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Harnessing Natures Palette: Bioengineering Yeast for Sustainable Textile Dyeing with Betalains

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The fast-paced digital era has sparked an alarming surge of micro-trends in the fashion industry. Many companies prioritize mass production of textiles to fuel this culture of disposable fashion. This mass production of dyed textiles relies heavily on the use of synthetic azo dyes and, in some processes, the use of heavy metals such as lead, mercury, and chromium VI. Consequently, our planet's landfills are inundated with an overwhelming volume of clothes that can leach toxic or carcinogenic chemicals into the environment. In response, scientists are researching sustainable alternatives that can harness living organisms to produce biosynthetic dyes that have less environmental impact. To contribute to this research effort, I aimed to develop a bioengineered strain of yeast that expresses red pigments called Betalains, a gene derived from beetroots. To achieve this, I cloned a three-enzyme polyprotein (RUBY) comprised of the requisite enzymes to express Betalains from a construct made for plant-based production into a plasmid for yeast expression. The expressed biosynthetic dye was then purified and its fabric-standing properties were characterized. Ultimately, this bioengineered yeast strain can be used to mass produce the red biosynthetic dye, Betalain, which could replace environmentally harmful synthetic dyes. This research project serves as a proof-of-concept study that could lay the foundation for broader adoption of more sustainable and ethically conscious textile dyeing processes for decades to come.

With the increase of fast fashion, people are increasingly aware of the textile industry's environmental impact. This impact includes the discharge of large quantities of chemical pollutants into the environment, such as sulphur, naphthol, vat dyes, nitrates, acetic acid, soaps, enzymes chromium compounds and heavy metals like copper, arsenic, lead, cadmium, mercury, nickel, and cobalt.1It is estimated that textile dyeing and finishing treatments contribute to 20% of the industrial wastewater pollution. Among the toxic chemicals in wastewater from textile producers, 72 substances have been detected, and due to inadequate effluent treatment methods, 30 of these cannot be removed.[1,2] If we look back 168 years before William H. Perkins developed the first synthetic, mass-produced chemical dye, we can see the potential to change the future with eco-friendly dyes derived from microorganisms, fungi, and plants.[3]

Turning to nature, inspiration is found in the wide pallet of color pigments beet plants provide, from red to violet and yellow. This project sought to take advantage of a pigment pathway in beets and clone the required enzymes into yeast cells.

Beetroot stands out as a notable source of betalains, including vivid red betacyanins and yellow betaxanthins. Derived from betalamic acid, betalains are nitrogen-containing compounds exclusive to plants within the Caryophyllales order. These water-soluble chromoalkaloids are primarily located within plant vacuoles, defining the unique coloration of Caryophyllales species. [4–10]

Many genes responsible for the biosynthesis of pigments have already been cloned, and are available, including those found in the UBQ: RUBY plasmid created by Zhang et al. The biosynthesis of betalains can be broken down into three required enzymatic reactions to convert the amino acid tyrosine to betalain. The three betalain biosynthetic genes required for this, Cytochrome P450 76AD1, l-DOPA 4,5-dioxygenase (DODA), and Glucosyltransferase were fused into a single open reading frame called RUBY, which has been expressed using a single promoter and terminator.[11,12]

The goal of this work was to bioengineer a yeast strain that produces betalain as a sustainable pigment for use as a dye. We obtained a version of the pPICZc yeast expression vector, MtPT4, consists of an AXO1 promoter and terminator flanking a multiple cloning site bearing the MtPT4 gene from Addgene.[13] Given the availability of the amino acid tyrosine, it was hypothesized that the RUBY cassette could be cloned into the pPICZc plasmid in place of the MtPT4 gene for expression of these enzymes in yeast to direct the synthesis of the red pigment, betalain.

The use of yeast over plants offers many benefits. Plant have a number of drawbacks including limited availability yearround and sensitivity to environmental conditions. Furthermore, extensive plant use may jeopardize a valuable species .[14] Production of pigments in yeast boasts advantages like easy and rapid growth in cost-effective mediums, simple processing methods, and independence from weather conditions, making them a compelling option for sustainable pigment production.[14]

Natural dyes often offer a limited and dull color range, low colorfastness, and no guarantee of safety over their synthetic counterparts. Their safety depends on factors like the mordant used which allows garments to maintain color. For example, the mordant chromium, is highly toxic and can significantly impact wastewater quality. We found a plant-based mordant alternative using the symplocos plant from Indonesia.

