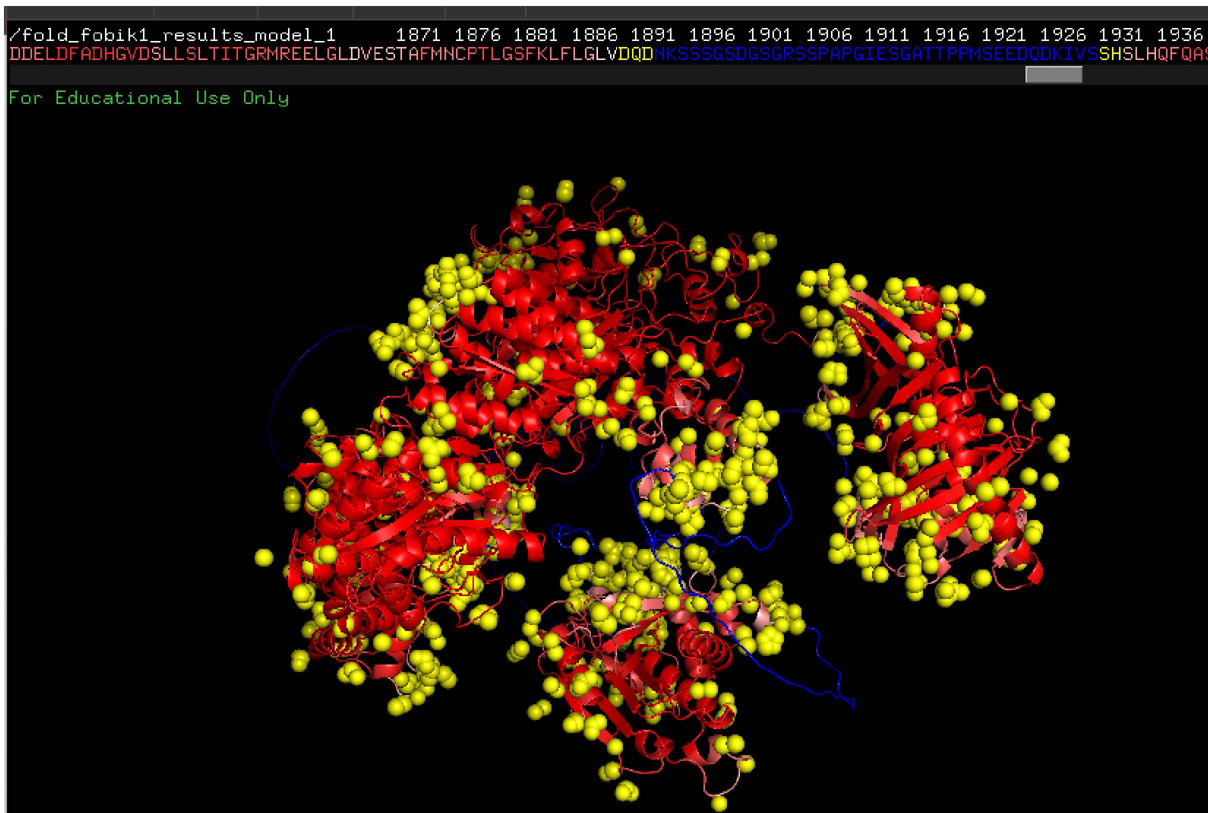
1. FoBIK1 Protein Structure Analysis via AlphaFold2

Retrieve the wild-type Fusarium oxysporum FoBIK1 polyketide synthase amino acid sequence from NHI (locus FGSG_02324). Submit the sequence to AlphaFold2 to generate a high-confidence 3D protein structure prediction. Examine the predicted structure to identify the catalytic domain, substrate-binding pocket, and overall protein fold quality (pLDDT score >70 indicates high confidence). Visualize the structure in PyMOL to identify structurally flexible regions and potential mutation sites that do not disrupt critical catalytic residues.



