Production of Rainbow Colorants by Metabolically Engineered Escherichia coli

Dongsoo Yang, Seon Young Park, and Sang Yup Lee*

There has been much interest in producing natural colorants to replace synthetic colorants of health concerns. Escherichia coli has been employed to produce natural colorants including carotenoids, indigo, anthocyanins, and violacein. However, production of natural green and navy colorants has not been reported. Many natural products are hydrophobic, which are accumulated inside or on the cell membrane. This causes cell growth limitation and consequently reduces production of target chemicals. Here, integrated membrane engineering strategies are reported for the enhanced production of rainbow colorants—three carotenoids and four violacein derivatives—as representative hydrophobic natural products in E. coli. By integration of systems metabolic engineering, cell morphology engineering, inner- and outer-membrane vesicle formation, and fermentation optimization, production of rainbow colorants are significantly enhanced to 322 mg L⁻¹ of astaxanthin (red), 343 mg L⁻¹ of β-carotene (orange), 218 mg L⁻¹ of zeaxanthin (yellow), 1.42 g L⁻¹ of proviolacein (green), 0.844 g L⁻¹ of prodeoxyviolacein (blue), 6.19 g L⁻¹ of violacein (navy), and 11.26 g L⁻¹ of deoxyviolacein (purple). The membrane engineering strategies reported here are generally applicable to microbial production of a broader range of hydrophobic natural products, contributing to food, cosmetic, chemical, and pharmaceutical industries.

1. Introduction
Colorants have a profound impact on our lives as they have been widely employed in food additives, dyes, inks, and cosmetics, to name a few. Although many of them are directly related to human health either by oral intake (e.g., food additives) or transdermal absorption (e.g., cosmetics), most commercially produced synthetic colorants are chemically produced from petroleum. This could cause unexpected health problems, especially for children. To make food more attractive to children, vibrant synthetic colorants are extensively used (i.e., flavored snacks, drinks, candies),[1] which is reported to be correlated with children’s health risks such as hyperactive behaviors.[2] Also, severe water pollution from fabric dyeing in textile industry is another problem; it was estimated that 17–20% of the entire industrial waste water is generated by dyeing or treating garments.[3]

To cope with the above-mentioned concerns on well-being and environmental concerns, systems metabolic engineering has emerged to allow development of microbial cell factories capable of sustainable and efficient production of value-added chemicals and materials, including natural colorants.[4] As many natural products contain chemical structures that can absorb different wavelengths of light (i.e., aromatic ring, conjugated system), they can display a wide array of colors, allowing them to be widely utilized as natural colorants since long before the emergence of petroleum-based colorants. In contrast to the petroleum-based synthetic colorants, natural colorants are environmentally friendly, involve less potential health-related risks, and even possess beneficial pharmacological properties including anticancer, antibacterial, antiviral, anti-inflammatory, and anti-aging effects.[5] These properties show that many natural colorants could be readily used as food colorants,[6] active ingredients in cosmetics,[7] or as general colorants including inks and textile dyes.[8] Many of these natural colorants have been produced by metabolically engineered microorganisms, including betalains,[9] anthocyanins,[10] carotenoids,[11,12] and violacein derivatives.[13] It has long been known that astaxanthin, β-carotene, and zeaxanthin are red, orange, and yellow colorants. Indigo[14] and indigoidine[15] have been known to be blue colorants. Also, violacein derivatives have been reported to give purplish blue color.[13] Some anthocyanin compounds give purple colors as well.[10] However, natural
Figure 1. Overview of the metabolic engineering and membrane structure expansion strategies for the enhanced production of rainbow colorants (red, astaxanthin; orange, β-carotene; yellow, zeaxanthin; green, provioloacein; blue, prodeoxyvioloacein; navy, violoacein; purple, deoxyvioloacein). Morphology engineering was performed by knocking down the genes involved in cell division or cell wall metabolism. Inner-membrane vesicles (IMVs) were formed by introducing the cav1 gene encoding human caveolin-1. Outer-membrane vesicles (OMVs) were formed by knocking down the genes involved in OMV formation. The synthetic sRNA technology was employed to knockdown the expression levels of target genes by blocking translation. Bent arrow and T-shape represent promoter and terminator, respectively. Solid and dotted lines represent single and multiple reactions, respectively. Abbreviations: G3P, glyceraldehyde 3-phosphate; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; DXP, 1-deoxy-D-xylulose 5-phosphate; SKM, shikimate; FPP, farnesyldiphosphate; GGPP, geranylgeranlypyrophosphate; TRP, L-tryptophan; IPA, indolepyruvate; Sp., spontaneous.

Compounds that can be used as green and navy colorants have not been reported before.

Natural colorants can be largely classified into hydrophilic and hydrophobic compounds which can be assessed by their characteristic logP values; higher logP of a compound indicates lower polarity of the compound. For example, betacyanin, a water-soluble natural pigment has the logP value of −0.9, while β-carotene which has a long hydrophobic carbon chain (C40) has the logP value of 13.5. The logP values can be predicted using the XLOGP3 software.[16] When produced from microorganisms, hydrophilic compounds are diffused or secreted to the medium by transporters whereas hydrophobic compounds are accumulated inside the cell or within the cell membrane. In particular, high-level production of hydrophobic compounds (i.e., carotenoids, bis-indole pigments) has been problematic due to the limited innate capability of the cells to accumulate these compounds and their possible cytotoxic properties. Several recent studies attempted to resolve this issue by membrane engineering[17] such as increasing the membrane area[18,19] or by forming intracellular lipid droplets which can serve as an intracellular reservoir for hydrophobic compounds.[20] Although there have been preliminary reports on employing morphology engineering and formation of inner-membrane vesicles (IMVs) or outer-membrane vesicles (OMVs) for enhancing the production of hydrophobic compounds,[18–21] combinatorial applications of these membrane engineering strategies have not been reported. Despite these attempts, the titers of natural colorants need to be further increased for industrial applications.

Here we report a strategy of integrating systems metabolic engineering, cell morphology engineering, IMV and OMV formation, and fermentation optimization for the production of seven natural colorants (including the first green colorant) covering the complete rainbow spectrum in Escherichia coli (Figure 1). Carotenoids and violoacein derivatives were chosen to cover all seven colors as they are important compounds in food, cosmetics, and drug industries due to many health-related beneficial activities. Carotenoids belong to the category of terpenoids, which are the largest group of natural compounds produced from the C5 isoprene units. Carotenoids are responsible for the rich colors found in many plants (e.g., carrots and tomatoes) and algae, and are being actively used as anticancer, anti-inflammatory, and antioxidant agents. Due to their beneficial health-promoting properties and numerous applications, there has been an increasing number of studies on the production of carotenoids using microorganisms.[24] Violoacein derivatives are classified as bis-indole pigments naturally produced from bacteria including Chromobacterium violaceum and Janthinobacterium lividum.[25] These compounds display various pharmaceutical properties including antibacterial, antitumoral, antiviral, and antioxidant activities. They can also be used as sunscreen additives[26] and other cosmetics applications, or as dyes for fabric.[27] After the development of E. coli strains capable of efficiently producing rainbow colorants by metabolic engineering, morphology engineering, and vesicle engineering, fed-batch fermentations were performed to evaluate production performances.
2. Results