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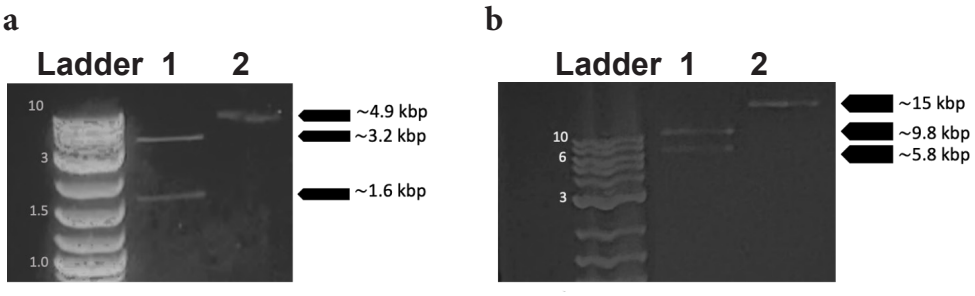

Figure 1 | Gel electrophoresis of restriction digests for plasmid verification of MtPT4 and UBQ:RUBY.
Agarose gel electrophoresis of **(a)** MtPT4 on a 1.5% gel. Lane 1 cut with restriction enzymes EcoRI and XhoI, lane 2 uncut and **(b)** UBQ:RUBY on a 1% gel. Lane 1 cut with XhoI, and HindIII, lane 2 uncut

Fallen leaves from symplocos are abundant in alum content, which is commonly used to treat cellulose fibers like cotton. Many beautiful, vibrant, and permanent colors can be achieved using only alum, a relatively safe mordant. Powdered leaves from symplocos trees offer a sustainable replacement for mined aluminum sulfate and alum acetate used in traditional dye recipes. This innovation not only expands the color palette but also reduces reliance on potentially harmful mordants like chromium, contributing to eco-friendlier dyeing practices. 1,[16–19]

The future of sustainable fashion is upon us, requiring exploration for eco-friendly replacements to synthetic dyes and mined mordants. As an alternative to plant-extracted betalains production of individual betalains by genetically engineered yeast could facilitate the continuous, controlled production of pigment with greatly reduced environmental dependencies and impacts. [11,20]

Materials and Methods

Plasmid growth and verification of MtPT4 and UBQ:RUBY

The UBQ:RUBY plasmid was a gift from Yunde Zhao via Addgene (plasmid # 160909).21 MtPT4 was a gift from Geoffrey Chang via Addgene (plasmid # 117096).13 Both plasmids were grown from separate bacterial stabs: MtPT4 was grown on Lennox-LB plates supplemented with 50 µg/mL Zeocin (ThermoFisher Scienctific, Cat. No. R25001), while UBQ:RUBY was grown on Miller-LB (Fisher Bioreagents Cat. No. BP1425-500) plates supplemented with 50 µg/mL Spectinomycin (ThermoFisher Scientific Cat. No. J66034-03).

Bacterial incubations were overnight at 37°C with shaking at 220 rpm. Single colonies, of each plasmid, were selected from their agar plate for bacterial cultures. The Monarch Miniprep Kit (NEB Cat. No. T1010S) was used to purify DNA from the liquid cultures and all plasmid isolations were verified by restriction digests.

The restriction enzymes XhoI (Cat. No. R0146S), EcoRI (Cat. No. R0101S), and HindIII (Cat. No. R0104S) were obtained from New England Biolabs. Digestion products were analyzed by agarose gel electrophoresis using a 2 and 1.2% agarose (Fisher Bioreagents Lot. No. 188373) stained with ethidium bromide. Gel electrophoresis was conducted at 100 V for 45 minutes in 1x TAE buffer. The resulting DNA fragments were visualized under UV light. The digestion pattern/bands were compared with an expected pattern based on the plasmid map and a 1 kb DNA Ladder (NEB Cat. No. N0550S).

