



Review

Biosynthesis and Extraction of Chlorophyll, Carotenoids, Anthocyanins, and Betalaine In Vivo and In Vitro

Xinxin Yu ^{1,2}, Hao Wang ^{1,2}, Xingchun Xiang ^{1,2}, Jingjing Fu ^{1,2}, Xin Wang ^{1,2}, Yuanhang Zhou ³ and Wang Xing ^{1,2,*}

¹ Key Laboratory of Sugar Beet Genetics and Breeding, College of Advanced Agriculture and Ecological Environment, Heilongjiang University, Harbin 150080, China; xinxinyuxx@163.com (X.Y.); wh2577002369@163.com (H.W.); xcxiang99@163.com (X.X.); bling199904@163.com (J.F.); wl190414@163.com (X.W.)

² National Beet Medium-Term Gene Bank, Heilongjiang University, Harbin 150080, China

³ Xinjiang Academy of Agricultural Sciences, Urumqi 830091, China; zhoyh1228@163.com

* Correspondence: 2014029@hlju.edu.cn

Abstract: As natural bioactive compounds, plant pigments play crucial roles not only in plant phenotype, growth, development, and adaptation to stress but also hold unique value in biotechnology, healthcare, and industrial applications. There is growing interest in the biosynthesis and acquisition of plant pigments. Thus, this paper explores emerging extraction methods of natural pigments and elucidates the biosynthesis pathways of four key plant pigments, chlorophylls, carotenoids, anthocyanins, and betalaine in vivo and in vitro. We comprehensively discuss the application of solvent, supercritical fluid [extraction], ultrasonic, and microwave-assisted extraction techniques, as well as introducing key enzymes, precursors, and synthetic pathways involved in pigment synthesis. δ -Aminolevulinic acid represents a pivotal initiating enzyme for chlorophyll synthesis, whereas isopentenylpyrophosphate, (IPP) and dimethylallyl pyrophosphate, (DMAPP) are closely associated with carotenoid biosynthesis. Phenylalanine and tyrosine are critical substances for anthocyanin and betalaine synthesis, respectively. Hence, crucial genes such as *chlI*, *crtB*, *PGT8*, *CYP76AD1*, and *BvDODA* can be employed for heterologous biosynthesis in vitro to meet the demand for increased plant pigment amount. As a pivotal determinant of plant coloration, an in-depth exploration into the high-quality acquisition of plant pigments can provide a basis for developing superior pigments and offer new insights into increasing pigment yield.

Keywords: biosynthesis; extraction; chlorophyll; carotenoids; anthocyanin; betalaine



Citation: Yu, X.; Wang, H.; Xiang, X.; Fu, J.; Wang, X.; Zhou, Y.; Xing, W. Biosynthesis and Extraction of Chlorophyll, Carotenoids, Anthocyanins, and Betalaine In Vivo and In Vitro. *Curr. Issues Mol. Biol.* **2024**, *46*, 10662–10676. <https://doi.org/10.3390/cimb46090633>

Academic Editor: Hany A. El-Shemy

Received: 19 August 2024

Revised: 17 September 2024

Accepted: 20 September 2024

Published: 23 September 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Plant pigments usually refer to chlorophyll, carotenoids, anthocyanins, and betalaine [1]. Chlorophyll is the primary photosynthetic pigment of green plants, which is employed in the conversion of solar energy into chemical energy. Chlorophyll can be classified into five distinct types, including chlorophyll a (Chl a), and chlorophyll b (Chl b) [2,3]. Carotenoids are naturally synthesized by all photosynthetic organisms and non-photosynthetic organisms, such as eukaryotes, archaea, and bacteria, all of which exhibit complex carotenoid metabolic processes [4]. Carotenoids can transfer the captured light energy to chlorophyll a, which can assist in photosynthesis [5], and can participate in regulating plant growth and development; as natural antioxidants and biosynthetic precursors of vitamin A (retinol), carotenoids are involved in human nutrition and health, preventing the occurrence of diseases [6]. Flavonoids are important non-nitrogenous plant pigments [7]. Research indicates that despite the similar distribution and functions of anthocyanins and beet pigments in plants, there is limited evidence suggesting these compounds have ever coexisted in the same plant species [8–10]. Anthocyanins are a type of flavonoid, whereas betalaine, a nitrogen-containing pigment derived from tyrosine, includes betacyanin and betaxanthin [11]. Anthocyanins are responsible for the red, purple, or blue pigmentation

in plants and fruits, with the intensity of this coloration depending on the position of substituents on the B ring [12,13].