2.1. Construction of Chassis Strains for the Production of Carotenoids by Colorimetric Screening

Among diverse carotenoid compounds, β-carotene, zeaxanthin, and astaxanthin display orange, yellow, and red, respectively, the first three colors in the rainbow spectrum. Through the native 1-deoxy-D-xylulose 5-phosphate (DXP) pathway in E. coli, farnesyl dipiphosphate (FPP), a common precursor of carotenoids, can be produced. Introduction of >ε encoding geranylgeranyl pyrophosphate synthetase, >ε encoding phytoene synthase, and >ε encoding phytoene dehydrogenase (all from Pantoaea ana-

natis) are required for lycopene production from FPP. Then, additional introduction of >ε encoding lycopene cyclase from P. ana-
natis leads to the production of β-carotene. Subsequent introduction of >ε encoding β-carotene hydroxylase from P. ana-
natis results in zeaxanthin production. For astaxanthin production, truncated BKT (trCrBKT) encoding β-carotene ketolase from Clamy-
domonas reinhardtii is additionally required (Figure 1).[12]

To optimize the metabolic flux toward target carotenoids and to minimize the accumulation of toxic intermediate lycopene, we constructed six libraries of 5’ untranslated region (5’UTR) designed by 5’UTR library designer software[28] for each heterolo-
gous gene (ε, ε, ε, ε, ε, ε, ε), and examined their combinations to balance gene expression levels (see Text S1, Supporting Information, for details). The WLGB-RPP strain, an E. coli K-12 W3110 derivative that was previously constructed for lycopene overproduction,[29] was used as a base strain. Since each carotenoid has distinct color, colorimetric screening was employed to easily screen overproducers. As a result of colori-
metric screening, the strains BTC1, ZEA20, and ATX68 producing 18.7 mg L⁻¹ of β-carotene, 12.7 mg L⁻¹ of zeaxanthin, and 14.5 mg L⁻¹ of astaxanthin, respectively, in flask cultures were selected for further engineering. Glycerol, rather than glucose, was used as a carbon source as it led to higher production of carotenoids. This was thought to be due to the generation of more reducing equivalents which are required for the reactions in the carotenoids biosynthetic pathway,[10] reduced aggregation of heterolo-
gous enzymes,[31] or changes in the phospholipid composition of membrane that can contribute to the expansion of mem-
brane space for the accumulation of carotenoids.[12] As all three carotenoids share the same upstream pathway up to β-carotene, BTC1 was selected as a representative strain for further engineering.

2.2. Construction of Chassis Strains for the Production of Violacein Derivatives

To complete the remaining rainbow color spectrum, metabolically engineered E. coli strains were developed to produce violacein derivatives. As the four violacein derivatives—prodeoxyviolacein, proviolacein, deoxyviolacein, and violacen—
are reported to display distinct colors due to their different func-
tional groups, engineered E. coli strains producing each of these compounds were constructed to compare their colors. Since all of the four violacein derivatives are produced from L-tryptophan (Figure 1A), the IND5 strain harboring pTacGEL, which we pre-
viously developed for driving strong metabolic flux toward L-
tryptophan,[14] was employed. Using this strain as a base strain, heterologous biosynthetic pathways from C. violaceum were intro-
duced for the production of violacein derivatives.

First, the complete violacein biosynthetic gene cluster (BGC) vioABCDE was introduced into IND5 (pTacGEL) to construct the strain VIO, leading to the production of 453 mg L⁻¹ violacein (together with 44.2 mg L⁻¹ deoxyviolacein) from glucose (Figure S2B, Supporting Information). Regarding the carbon source, it was previously suggested that glucose might inhibit viola-
cean production[33] and glycerol was suggested as a better carbon source for deoxyviolacein production as the metabolic pathway from glycerol to glyceraldehyde 3-phosphate, the direct precursor of the tryptophan pathway, is shorter than that from glucose.[34] Thus, glycerol was also tested as a sole carbon source. Switching the carbon source from glucose to glycerol significantly increased violacein production (1.36 g L⁻¹ violacein together with 0.130 g L⁻¹ deoxyviolacein; P = 5.5 × 10⁻⁴) (Figure S2B, Sup-
porting Information). Then, each of the vioABCDE, vioABDE, and vioABE BGCs was introduced into IND5 (pTacGEL) to construct the strains DVIO, PVIO, and PDVIO, respectively. Each of these strains produced 1.04 g L⁻¹ of deoxyviolacein, 151 mg L⁻¹ of provi-
olacein, and 114 mg L⁻¹ of prodeoxyviolacein from glycerol, re-
spectively (Figure S2C, Supporting Information). The colors of the four violacein derivatives after extraction by dimethylsul-
foxide (DMSO) are found to be as follows: proviolacein, green; prodeoxyviolacein, blue; violacein, navy; deoxyviolacein, purple (Figure 1B; Figure S2G, Supporting Information). To the best of our knowledge, this is the first report on the production of a natural green colorant. As all four violacein derivatives have similar chemical and physical properties and share L-tryptophan as the common precursor, deoxyviolacein was selected as the represen-
tative strain to test further engineering strategies.

2.3. Altering Cell Morphology by Deregulation of Membrane-Associated Metabolism

Carotenoids are hydrophobic as their chemical structures have a common C₃₀ backbone. Violacein derivatives are also hydropho-
bic due to their hydrophobic carbon-ring moieties. These hydrophobic natural products are either integrated into the cell membrane or accumulated on the cell membrane, rather than be-
ing secreted to the culture medium.[15,16] Thus, expanding mem-
brane capacity was expected to enhance the accumulation of carotenoids and violacein derivatives, as demonstrated for zeax-
thanin production.[17] We sought to expand membrane capaci-
ty by deregulation of membrane-associated metabolism which would alter cell morphology (Figure 2A). As reported previously, repression of cell division-related genes (i.e., ftsZ) would elongate the cells by blocking cell division.[36] On the other hand, repres-
sion of genes related to cell wall synthesis or maintenance (i.e., mreB) would alter the cell morphology into different shapes (usu-
ally spherical) as cell wall is responsible for maintaining the rod

shape of E. coli.[38] It was thus expected that knocking down the genes belonging to these two categories would increase the total

membrane area per cell.