DNA manipulation of RUBY construct from UBQ:RUBY plasmid

The RUBY construct (bases 2,140-6,096) was PCR amplified using a Mastercycler Nexus Gradient from Eppendorf (serial no. 6331ER710937) The PCR reaction mixture consisted of a forward cloning primer, RUBYf, (Integrated DNA Tech Cat. No 472031126), 5'-CC-ATGGATCTGCGAC-CCTCGCCATGATCC-3', reverse cloning primer, RUBYr (Integrated DNA Tech Cat. No. 472031127), 5'-GCGGCCGC-CTAT-CACTGGAGGCTTGGCTCAAG-3', for a 1 µM concentration of each, 1 µL Taq Polymerase (Promega Cat. No. M712B), and 1.5 µL (~10ng) of UBQ:RUBY DNA from the miniprep mentioned previously. The cycling conditions were as followed: initial denature at 95°C for 2 minutes, followed by 30 cycles of denaturing at 95°C for 15 seconds, annealing was at 53°C for 15 seconds, and extension at 72°C for 5 minutes. A final extension step was run at 72°C for 1 minute. The PCR products were analyzed by gel electrophoresis using a 1.5% agarose gel in 1x TAE. The product bands were excised and purified using the Monarch Gel Purification kit (NEB Cat. No. T1020S).

Generation of Betalains

Betalains were extracted from the beet plant by washing and peeling the top layers then mashed in a mortar pestle. 10g of beet peels were subjected to a nonpolar extraction in a beaker with 100 mL of MeOH: H2O (80/20, v/v) as the matrix. The beaker was wrapped in tinfoil and left to incubate in an orbital shaker for 120 minutes at 45°Cshaking at 100 rpm. The liquid was filtered using a Q8 filter paper (Fisher Scientific Cat No.09-790-B). The polar extraction was done by blending 100g of beets with Reverse Osmosis (RO) water to create a homogenous mixture and spilt into four 50 mL conical tubes. All tubes were centrifuged for 30 minutes at 3500 rpm to form a pellet then the supernatant was filtered with Q8 filter paper. Both extraction methods underwent a 2-fold dilution scheme a total of seven times in a 96-well plate before an absorbance spectrum from 350nm to 750nm was ran using a spectrophotometer (Molecular devices SpectraMax M3).

Biphasic Extraction and Crude Purification of Betaxanthins

10g of beet peels were weighed and submerged in a beaker with 50 mL of RO water and 50 mL of chloroform. The solution was covered in tinfoil, mixed, and left to form two layers while incubating in an orbital shaker for 90 minutes at 45°C shaking at 100 rpm. The top organic layer containing betaxanthins was filtered with Q8 filter paper into a 50 mL Erlenmeyer flask for further purification by evaporating the chloroform at 100°C to create a crude product. This was reconstituted in 10 mL of RO water. The betaxanthin's underwent the same dilution scheme and absorbance spectrum as above.

Polar Extraction of Betacycanins

10g of beet peels were weighed and submerged in a beaker with 100 mL of DI water and covered in tinfoil to incubate in an orbital shaker for 90 minutes at 45°C shaking at 100 rpm. After incubation the liquid

was filtered with Q8 filter paper to remove any insoluble materials. The betacycanins underwent the same dilution scheme and absorbance spectrum described above.

Symplocos mordant treatment and beet dye

This method established four groups; fabric 1: untreated fabric, fabric 2: symplocos treated fabric, fabric 3: untreated dyed fabric, fabric 4: dyed symplocos treated fabric. The mordant was made by boiling 22g of symplocos in water for an hour. Fabrics 2&4 were submerged in the pot with the symplocos to simmer for an hour, rung out, rinsed to remove any residual powder, and air dried. The beet dye was generated by boiling chopped, fresh beets for an hour. Beets were removed, leaving a solution of pigments. Fabrics 3&4 were submerged in the beet dye for an hour, then left to dry and rinsed with warm sink water the next day.