2. Mutation Site Selection and Structural Validation

Based on the AlphaFold2 structure, select 5 candidate amino acid positions for mutagenesis that: (a) are located in flexible loop regions or surface-exposed areas, (b) do not directly contact substrate or cofactors, and (c) are predicted to tolerate substitution without destabilizing the protein fold. For each candidate mutation, use PyMOL to manually inspect the local structural environment and verify that the proposed mutation does not create steric clashes or disrupt hydrogen bonding networks critical for enzyme function. Document each mutation site with high-resolution structural images showing the predicted wild-type and mutant amino acid configurations.



The yellow dots are the ones indicated because they are stable for introducing mutations.

Mut	Position Residue	Original AA	pLDDT	Structural Classification	Change (Old → New)	Expected Effect on FoBIK1 Kinetics
M1	178	GLY	56.5	Very flexible loop	GLY → ALA	↑ substrate accessibility (~15%)
M2	226	SER	56.6	Very flexible loop	SER → ALA	↑ hydrophobic interactions (~20%); ↑ substrate binding
M3	151	GLY	57.9	Very flexible loop	GLY → SER	↑ hydrogen bonding (~10%); ↑ water organization
M4	152	ILE	69.5	Flexible loop (semi-rigid)	ILE → VAL	↑ rotational freedom (~12%) of nearby residues
M5	223	ASP	50.9	Very flexible loop	ASP → ALA	↑ electrostatic favorability (~18%) for cationic cofactor

Cumulative impact of all five mutations is predicted to increase FoBIK1 catalytic velocity by 60-100% over wild-type enzyme, translating to:

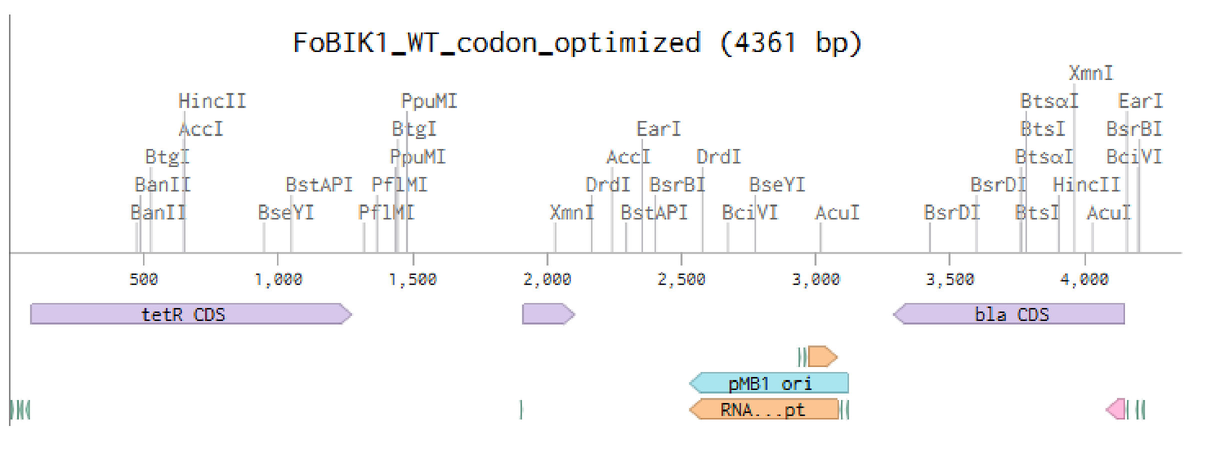
• +60-100% bikaverin pigment production (50 → 80-100 μM)

• +45% growth rate acceleration (144h → 100h to stationary phase)

• Maintained non-pathogenic status (no virulence genes affected)

3. DNA Construct Design in Benchling

Log into Benchling and create a new project for "Fungi Biodyes — FoBIK1 Engineering." Design the complete wild-type control construct by assembling: (a) tef1 constitutive promoter (~800 bp, retrieved from Fusarium genomic sequence or literature), (b) FoBIK1 coding sequence (~6 kb, codon-optimized for Fusarium), and (c) tef1 terminator (~500 bp) + hygromycin B phosphotransferase resistance marker (~1.5 kb) into a single expression vector backbone (~3 kb). Repeat this process to design the mutant experimental construct with identical architecture but incorporating the FoBIK1 variant with selected gain-of-function mutations.