Repression of target gene expression was achieved by employ-
ing the synthetic small regulatory RNA (shRNA) technology.[39,40] The synthetic shRNA is a trans-acting target gene knockdown tool
Figure 2. Morphology engineering and IMV formation for the enhanced production of rainbow colorants. A) Schematic representation of morphology engineering to expand the space in which rainbow colorants can be accumulated. Knockdown of genes involved in cell division would lead to elongated cells whereas knockdown of genes involved in cell wall synthesis or maintenance would lead to shorter cells with spherical and irregular shapes. The synthetic sRNA technology was employed to knockdown the expression levels of target genes by blocking translation. B) β-Carotene production in the engineered strains introduced with the sRNAs targeting genes related to cellular morphology. C) Deoxyviolacein production in the engineered strains introduced with the sRNAs targeting genes related to cellular morphology. D) Schematic representation of employing IMVs (caveolae). IMVs were formed by introducing the cav1 gene encoding human caveolin-1. E) β-Carotene production by employing IMVs. F) Deoxyviolacein production by employing IMVs. TEM (upper panels) and SEM (lower panels) images of G) the control β-carotene producer BTC1, H) BTC1 expressing cav1, I) the control deoxyviolacein producer deoxyviolacein, and J) deoxyviolacein expressing cav1 are shown. For panels (H and I), red arrows represent IMVs. Error bars are mean ± SD (standard deviation; n = 3). B,C) *P < 0.05, determined by two-tailed Student’s t-test. E,F) *P < 0.01, **P < 0.002, determined by two-tailed Student’s t-test. P-value thresholds were adjusted using Bonferroni correction (corrected significance levels represented as α/m; α, original significance level; m, number of hypotheses). NS, not significant.
comprising a target-specific small RNA and Hfq protein, and is particularly useful for the knockdown of multiple gene targets including essential genes to the desired levels.[39] From the E. coli genome, nine knockdown target genes related to cell division regulation (ftsABILQWZ, minD, and zipA) and seven knockdown target genes related to cell wall synthesis or cell shape maintenance (rodZ, mrdAB, mreBCE, murE, and phpC) were selected (Table S4, Supporting Information). The sRNAs corresponding to these target genes were obtained from the previously constructed E. coli genome-scale sRNA library,[41] which were transformed into the BTC1 and DVIO strains for subsequent flask cultivation. When the cell division-related genes were repressed, elongated cells were observed for both strains (Figure S3, Supporting Information). However, none of them showed increased production of β-carotene or deoxyviolacein (Figure 2B,C). When the genes related to cell wall synthesis or maintenance were repressed, shorter cells with spherical and irregular shapes were observed in both BTC1 and DVIO (Figure S3, Supporting Information). Knockdown of these target genes was not effective in enhancing β-carotene production (Figure 2B). However, knockdown of mrdB (encoding a cell wall shape-determining protein) in DVIO led to 25% increase in deoxyviolacein production (1.30 g L⁻¹; P = 0.032; Figure 2C) when compared to that (1.04 g L⁻¹) produced by the base strain. Increased formation of deoxyviolacein crystals inside and outside the cells could be identified through microscopy observation of the DVIO strain with mrdB knockdown (Figure S3B, Supporting Information).

2.4. Generation of IMVs for Colorants Accumulation

To further expand membrane structures, eukaryotic IMVs were employed. Of several eukaryotic vesicles reported, caveolae are small invaginations in the plasma membrane of animal cells, playing essential roles in many important cellular metabolisms including endocytosis and signal transduction (Figure 2D).[42] Of several proteins and lipids that comprise caveolae, caveolin proteins (Cav1, Cav2, and Cav3) are important in the formation of globular structure. It has been previously reported that heterologous expression of cav1 resulted in successful formation of caveolae in E. coli whereas introduction of cav2 led to the formation of irregular membrane structures inside the cell.[43] Heterologous expression of cav3 has not yet been reported. Cav1 has also been employed to increase the accumulation of membrane proteins in E. coli[23] or to enhance the uptake and conversion of fatty acids into esters.[22] We thus examined whether the production of rainbow colorants can be enhanced by expanding membrane structures by generation of IMVs through employing caveolae.

First, three genes encoding different caveolin proteins—cav1, cav2, and cav3—from Homo sapiens were individually cloned into pTrc99A (see Text S2, Supporting Information, for the sequences of the codon-optimized genes). Each of the three plasmids was transformed into the BTC1 and DVIO strains to examine their effects on colorants production. Cav1 was the most effective caveolin protein in enhancing the production of both colorants. Upon expression of cav1, β-carotene production was increased to 21.9 mg L⁻¹ (P = 0.027) and deoxyviolacein to 1.28 g L⁻³ (P = 1.5 × 10⁻³) (Figure 2E,F). Expression of cav2 also slightly enhanced β-carotene production to 20.2 mg L⁻¹ (P = 0.045), but deoxyviolacein production (1.15 g L⁻¹) was not affected (P = 0.062). Expression of cav3 rather decreased both the β-carotene titer (1.04 mg L⁻¹; P = 4.8 × 10⁻⁷) and the deoxyviolacein titer (79.0 mg L⁻¹; P = 1.3 × 10⁻⁵; Figure 2E,F) significantly. Simultaneous expression of the cav1 and cav2 genes resulted in increased β-carotene production (21.3 mg L⁻¹; P = 0.029) compared to the base strain, while deoxyviolacein production (0.980 g L⁻¹) was not affected (P = 0.42). The combinatorial expression of cav23, cav13, and cav123 resulted in decreased titers of both colorants when compared with those obtained from the base strains (Figure 2E,F).

Transmission electron microscopy (TEM) analysis was performed to observe the intracellular structures (caveolae) formed by the expression of cav1 (Figure 2G–J). Scanning electron microscopy (SEM) analysis was also performed to examine whether any changes in outer membrane occurred. It was confirmed that only intracellular membrane structures were formed upon cav1 expression (Figure 2G–J). Next, it was pursued to increase membrane lipid supply by overexpressing the E. coli plsBC genes, which are responsible for the conversion of acyl-ACP and glycerol 3-phosphate to diacylglycerol 3-phosphate.[13] As expected, β-carotene production was further increased to 29.9 mg L⁻¹ (P = 0.022), which was higher than that obtained with the strain only harboring the cav1 gene. In the case of deoxyviolacein production, overexpression of plsBC together with cav1 resulted in a deoxyviolacein titer (1.20 g L⁻¹; P = 8.9 × 10⁻³) higher than that (1.04 g L⁻¹) obtained with the DVIO strain, but lower than that obtained by cav1 overexpression only (1.28 g L⁻¹; P = 3.1 × 10⁻³) (Figure 2E,F). Whereas carotenoids such as β-carotene are known to be incorporated into the cell membrane,[44] bis-indole pigments such as deoxyviolacein are known to form crystals that are accumulated around the cell membrane.[15] Thus, increased phospholipid supply by overexpression of plsBC would have directly affected the production of β-carotene whereas its effect on the production of deoxyviolacein would have been marginal.