Results

Confirmation of Plasmid Identity via restriction digest and gel electrophoresis

To ensure the integrity of our experimental materials, a thorough verification of the identity of the two plasmids was crucial to our bioengineering of the yeast. Utilizing restriction digest and gel electrophoresis techniques, we confirmed the identity of the MtPT4 and UBQ: RUBY plasmids, by generating restriction fragments consistent with the pattern expected for these vectors. MtPT4 was digested with EcoRI and XhoI then electrophoresis of the digest was performed on an agarose gel against the 1kbp+ DNA ladder and imaged under UV light. As expected, two bands in lane 1 were observed at 3.2 kbp and 1.6 kbp on the gel **(Figure 1a)**. UBQ:RUBY was digested with HindIII and XhoI then followed the same visualization steps as MtPT4. As expected, two bands in lane 1 were observed at 9.8 kbp and 5.8 kbp on the gel **(Figure 1b)**. For each digest an uncut sample of the plasmid was run in lane 2 to serve as a control and reference point. These results help confirm that each plasmid of interest is present and suitable for further manipulation.

RUBY cassette amplification via PCR and gel electrophoresis

The UBQ:RUBY plasmid was engineered for betalanin synthesis in plants cells via the enzymes contained in the RUBY cassette, Cytochrome P450 76AD1, l-DOPA 4,5-dioxygenase (DODA), and Glucosyltransferase. We amplified this cassette for cloning into a yeast expression vector (MtPT4) for betalanin production in yeast cells. PCR amplification targeting bases 2140 to 6096 of the RUBY cassette yielded a distinct band of 3965 base pairs on gel electrophoresis, indicative of successful amplification **(Figure 2)**.

Qualitative Analysis of betalains presented in beets using different extraction techniques via spectrophotometer

The qualitative analysis of betalains and its subclasses was performed using extractions using both polar and nonpolar techniques, followed by the measurement of absorbance between 350 and 750 nm at 10 nm intervals on the spectrophotometer. Betalain was first extracted with a nonpolar technique by incubating beet peels in MeOH: H2O (80/20, v/v) at 45°C for 120 minutes while shaking at 100 rpm. Two distinct peaks are observed for the two subclasses of pigment. The higher absorbance peak at 480 nm corresponds to betaxanthin, while the lower peak at 540 nm corresponds with betacyanin **(Figure 3a).** The presence of these two peaks in the spectrum was consistent with expected data confirming a successful extraction of both betacyanin and betaxanthin. The second betalain extraction method used a polar technique by blending beets with water to create a homogenous mixture that was centrifuged at 3350 rpm for 30 minutes then filtered. We expected to visualize peaks on the absorbance spectrum to reflect the differential polarity of these substances compared to the previous non-polar extraction. As predicted the betaxanthin peak at 480 nm was slightly smaller than the betacyanin peak at 540 nm (**Figure 3b**). Overall, these results suggest that both betalain molecules were present in a raw extract from beets. To further investigate the subclasses of pigments in beets, betaxanthin and betacyanin, were differentially purified with two different extraction techniques developed based on chemical structures and their respective polarities. Of the two subclasses, betacyanin is more polar, due to the sugar moiety, making it favorable in a polar extraction method with an expected single peak at 540nm based on data from the second extraction method above. Betacyanin was extracted by incubating beet peels in water at °C for 90 minutes while shaking at 100 rpm. Another absorbance spectrum was run. As expected, one large peak at 540nm was observed, indicating a successful isolation via extraction **(Figure 3c)**. Conversely, a nonpolar organic solvent will favor the extraction of betaxanthin over betacyanin. Betaxanthin was extracted through a biphasic extraction, with equal parts chloroform and water, following the same incubation steps as betacyanin. The organic phase of chloroform, containing betaxanthin, was removed, filtered, and heated to 100°C to evaporate the chloroform, resulting in a crude purification of betaxanthin. This was reconstituted in water and reanalyzed by spectrophotometry. The absorbance spectrum resulted in a large peak at 480nm, as expected based on the data from the first extraction, the slight shoulder observed shows this extraction method didn't result in a pure product **(Figure 3d)**.