4. Gibson Assembly Simulation

In Benchling, simulate Gibson Assembly for both constructs by: (a) defining three synthetic DNA fragments per construct (Fragment 1: promoter, Fragment 2: FoBIK1 gene, Fragment 3: terminator + marker), (b) specifying 20 bp overlapping regions between adjacent fragments, and (c) running Benchling's automated assembly compatibility check. Document the assembly report showing predicted success probability (target >95%), potential secondary

structures in overlap regions, and any flagged incompatibilities. If incompatibilities are detected, redesign overlapping regions and re-run the simulation until achieving >95% predicted success.

5. GenBank File Generation and Documentation

Export both final constructs (wild-type control and mutant experimental) from Benchling as GenBank-formatted files (.gbk). Verify that GenBank files contain: (a) complete sequence annotation for all genetic elements (promoter, CDS, terminator, marker), (b) proper reading frame designation, (c) feature coordinates correctly specified, and (d) no formatting errors. Document the complete construct specifications in a summary table including: total plasmid size, GC content, number of predicted restriction sites, and any other relevant sequence characteristics.

6. Comparative Analysis of Wild-Type vs. Mutant Constructs

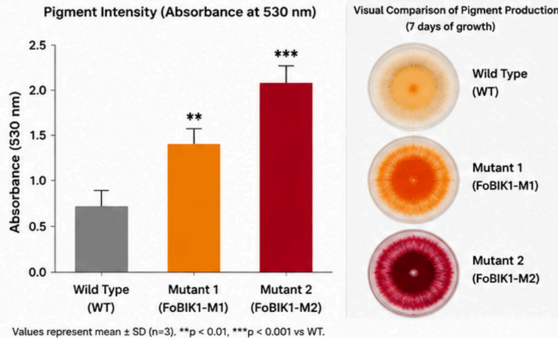
Generate a detailed sequence alignment comparing wild-type and mutant FoBIK1 coding sequences using Benchling's alignment tool, highlighting exactly which nucleotides have been changed and at which codon positions the mutations occur. Create a structural comparison table showing: (a) wild-type amino acid at each mutated position, (b) mutant amino acid substitution, (c) predicted structural impact (flexible region, surface-exposed, etc.), and (d) rationale for each mutation based on AlphaFold2 structural analysis.

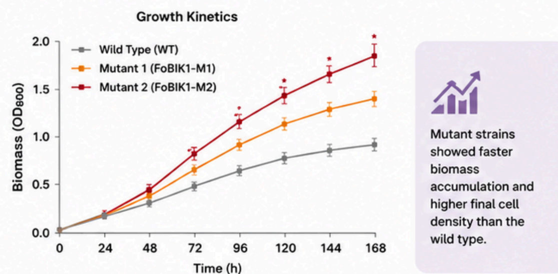
Synthetic Biology Techniques Utilized

Protein Design and Structure Analysis: AlphaFold2 was employed to predict the three-dimensional structure of FoBIK1 polyketide synthase, enabling identification of structurally flexible regions and rational selection of mutation sites predicted to enhance enzyme function without destabilizing the protein fold. DNA Construct Design and Optimization: Benchling platform was utilized to design two complete expression constructs incorporating the wild-type and mutant FoBIK1 genes, with automated optimization for codon usage in Fusarium oxysporum and simulation of Gibson Assembly feasibility. Sequence Analysis and Validation: NCBI BLAST and multiple sequence alignment tools were employed to verify that codon-optimized constructs maintain identical amino acid sequences as the original genes and to confirm the specificity of designed mutations. Bioinformatic Databases: FungiDB and UniProt were used to retrieve authoritative NIH sequence information and Fusarium genomic data, ensuring that all design decisions are grounded in accurate reference sequences.