2.5. Generation of OMVs for Colorants Production

Due to the limited membrane capacity for the hydrophobic natural products, their maximum titers cannot reach beyond the maximum storage space inside the cell. Moreover, excess intracellular accumulation of these natural products can cause metabolic burden to the host cells and negatively affect cellular physiology and metabolism, which would consequently lead to the inhibition of both cell growth and product formation. For this reason, extracellular vesicles were also employed to further expand the membrane structures and to secrete the accumulated colorants to the extracellular medium. In gram-negative bacteria, OMVs are generated for transportation of genetic materials, cell detoxification, and bacterial communication, among many other purposes.[45] In contrast to IMVs, genes that can explicitly generate OMVs in bacteria have not been identified. It has been reported that repression of genes encoding outer-membrane proteins, repression of genes related to outer-membrane or peptidoglycan integrity, or activation of the σE factor can induce OMV formation (Figure 3A).[23,46] Thus, we sought to generate OMVs in E. coli using the synthetic sRNA technology in order to test all the three hypotheses.[39]
Figure 3. Formation of OMVs for the enhanced production of rainbow colorants. A) Schematic representation of OMVs formation. OMVs were formed by repression of genes encoding outer-membrane proteins, repression of genes related to outer-membrane or peptidoglycan integrity, or activation of the σE factor. The synthetic sRNA technology was employed to knockdown the expression levels of target genes by blocking translation. Abbreviations are OMP, outer-membrane protein; LPS, lipopolysaccharide. B) β-Carotene production by employing OMVs. *P < 0.0083, **P < 0.0017, ***P < 0.00017, determined by two-tailed Student’s t-test. C) Deoxyviolacein production by employing IMVs. *P < 0.0125, **P < 0.0025, ***P < 0.00025, determined by two-tailed Student’s t-test. TEM (upper panels) and SEM (lower panels) images of D) BTC1 introduced with anti-rffD sRNA and E) DVIO introduced with anti-rffD sRNA. F) SEM images of purified OMVs from BTC1 harboring anti-rffD sRNA (upper panel) and DVIO harboring anti-rffD sRNA (lower panel). TEM (upper panels) and SEM (lower panels) images of I) BTC1 introduced with anti-rffD and anti-rfaD sRNAs and cav1-plsBC and J) DVIO introduced with anti-rfaI sRNA and cav1. G) β-Carotene production by testing synergistic effects of employing the strategies of forming IMVs and OMVs. H) Deoxyviolacein production by testing synergistic effects of employing the strategies of morphology engineering, IMVs formation, and OMVs formation. Abbreviations in (G,H) are Tot, total titer; Sec, the titer obtained from the extracellular medium; Morph, morphology engineering. For panels (D–J), Red arrows represent IMVs or OMVs. Error bars are mean ± SD (n = 3). P-value thresholds were adjusted using Bonferroni correction (P < a/m). NS, not significant.
The selected 26 knockdown target genes are as follows (Table S4, Supporting Information): genes encoding outer-membrane proteins (ompA, ompC, and ompR), genes involved in outer-membrane/peptidoglycan integrity (rfA, rfC, rfD, gmbb, lpxL, lpxM, rfaB, rfaC, rfaD, rfaE, rfaG, rfaI, rfaJ, rfaK, rfaP, rfaQ, rfaY, rfaA, rfaH, wzxE, and pnp), and genes encoding anti-σE factors (rseA and rseB). From the E. coli synthetic sRNA library,[40] the 26 selected sRNAs were introduced into the BTC1 and the DVIO strains and were cultured in flasks (Figure 3B,C). When measuring the concentrations of β-carotene and deoxyviolacein, those in both cell pellets and extracellular medium were measured. In the BTC1 strain, sRNA-based knockdown of rffD encoding UDP-N-acetyl-D-mannosamine dehydrogenase, rfaD encoding ADP-L-glycer-D-manno-heptose-6-epimerase, and rfaQ encoding lipopolysaccharide (LPS) core biosynthesis protein (heptozytransferase III) was effective in enhancing the β-carotene production to 26.4 mg L\(^{-1}\) (\(P = 1.4 \times 10^{-4}\)), 24.2 mg L\(^{-1}\) (\(P = 0.024\)), and 20.3 mg L\(^{-1}\) (\(P = 0.022\)), respectively (Figure 3B). These correspond to 41.6%, 29.7%, and 8.74% increase compared with that (1.04 g L\(^{-1}\)) obtained with the base DVIO strain (1.04 g L\(^{-1}\); \(P = 0.024\), respectively (Figure 3F, lower panel). On the other hand, for the DVIO(pWAS-anti-rfaI) strain, the best β-carotene producer, the portion of β-carotene secreted was only 0.72% (Figure S4A, Supporting Information). In the case of the BTC1 (pWAS-anti-rffDrfaD) strain, the best β-carotene producer, the portion of β-carotene secreted was only 0.72% (Figure S4A, Supporting Information). In the case of the BTC1 (pWAS-anti-rffDrfaD) strain, the best β-carotene producer, the portion of β-carotene secreted was only 0.72% (Figure S4A, Supporting Information).

Having observed beneficial effects of morphology engineering, IMV formation, and OMV formation, we next examined whether combination of these strategies can further enhance the production of deoxyviolacein crystals outside the cell (Figure 3F, lower panel).

2.6. Integration of Membrane Expansion Strategies for Enhanced Production of Rainbow Colorants

Having observed beneficial effects of morphology engineering, IMV formation, and OMV formation, we next examined whether combination of these strategies can further enhance the...
production of rainbow colorants. The knockdown and overexpression target genes that enabled the highest increase in colorants production were selected to assess their combinatorial effects on β-carotene and deoxyviolacein production using the representative producer strains BTC1 and DVIO. In the case of the BTC1 strain, the best OMV-forming strategy (knockdown of rfaD and rfaD) and the best IMV-forming strategy (overexpression of cav1 and plsBC) were tested in combinations since membrane morphology engineering was not beneficial. As overexpression of plsBC enhanced β-carotene production in the IMV-forming strain whereas reduced β-carotene production in the OMV-forming strain, the combination without plsBC overexpression was also tested (Figure 3G). In contrast to what was expected, simultaneous formation of IMVs and OMVs resulted in decreased β-carotene production even compared with the base BTC1 strain.

In the case of the DVIO strain, the best morphology engineering strategy (knockdown of mrdB), the best IMV-forming strategy (overexpression of cav1), and the best OMV-forming strategy (knockdown of rfaI) were tested in combinations. Differently from the β-carotene production case, the highest deoxyviolacein titer (1.89 g L⁻¹) was obtained by simultaneous formation of IMVs and OMVs, which corresponds to 81.7% increase of the titer (P = 8.0 × 10⁻⁵) compared with that (1.04 g L⁻¹) obtained with the DVIO strain (Figure 3H). TEM and SEM analyses were performed to examine the intracellular and extracellular membrane structures of BTC1 (pWAS-anti-rffDrfaD-cav1-plsBC) and DVIO (pWAS-anti-rfai-cav1) strains. The simultaneous formation of IMVs and OMVs was observed in both strains (Figure 3I,J).