Dyeing cotton with Symplocos mordant and beet dye

The treatment of fabric with a mordant, typically a heavy metal, is a crucial step for a garment's colorfastness. Here, the typical heavy metal mordant was replaced with one derived by the symplocos plant from Indonesia. The symplocos plant represents a sustainable source of alum, used as a mordant by grinding the leaves into a fine powder. To treat fabric, a symplocos bath was made by boiling the symplocos powder at 60°C for an hour. Fabric 2 was treated by simmering in the symplocos bath, allowing the alum to etch away at the fabric's fibers exhibiting in a tinted pale-yellow hue. Upon comparison with the untreated fabric

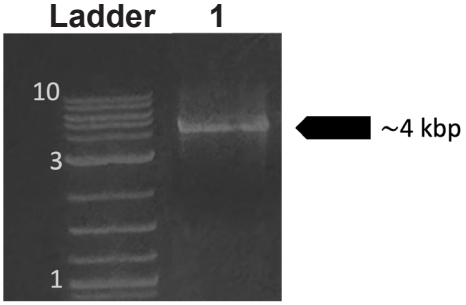

Figure 2 | The RUBY fragment was amplified properly through PCR. Agarose gel electrophoresis of RUBY PCR fragment amplified from UBQ:RUBY plasmid using Ruby forward and reverse primers. Electrophoresis. was performed on a 1.5% gel. A 1 kb plus DNA ladder was used. The single band at 4 kb confirms the RUBY cassette was amplified during PCR.

1 we can confirm the symplocos mordant treatment was successful **(Figure 4a)**. The beet dye was made by boiling chopped beets for an hour then removing it from the heat. Fabric 4 was treated with the mordant as described for Fabric 2 above. While fabric 4 dried, fabric 3 was submerged in the beet dye bath for an hour and rinsed with warm water until the water ran clear then air dried. After fabric 4 was dry it underwent the same dyeing steps as fabric 3. A comparison of the fabric samples revealed that fabric 4 showed superior retention of red pigment, observed by the brighter hue compared to the salmon color of fabric 3 **(Figure 4b)**. This was also evident by the shorter rinse time until the water ran clear. The enhanced colorfastness in fabric 4 can be attributed to the formation of a coordination complex between fabrics fibers and the beet dye facilitated by the alum treatment in the symplocos mordant. These results suggest the symplocos plant-based could serve as a good replacement for conventional heavy-metal dyes.

Discussion

Beets are known to produce betalain, which serves as basis for the RUBY cassette, utilized in this experiment. To establish a refence for my bioengineered yeast dye, the spectral properties of dye extracted from beets was characterized. Whole beets were obtained from a local grocery store to be washed, peeled, chopped, and blended. The two expected betalain pigment types were successfully extracted with absorbance maxima for betacyanin at 540 nm and betaxanthin at 480 nm.[4,5,8,9,20]

We examined manipulating betalain production yields to obtain different color pigments from a single source. Through analysis of the chemical structures of betaxanthin and betacyanin, two techniques were created to develop to standardize each specific class. The polar extraction for betacyanin was successful, while the biphasic extraction for betaxanthin needs to be further investigated to create a pure product, potentially with a second round of extraction.