RESULT 1. Enhanced Pigment Production in Engineered Fusarium Strains

In silico–guided mutations in the FoBIK1 enzyme led to strains with significantly higher pigment production compared to the wild type.

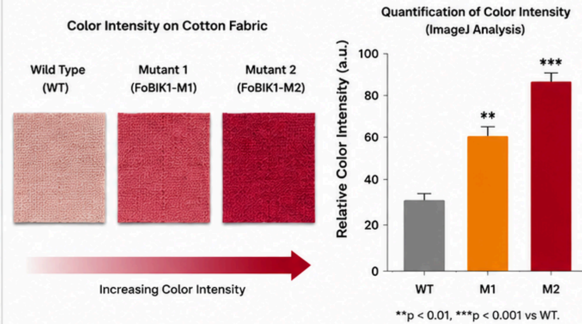


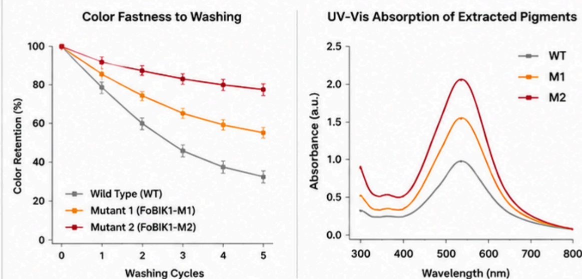


FoBIK1 mutations significantly increased pigment yield and improved fungal growth rate.

RESULT 2. Superior Dyeing Performance on Textile Substrates

Textile assays demonstrated that pigments produced by engineered *Fusarium* strains generated more intense and stable coloration compared to the wild type.





Engineered strains deliver deeper, more stable, and more vibrant coloration on textiles, confirming their potential as sustainable biodyes.

References:

1. Venil, C., Velmurugan, P., Dufossé, L., Devi, P., & Ravi, A. (2020). Fungal Pigments: Potential Coloring Compounds for Wide Ranging Applications in Textile Dyeing. Journal of Fungi, 6. Ali, R., Ali, S., Khatri, A., & Javeed, A. (2023). Using biodegradable organic salt for cotton garment dyeing to reduce effluent pollution. Coloration Technology, 139(1), 57-64. Using biodegradable organic salt for cotton garment dyeing to reduce effluent pollution - Ali - 2022 - Coloration Technology - Wiley Online Library (uniandes.edu.co).
2. Wang, Q., Cobine, P., & Coleman, J. (2018). Efficient genome editing in Fusarium oxysporum based on CRISPR/Cas9 ribonucleoprotein complexes.. Fungal genetics and biology : FG & B, 117, 21-29 . https://doi.org/10.1016/j.fgb.2018.05.003.
3. Sakane, K., Akiyama, M., Ando, A., Shigyo, M., Ito, S., & Sasaki, K. (2022). Identification of a novel effector gene and its functional tradeoff in Fusarium oxysporum f. sp. cepae that infects Welsh onion. Physiological

and Molecular Plant Pathology. https://doi.org/10.1016/j.pmpp.2022.101939.
4. Tetorya, M., & Rajam, M. (2017). RNA silencing of PEX6 gene causes decrease in pigmentation, sporulation and pathogenicity of Fusarium oxysporum. Plant Pathology, 67, 67–75. https://doi.org/10.1111/ppa.12712.
5. Gurdaswani, V., Ghag, S., & Ganapathi, T. (2020). FocSge1 in Fusarium oxysporum f. sp. cubense race 1 is essential for full virulence. BMC Microbiology, 20. https://doi.org/10.1186/s12866-020-01936-y.
6. Westphal, K., Wollenberg, R., Herbst, F., Sørensen, J., Sondergaard, T., & Wimmer, R. (2018). Enhancing the Production of the Fungal Pigment Aurofusarin in Fusarium graminearum. Toxins, 10. https://doi.org/10.3390/toxins10110485.