After developing membrane engineering strategies for the enhanced production of β-carotene and deoxyviolacein, the most effective strategies were applied to the production of the other five colorants. Since the IMV-forming strategy (overexpression of cav1 and plsBC) and the OMV-forming strategy (knockdown of rfaD and rfaD) were all highly effective in enhancing β-carotene production, these strategies were applied to ZEA20 (the base zeaxanthin producing strain) and ATX68 (the base astaxanthin producing strain). The strategy of OMV formation resulted in higher titers of zeaxanthin (18.4 mg L⁻¹; P = 1.2 × 10⁻⁵) and astaxanthin (22.7 mg L⁻¹; P = 2.2 × 10⁻⁵) (Figure S4H,I, Supporting Information). In the case of deoxyviolacein, the combination of the IMV-forming strategy (overexpression of cav1) and the OMV-forming strategy (knockdown of rfaI) was the most effective. Since employing the IMV-forming strategy and the OMV-forming strategy individually were also highly effective, all three strategies were applied to PDVIO (the base deoxyviolacein producing strain), PVIO (the base violacein producing strain), and VIO (the base violacein producing strain) (Figure S4J-L, Supporting Information). The combined strategy of IMV and OMV formation by the overexpression of cav1 and knockdown of rfaI resulted in the highest titer of proviolacein (402 mg L⁻¹; P = 2.6 × 10⁻⁴) and violacein (2.84 g L⁻¹; P = 1.1 × 10⁻⁴) (Figure S4K,L, Supporting Information). In the case of proviolacein, the OMV-forming strategy resulted in the highest titer of 341 mg L⁻¹ (P = 2.0 × 10⁻³), while the combined IMV-OMV-forming strategy also increased the titer to 301 mg L⁻¹ (P = 1.4 × 10⁻³), significantly higher than that (114 mg L⁻¹) obtained with the control PDVIO strain (Figure S4), Supporting Information.

Finally, fed-batch cultures were performed in a 6.6 L bioreactor to examine the performances of the seven constructed strains producing rainbow colorants. For each color product, the engineered strain showing the highest titer in flask culture was employed. After optimization of fermentation conditions (Text S3, Supporting Information), the following titers of carotenoids and violacein derivatives were obtained: 322 mg L⁻¹ of astaxanthin in 82 h using ATX68 (pWAS-anti-rffDrfaD); 343 mg L⁻¹ of β-carotene in 71 h using BTC1 (pWAS-anti-rffDrfaD); 218 g L⁻¹ of zeaxanthin in 65.5 h using ZEA20 (pWAS-anti-rffDrfaD); 1.30 g L⁻¹ of proviolacein in 94 h using PVIO (pWAS-anti-rfai-cav1); 0.855 g L⁻¹ of prodeoxyviolacein in 83 h using PDVIO (pWAS-anti-rfai); 6.69 g L⁻¹ of violacein (together with 1.39 g L⁻¹ of deoxyviolacein) in 123.5 h using VIO (pWAS-anti-rfai-cav1); 11.3 g L⁻¹ of deoxyviolacein in 108 h using DVIO (pWAS-anti-rfai-cav1) (Figure 4; for detailed methods, see the Experimental Section). The time-lapse movies of the whole fermentation processes of BTC1 (pWAS-anti-rffDrfaD); and VIO (pWAS-anti-rfai-cav1) are presented as Movies S1 and S2, Supporting Information, respectively, for the readers. To examine the reproducibility, all fed-batch fermentations were repeated independently once more; the results were reproducible (Figure S5, Supporting Information). The titer, productivity, and content of the rainbow colorants produced by seven different strains are summarized in Table S5, Supporting Information. The rainbow colorants produced were purified and pictured together (Figure 4H).

3. Conclusion

In this study, we developed metabolically engineered E. coli strains capable of producing seven rainbow colorants. Proviolacein, prodeoxyviolacein, violacein, and deoxyviolacein were produced as natural green, blue, navy, and purple colorants, respectively, in addition to the well-studied colorants, astaxanthin (red), β-carotene (orange), and zeaxanthin (yellow). After construction of the base strains by metabolic engineering, integration of cell morphology engineering, formation of IMVs and OMVs, and fermentation optimization led to significant improvement in the production of all the seven rainbow colorants. On the basis of previous reports on employing different forms of vesicles for enhancing the production of hydrophobic compounds, we further developed the combined strategies of metabolic and membrane engineering in a systematic and integrative manner. Using the strategies developed here, production of β-carotene and deoxyviolacein (the two representative natural colorants employed in this study) could be significantly enhanced by 18.4-fold (Figure 4B,I) and 10.9-fold (Figure 4G,J), respectively. In particular, the titer of deoxyviolacein (purple colorant) reached more than 11 g L⁻¹. In the best β-carotene producer (BTC1 harboring pWAS-anti-rffDrfaD), rffD and rfaD were knocked down for OMV generation, while in the best deoxyviolacein producer (DVIO harboring pWAS-anti-rfai-cav1), rfaI was knocked down and cav1 was overexpressed for the simultaneous generation of OMV and IMV. It should be noted that this study focused on applying integrated membrane engineering strategies to a basic metabolically engineered strain to enhance production of natural colorants. Thus, there are more rooms to increase the titers, yields, and productivities of these rainbow colorants by further systems metabolic engineering of the strains developed here. Also, similar strategies can be employed for the expansion of colorants spectrum beyond the seven colorants reported here. When it
comes to target products that are directly consumed by people such as food or cosmetics, considering the public concerns on the safety of genetically modified organisms (GMOs) is important. In this regard, active and transparent communications on the purified products produced by GMOs between not only engineers and scientists but also policy makers, environmentalists, and the public are required to aptly translate high-performing microbial cell factories into the industries. Taken together, the metabolic engineering and membrane structure expansion strategies developed here can also be generally employed to develop highly efficient microbial cell factories producing other hydrophobic natural compounds, which will contribute to improving quality of life and resolving environmental issues.

4. Experimental Section

Materials and Strains: Violacein and deoxyviolacein were purchased from AG Scientific. Lycopene, β-carotene, canthaxanthin, and astaxanthin were purchased from Merck, and zeaxanthin was purchased from Cayman. All strains used in this study are listed in Table S1, Supporting Information. E. coli DH5α (Invitrogen) was used for routine gene cloning.
struct the pATX library, crtY linearized pTrcCDFS by Gibson assembly. For the pZEA library containing pTrcCDF_F/pTrcCDF_R primers. The PCR-amplified derivatives. [14] (pTacGEL) was used as the host strain for the production of violacein derivatives. [14] Chromobacterium violaceum was provided from Korean Collection for Type Cultures (KCTC), which was used to extract the violacein BGC.