Continuing the analysis, we investigated whether the pigment was

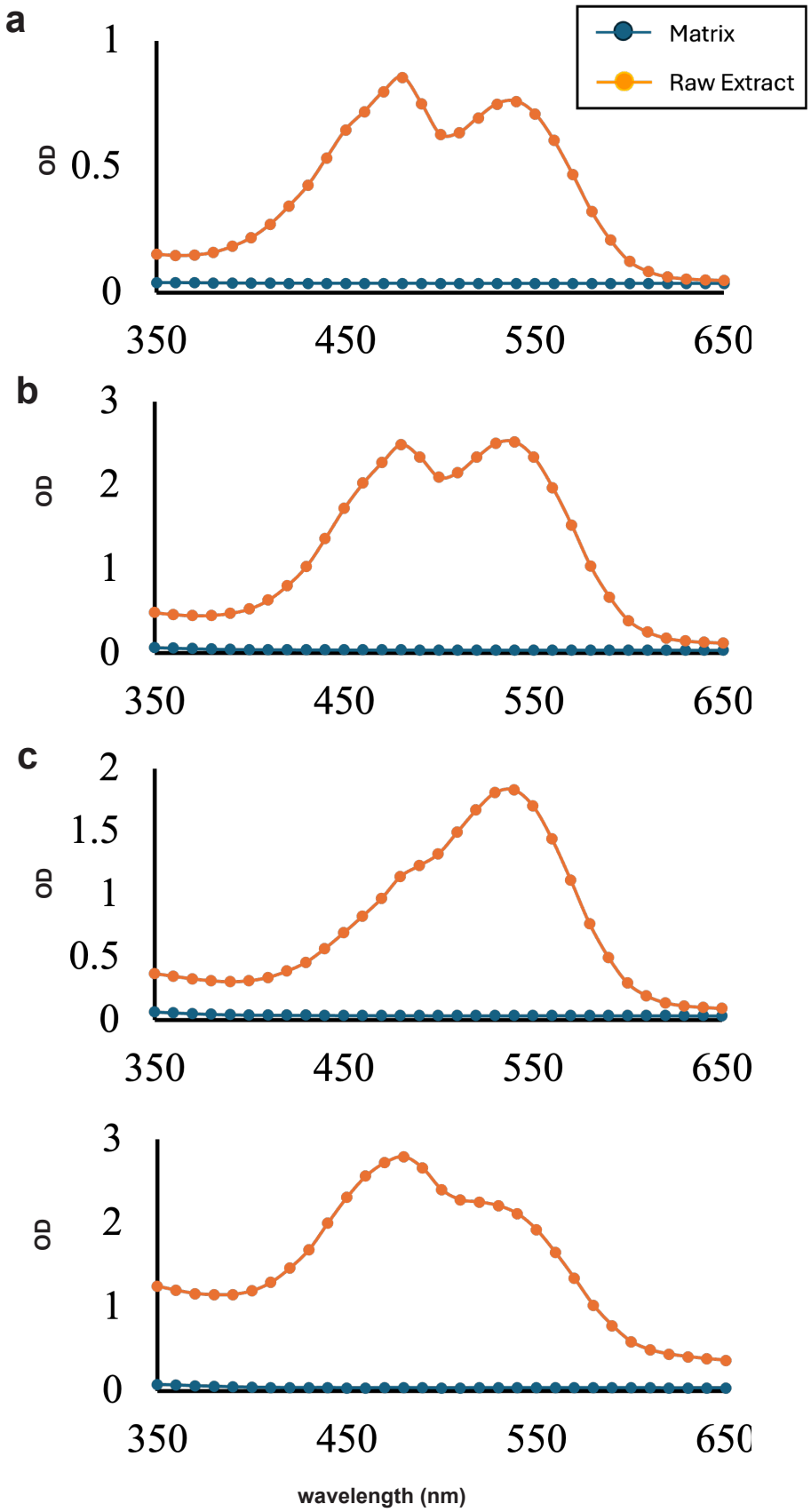

Figure 3 | Qualitative Analysis of betalains present in beets using different extraction techniques via spectrophotometer. 150 µL of each pigment classes, extracted from beet peels, underwent a 2-fold dilution scheme six times in a 96-well plate using the proper matrix. An absorbance spectrum from 350nm until 750nm was ran on a spectrophotometer, picking the best dilution factor (DF) to represent the data (a) Nonpolar extraction; 1:2 dil. of betalains extracted with a nonpolar matrix of MeOH: H2O (80/20, v/v) from peeled beets (b) Polar extraction; 1:8 dil. of betalains extracted with a polar matrix of di-water from blended beets (c) 1:8 dil. of betacyanins extracted with a polar matrix of di-water from peeled beets (d) 1:16 dil. of betaxanthins extracted with chlorofom during a bipahsic extraction then purfied and reconstiituted in water.