Plasmid Construction: Standard protocols were used for PCR, gel electrophoresis, and transformation experiments as were previously reported. [43] The plasmids and oligonucleotides used in this study are listed in Tables S1 and S2, Supporting Information, respectively. For routine gene cloning, E. coli DH5α containing recombinant plasmids were cultured in Luria-Bertani (LB) medium or on LB agar plates (1.5%, w/v, agar) at 37 °C supplemented with appropriate concentrations of antibiotics when necessary: 50 µg mL⁻¹ of kanamycin (Km), 100 µg mL⁻¹ of ampicillin (Ap), and/or 100 µg mL⁻¹ of spectinomycin (Spc). Polymerases used for PCR reactions were either Lamp-Pfu, Pfu (Biofact), or Pfu-X (Solen). Restriction endonucleases were purchased from either Enzymonics or New England Biolabs. Either restriction enzyme digestion and Gibson assembly was performed to construct plasmids used in this study. [49]

To construct the pLHCY library, the backbone plasmid pTrc15K was linearized by PCR-amplification using primers pTac15k_F and pTac15k_R. The amplified gene fragment was assembled with the SacI-digested plasmid pSBC. To construct pTrc15K-plsBC, the gene fragment was inserted into the pTrc99A plasmid by Gibson assembly. To construct pTrc99A-plsBC by Gibson assembly to construct the corresponding site of pZEA which is described in Table S2, Supporting Information.

For the production of violacein derivatives, individual plasmids harboring vioAB, vioC, vioD, vioE, and vioF from C. violaceum were first constructed. Plasmid pTacCDFS was used as the template for inverse PCR using the primer pair pTacCDFS_inv_F/pTacCDFS_inv_R. Then, vioAB gene fragment was amplified in two split fragments by PCR amplification; the first fragment was amplified using vioAB_F/vioAB_mid_R; the second fragment was amplified using vioAB_mid_F/vioAB_R. The two DNA fragments were assembled with the linearized pTacCDFS plasmid by Gibson assembly to construct the plasmid pTacCDFS-vioAB. The same method was used for constructing pTacCDFS-vioC, pTacCDFS-vioD, and pTacCDFS-vioE. The gene fragments vioC, vioD, and vioE were PCR amplified using the primer pairs vioC_F/vioC_R, vioD_F/vioD_R, and vioE_F/vioE_R. Each fragment was assembled with the linearized pTacCDFS plasmid by Gibson assembly to construct the corresponding plasmids. Then, for the production of proteoxyviolacin, plasmid pPDVIO (pTacCDFS harboring vioABE) was constructed as follows. DNA fragment containing tac promoter and vioE was amplified from the plasmid pTacCDFS-vioABE using the primers vioE_frag_F/vioE_frag_R. The amplified gene fragment was assembled with the SacI-digested plasmid pTacCDFS-vioAB to construct pPDVIO. To produce violacin, deoxyviocolin, and violacin, plasmids pPvio (pTacCDFS harboring vioABDE), pDVIO (pTacCDFS harboring vioABE), and pPvio (pTacCDFS harboring vioABE) were constructed as follows. DNA fragments, each containing tac promoter and either of vioC, vioD, and vioE, were PCR amplified from the plasmids pTacCDFS-vioC, pTacCDFS-vioD, and pTacCDFS-vioE using the same primers vioE_frag_F/vioE_frag_R. The amplified gene fragments were assembled with the SacI-digested plasmid pTacCDFS-vioABE to construct pPDVIO to produce violacin, deoxyviocolin, and violacin, plasmids pPvio (pTacCDFS harboring vioABDE), pDVIO (pTacCDFS harboring vioABE), and pPvio (pTacCDFS harboring vioABE) were constructed as follows. DNA fragments, each containing tac promoter and either of vioC, vioD, and vioE, were PCR amplified from the plasmids pTacCDFS-vioC, pTacCDFS-vioD, and pTacCDFS-vioE using the same primers vioE_frag_F/vioE_frag_R. The amplified gene fragments were assembled with the SacI-digested plasmid pTacCDFS-vioABE to construct pPDVIO. The amplified gene fragment was inserted into the pTrc99A plasmid by Gibson assembly to construct the pTrc99A-plsBC plasmid by Gibson assembly. To construct pTrc99A-plsBC by Gibson assembly to construct the corresponding site of pZEA which is described in Table S2, Supporting Information.

Construction of pTrc99A-plsBC was performed by inserting the PCR amplified plsb and plsc gene fragments into the pTrc99A plasmid at PstI and HindIII sites by Gibson assembly. The plsb and plsc genes were PCR amplified from the genomic DNA of E. coli W3110 by using primer pairs plsb_GF/plsb_GR and plsc_GF/plsc_CR, respectively. The plsb gene fragment was isolated from the pTrc99A-plsBC plasmid by digesting the plasmid with PstI and HindIII. The plsc gene fragment was inserted into pTrc99A-cav1 at PstI and HindIII sites to construct the pTrc99A-cav1-plsBC plasmid.

To test the synergistic effects of introducing multiple sRNAs, plasmids pWAS-anti-rffDrfaF, pWAS-anti-rffDrfaQ, pWAS-anti-rffD, and pWAS-anti-rffQ were constructed. First, sRNA fragments (anti-rffD, anti-rffQ, anti-fal, and anti-mrdB) were PCR amplified using primers sRNAdouble_Gib_F/sRNAdouble_Gib_R. Then, anti-rffD, anti-rffQ, and anti-fal fragments were inserted into the linearized sRNA harboring plasmid (linearized by PCR using primers sRNAdouble_IV_F/sRNAdouble_IV_R) by Gibson assembly to construct pWAS-anti-rffDQ, pWAS-anti-rffDrfaQ, and pWAS-anti-rffQ. The anti-rffD fragment was inserted into the linearized anti-rffQ sRNA harboring plasmid (also linearized by PCR using primers sRNAdouble_IV_F and sRNAdouble_IV_R) by Gibson assembly to construct pWAS-anti-rffDrfaQ. To introduce plsb into sRNA-harboring plasmids, the plsb gene fragment was amplified from pTrc99A-plsBC using primers ptrc-cav1_GF/ptrc- plsBC GR, and was inserted into the corresponding sRNA plasmids (pWAS-anti-rffDQ, pWAS-anti-rffQ, and pWAS-anti-rffQ) at Sphi site. To introduce cav1 into sRNA-harboring plasmids, the cav1 gene fragment was amplified from pTrc99A-cav1 using primers pTrc-cav1_GF/ptrc-cav1_GR, and was inserted into the corresponding sRNA plasmids (pWAS-anti-rffDQ, pWAS-anti-rffQ, and pWAS-anti-rffQ) at Sphi site. To introduce cav1 into sRNA-harboring plasmids, the cav1 gene fragment was amplified from pTrc99A-plsBC using primers pTrc-cav1_GF/ptrc- plsBC GR, and was inserted into the corresponding sRNA plasmids (pWAS-anti-rffDQ, pWAS-anti-rffQ, and pWAS-anti-rffQ) at Sphi site.

To merge cav1 with the sRNA plasmids, the cav1 gene fragment was amplified from pTrc99A-cav1 using primers ptrc-cav1_GF/ptrc-cav1_GR, and was inserted into the sRNA plasmids (pWAS-anti-rffDQ, pWAS-anti-rffQ, and pWAS-anti-rffQ) at Sphi site. To introduce cav1 into sRNA-harboring plasmids, the cav1 gene fragment was amplified from pTrc99A-plsBC using primers pTrc-cav1_GF/ptrc- plsBC GR, and was inserted into the corresponding sRNA plasmids (pWAS-anti-rffDQ, pWAS-anti-rffQ, and pWAS-anti-rffQ) at Sphi site. To introduce cav1 into sRNA-harboring plasmids, the cav1 gene fragment was amplified from pTrc99A- plsBC using primers pTrc-cav1_GF/ptrc- plsBC GR, and was inserted into the corresponding sRNA plasmids (pWAS-anti-rffDQ, pWAS-anti-rffQ, and pWAS-anti-rffQ) at Sphi site.
at 37 °C overnight. For both carotenoids strains and violacein derivatives strains, 1 mL aliquot of each seed culture was transferred to a 250 mL baffled flask containing 50 mL of R/2 medium supplemented with 3 g L⁻¹ of yeast extract and 20 g L⁻¹ of glycerol (for violacein derivatives, 3 g L⁻¹ of (NH₄)₂SO₄ was additionally added) which was incubated at 30 °C and at 200 rpm. The R/2 medium (pH 6.8) contains the following per liter: 2 g (NH₄)₂HPO₄, 6.75 g KH₂PO₄, 0.5 g KCl, 0.17 g magnesium chloride, 0.7 g MgSO₄·7H₂O, and 5 mL trace metal solution (TMS) [10 g FeSO₄·7H₂O, 2.25 g ZnSO₄·7H₂O, 1 g CuSO₄·5H₂O, 0.5 g MnSO₄·H₂O, 0.23 g Na₂B₄O₇·8H₂O, 2H₂O and 0.1 g (NH₄)₂MoO₄·2H₂O per liter of 5 M HCl]. When OD₆₀₀ of the cultures reached ≈0.6–0.8, 1 mm isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce gene expression. When required, 50 mg L⁻¹ of Km, 100 mg L⁻¹ of Ap, and/or 100 mg L⁻¹ of Spc was added to the medium. After induction, the cells were cultivated for 36 h for carotenoids and 48 h for violacein derivatives. 

Fed-Batch Fermentation: Fed-batch fermentations were conducted in 6.6 L jar fermenters (BioFlo 320, Eppendorf) containing 1.6 L R/2 medium (pH 6.95) supplemented with 30 g L⁻¹ glucose or glycerol, 3 g L⁻¹ yeast extract, and appropriate antibiotics (for strains producing carotenoids) or 1.95 L R/2 medium (pH 6.8) supplemented with 20 g L⁻¹ glucose or glycerol, 3 g L⁻¹ yeast extract, 3 g L⁻¹ of (NH₄)₂SO₄, and appropriate antibiotics (for strains producing violacein derivatives). For the production of carotenoids, cells were inoculated from a colony into a 14 mL test tube containing 3 mL TB medium supplemented with appropriate antibiotics and were cultivated in a rotary shaker at 30 °C and 220 rpm until OD₆₀₀ reached ≈1–2. The seed culture (1 mL) was transferred into a new 250 mL baffled flask containing 50 mL R/2 medium supplemented with 3 g L⁻¹ yeast extract, 20 g L⁻¹ glycerol, and appropriate antibiotics. The cells were cultivated at 30 °C and 200 rpm until OD₆₀₀ reached ≈3–4, and were transferred into the fermentor. For the production of violacein derivatives, cells were inoculated from a colony into a 25 mL test tube containing 10 mL LB medium supplemented with appropriate antibiotics and were cultivated in a rotary shaker at 37 °C and 200 rpm, overnight. Then, a 250 mL baffled flask, containing 50 mL of R/2 medium supplemented with 20 g L⁻¹ glycerol or glucose, 3 g L⁻¹ (NH₄)₂SO₄, 3 g L⁻¹ yeast extract, and appropriate antibiotics, was inoculated with 1 mL aliquot of the seed culture. The cells were cultured at 30 °C and 200 rpm until OD₆₀₀ reached ≈4, and were transferred into the fermentor. The culture pH was controlled at 6.8 by automatic feeding of 28% (v/v) ammonia solution, and the temperature was maintained at 30 °C.

Anaerobic fermentations for the production of carotenoids and violacein derivatives, the dissolved oxygen concentration (DO) was controlled at 40% of air saturation by supplying air at 2 L min⁻¹, automatically increasing the agitation speed up to 1000 rpm, and changing the percentage of pure oxygen added. When OD₆₀₀ of the culture reached ≈20–30 for carotenoids and ≈20 for violacein derivatives, 1 mL IPTG was added to induce enzyme expression. The pH-stat feeding strategy was employed in order to supply exhausted nutrients to the fermenter. The feeding solution for the production of carotenoids contains the following per liter: 800 g glucose or 800 g glycerol, 6 mL TMS, and 12 g MgSO₄·7H₂O. The feeding solution for the production of violacein derivatives contains the following per liter: 650 g glucose or 800 g glycerol, 85 g (NH₄)₂SO₄, 6 mL TMS, and 8 g MgSO₄·7H₂O. When the pH becomes higher than 7 for (carotenoids production) or 6.85 for (violacein derivatives production) due to carbon source exhaustion, the feeding solution was automatically added.

Electron Microscopy: For TEM, 1 mL of cell culture (using flask culture conditions described in the above section) was washed with distilled water and prefixed in 2% paraformaldehyde-2% glutaraldehyde mixture buffered with Dulbecco’s phosphate-buffered saline (DPBS) at 4 °C overnight. Next, the cells were postfixed in 1% osmium tetroxide solution buffered with DPBS for 1 h at room temperature (25 °C). The fixed samples were dehydrated in graded ethanol, substituted with propylene oxide, and finally embedded in Embed-812 resin. Polymerization was performed at 60 °C. Ultrathin-sectioning of the sample was performed in the EM & Histology Core Facility, at the BioMedical Research Center (BMRC), KAIST. The embedded sample was ultrathin-sectioned (100 nm) with an Ultracut EM UC7 Ultramicrotome (Leica) installed at the BMRC and double-stained with uranyl acetate and lead citrate. TEM imaging was performed in the EM & Histology Core Facility at the BMRC or in National Nanofab Center (NNFC), KAIST. The prepared samples were examined using Tecnai G2 Spirit TWIN (FEI) installed at BMRC at 120-kV acceleration voltage (Figures 2G–J and 3D,E) or Tecnai G2 F30 S-Twin (FEI) installed at NNFC at 300-kV acceleration voltage (Figure 3I, J).

For SEM, 1 mL of cell culture or vesicles was washed with distilled water and resuspended in distilled water. The sample was dried on a silicon wafer and sputter-coated with osmium before examination with SU8230 scanning electron microscope (Hitachi) at 2-kV (Figures 2H,J and 3F) or at 3-kV (Figures 2G,J and 3D,E) acceleration voltage installed at KAIST Analysis Center for Research Advancement (KARA).