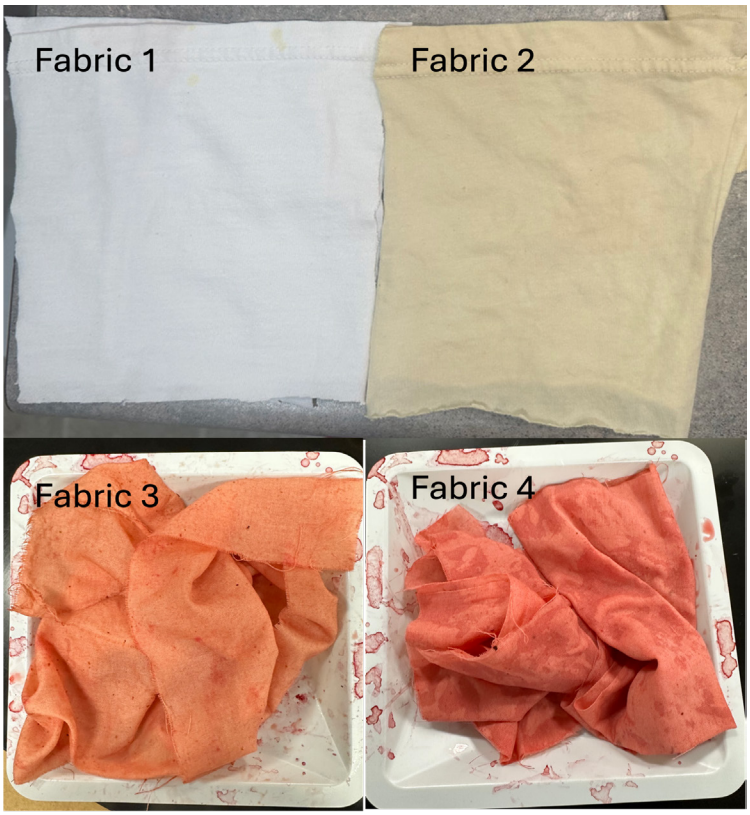

Figure 4 |Dyeing cotton with symplocos mordant and beet dye.
Comparison of 22 grams of fabric treated with or without symplocos then dyed. Untreated samples on the left, treated on the right (a) Undyed comparison to test symplocos mordant; fabric 1 untreated, fabric 2 was treated for an hour in the symplocos mordant bath (b) comparison of dyed fabric that had been treated against fabric that hasn't, both fabric samples were dyed in the beet bath for 1 hour; fabric 3 untreated and dyed appears more salmon and dull, fabric 4 treated with symplocos and dyed appears brighter red

pH-dependent by exposing the liquid from polar and nonpolar extractions to various pH buffers ranging from 1 to 12. Due to the stability of betalain within the pH range 3 to 7 [4,8,9,22], the only observed color change occurred at pH of 12. Suggesting the pigment is pH-dependent where pH 12 marked the transition from red and purple to orange to yellow (data not shown).

The plant-based mordant, symplocos, was obtained as dried leaf powder from the forests of Indonesia. The yellow-tinted fabric and enhanced colorfastness observed indicates symplocos offers an alternative for a commercially available alum mordant. By using the fallen leaves, we supported a sustainable crop rather than industrial mining.[23]

Overall, this work serves as progress toward a proof of concept study with numerous potential applications.

Future Directions

Future work will continue to develop the reagents and processes required for the production of pigment dyes in yeast. We expect that once the RUBY cassette is under a yeast promoter and terminator, yeast cells will exhibit a red phenotype, confirming a successful transformation. Liquid cultures of the bioengineered yeast cells will be assessed for pigment expression followed by the development of a purification scheme for one or both betalains. Betalain extraction from the supernatant or cell portion of harvested cultures using various extraction methods mentioned will confirm their presence, which will then be to be further divided into betacyanin and betaxanthin. To assess the purity of the red dye derived from betalains chemical fingerprinting must be conducted by HPLC analysis against a known standard.

The observed color shift at pH 12 presents promising options for further exploration and warrants further investigation which would extend the potential for these pigments. Once the purity of the dye is confirmed it will be tested in different applications with various textiles, evaluating the colorfastness of these dyes in combination with symplocos or other plant-derived other plant-based mordants.

Overall, these future steps aim to advance of the capacity for betalain-based dye production in a sustainable, scalable manner and explores its potential application in textile dyeing.

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Author Biography

In May 2024, Bridget Ruhme graduated with a Bachelor of Applied Science in Biotechnology from The University of Kansas. Bridget plans to work in the biotech industry during which time she will seek research residencies within the new sustainable design practices from bio-mediums to expand her capstone work.

Author Contributions

B.R.R. contributed to the design, experimental, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

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