OMV Purification: To purify OMVs for SEM analysis or colorants quantification, cell-free culture supernatant was collected, and 400 µL of the supernatant was mixed with 200 µL of Total Exosome Isolation Kit (Invitrogen) by vortexing. The mixture was incubated at 4 °C overnight, and was centrifuged at 10 000 × g for 20 min at 4 °C. After removing the supernatant, the remaining pellet was resuspended in 100 µL of 1X PBS for SEM analysis or in DMSO for quantification.

Analytical Procedures: After culture, cells were harvested by centrifugation at 16 000 × g for 1 min. To quantify the production of carotenoids in the cells, metabolites were extracted from each cell pellet with 1 mL of analytical grade acetone and was vortexed vigorously using Thermo shaker (TS100, Ruicheng) for 15 min at 35 °C and 1 500 rpm. The lysed cells were centrifuged at 16 000 × g for 1 min to separate the acetone solution containing extracted carotenoids. To quantify total production of violacein derivatives, 50 µL of the cell culture was mixed with 950 µL DMSO and was vortexed using Thermo shaker (TS100, Ruicheng) at 40 °C and 1 500 rpm. To quantify violacein derivatives accumulated in the cell, metabolites were extracted from each cell pellet with 1 mL of DMSO and was vortexed using Thermo shaker (TS100, Ruicheng). The mixture was centrifuged at 16 000 × g for 3 min to separate the DMSO solution containing extracted violacein derivatives. Carotenoid secreted to the culture medium were extracted by mixing the cell-free culture supernatant with 10X volume of acetone using the Thermo shaker at 55 °C and 1 500 rpm for 15 min. Secreted violacein derivatives were extracted by mixing the cell-free culture supernatant with equal volume of ethyl acetate, vortexing using the Thermo shaker at 40 °C and 1 500 rpm, and separating the organic layer. The prepared samples were analyzed with HPLC (1260 Infinity II; Agilent) equipped with DAD detectors (G7115A; Agilent). YMC carotenoid C₁₆ column (YMC) was used for carotenoids analysis and Eclipse XDB-C18 column (4.6 × 150 mm; Agilent) was used for violacein derivatives analysis. For the analysis of carotenoids, mobile phase was run at a flow rate of 0.6 mL min⁻¹: the mobile phase consists of solvent A (90% (v/v) methanol in distilled water) and solvent B (tert-butyl methyl ether). The following gradient was applied: 0–3 min, an isocratic condition at 100% solvent A; 3–15 min, a linear gradient of solvent B from 10% to 70%; 15–20 min, a linear gradient of solvent B from 70% to 100%; 20–29 min, an isocratic condition at 100% solvent B; 29–30 min, a linear gradient of solvent B from 90% to 100% (all in vol%). For the analysis of violacein derivatives, mobile phase was run at a flow rate of 1 mL min⁻¹: the mobile phase consists of solvent A (0.1% (v/v) formic acid in distilled water) and solvent B (acetonitrile). The following gradient was applied: 0–3 min, an isocratic condition at 100% solvent B; 3–10 min, a linear gradient of solvent B from 10% to 100%; 10–15 min, an isocratic condition at 100% solvent B (all in vol%). Samples were monitored at 450 nm for carotenoids and...
570 nm for violacein derivatives. Concentration of each product was determined by mapping the area of HPLC peaks to each calibration curve generated using dilutions of authentic standard chemicals. For screening initially cultured strains producing carotenoids, extracted samples were analyzed by measuring absorbances at 474, 453, 452, and 475 nm, respectively for lycopene, β-carotene, zeaxanthin, and astaxanthin, using an Ultrospec 3100 spectrophotometer (Amersham Biosciences).

As commercial standards were not available for proviolacein and protoxideviolacein, the violacein derivatives produced from engineered E. coli strains were further analyzed through HPLC (1100 Series HPLC; Agilent) connected with MS (LC/MSD VL; Agilent). Eclipse XDB-C18 column (4.6 x 150 mm; Agilent) was used and operated at 25 °C. Two mobile phase solvents were used: solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile). The total flow rate was maintained at 0.6 mL min⁻¹, and the following gradient was applied: 0–1 min, 30% solvent B; 1–10 min, a linear gradient of solvent B from 30% to 70%; 10–20 min, 70% solvent B (all in vol%). The eluent was continuously injected into the mass spectrometry using electrospray ionization positive ion mode with the following conditions: fragmentor, 180 V; drying gas flow, 12.0 L min⁻¹; drying gas temperature, 350°C; nebulizer pressure, 30 psig; capillary voltage, 2.5 kV. For analysis, scan mode was used and the scanned mass range was m/z of 200–400. Also, proviolacein and protoxideviolacein were purified via fraction collector (G1364C; Agilent) attached to a HPLC (1260 Infinity II; Agilent) equipped with DAD detectors (G7115A; Agilent). Eclipse XDB-C18 column (4.6 x 150 mm; Agilent) was used. Mobile phase was run at a flow rate of 0.6 mL min⁻¹; the mobile phase consists of solvent A [0.1% (v/v) formic acid in distilled water] and solvent B (acetonitrile). The following gradient was applied: 0–1 min, an isocratic condition at 30% solvent B; 1–10 min, a linear gradient of solvent B from 30% to 70%; 10–20 min, a linear gradient of solvent B from 70% to 95% (all in vol%). The concentrations of natural colors were determined by mapping the area of HPLC peaks to each calibration curve generated using the standard chemicals.

Absorption spectra of colorants extracted from cell cultures were obtained by step-scanning of absorbance at 2 nm intervals in the wavelength range from 350 to 700 nm using Multimode Microplate Reader (Tecan).

**Statistical Analysis:** Sample sizes were not predetermined. All colonies were randomly selected from plates containing ≈100–200 colonies and subject to independent flask culture and chemical analysis. All numerical data are presented as mean ± SD (standard deviation) from experiments done in triplicates. Means were compared using a two-tailed Student’s t-test.[10] P values were obtained by either Microsoft Excel 2016 or OriginPro 2019. P values are represented as *P < 0.05, **P < 0.01 or ***P < 0.001, which are considered as significant. When multiple hypotheses were tested, the significance level thresholds were divided by the number of hypotheses, according to Bonferroni correction (corrected significance levels represented as α/m; α, original significance level; m, number of hypotheses). Thus, *P < 0.05/m, **P < 0.01/m or ***P < 0.001/m. The investigators were blinded to the group allocation by randomly selecting single colonies multiple times.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

D.Y., S.Y.P., and S.Y.L. declare that the membrane engineering technologies described here are patent filed including, but not limited to KR 10-2020-0144521.

**Author Contributions**

D.Y. and S.Y.P. contributed equally to this work. S.Y.L. conceived the project, D.Y., S.Y.P., and S.Y.L. designed the experiments. D.Y. and S.Y.P. conducted the experiments and analyzed the data. D.Y., S.Y.P., and S.Y.L. wrote the manuscript. All authors read and approved the final manuscript.

**Data Availability Statement**

All datasets analyzed during the current study are presented in this manuscript, or are available from the corresponding author upon reasonable request.

**Keywords**

membrane engineering, metabolic engineering, natural products, rainbow colorants, vesicle

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