Natural pigments contain a large number of vitamins, minerals, plant active compounds, and other nutrients, and exhibit various biological activities including antioxidant, anti-inflammatory, anti-cancer, neuroprotection, and cardiovascular protection [14,15]. Therefore, plant pigments have great potential in the cosmetics, pharmaceuticals, and food industries [15]. In recent years, driven by increasing health awareness and the discovery of new pharmacological effects associated with various natural pigments, natural colors are facing a rapidly growing global market [16]. However, traditional production methods are source-dependent and extraction is a key step in recovering purified bioactive pigments from specific plants. Conventionally, the extraction of natural plant pigments typically involves solid–liquid extraction, where plant tissues are processed by grinding and soaking to facilitate the diffusion of the pigments into the extraction solvent. The mechanical breakdown of tissues is associated with the release of cell walls and other cellular components, which require further purification. However, solvent extraction is limited by long processing times, low extraction rates, and high solvent requirements, and often constrained by the low content of target pigment compounds and their antagonistic effects on the environment and human health [17–19]. To address this issue, researchers have refined commonly used methods (e.g., Soxhlet extraction [20,21], extraction, etc.). New techniques, including ultrasound and microwave extraction, high-pressure extraction, supercritical fluid extraction, and electric field and pulsed electric field extraction, have also been introduced and are gradually being applied in this field [17,22,23].

Much effort has been made to increase pigment yields from “natural producers” by developing culture systems of plant cells and tissues *in vitro*, as well as optimizing microbial cultivation methods. Synthetic dyes demonstrate better and more stable performance in product development compared to natural dyes [15]. However, the synthetic pigment side effects and toxic effects [24] make people more focused on the safety of the product, so adopting more reliable development approaches is necessary. Synthetic biology makes it possible for the heterologous biosynthesis of pigments by designing and reconstructing novel biological modules and biosystems. This paper reviews recent advancements in major pigment extraction processes and discusses synthetic pathways for the four primary plant pigments. Finally, it addresses future challenges in the application safety of plant pigments.

2. Extraction of Plant Pigments

2.1. Liquid–Liquid Extraction or Solid–Liquid Extraction

The solvent extraction of plant pigments involves several steps such as drying, extraction, filtration, and evaporation. Depending on the nature of the material to be extracted, it can be classified as liquid–liquid extraction (LLE) or liquid–solid extraction (LSE) [25]. The commonly used methods of LSE include the impregnation method, infiltration method, decoction method, exudation method, Soxhlet extraction method, and steam distillation method. The extraction solvents typically used are water, ethanol, methanol, acetone, and other organic solvents. For decades, Soxhlet extraction has been described as a general chemical extraction technique [26]. Soxhlet extraction employs a special extraction apparatus that recycles the solvent in the biomass by condensing it in many cycles [25]. Whatever the extraction method, they share one common feature: the reliance on solvents. As a result, they all face a common drawback: the toxicity of organic solvents causes a certain amount of environmental damage [25,27,28]. For most plant pigments, the most commonly used solvents are water and aqueous alcohol. For beet glycosides, water is the optimal extraction solvent [15], and for anthocyanins, a combination of water and alcohol is the most suitable solvent [29]. In addition, other organic solvent systems can also efficiently extract plant pigments. Ashenafi [30] used different organic solvent systems (e.g., 80:20 *v/v* acetone:ddH₂O, 80:20 *v/v* methanol:ddH₂O water, 80:20 *v/v* acetonitrile:ddH₂O water, and 40:40:20 *v/v* acetone:methanol:ddH₂O) and found that β -carotene was only stable

in the 80:20 (*v/v*) acetone:ddH₂O solvent system after storage at standard refrigerator temperatures (4 to 8 °C).

2.2. Supercritical Fluid [Extraction], SFE

SFE technology relies on utilizing a substance called a supercritical fluid, which demonstrates characteristics of both gas and liquid states when kept above its critical temperature and pressure [31]. As shown in Figure 1, the advantage of this technology lies in its low cost and the ease with which supercritical fluids can be removed from the extracted compounds [29,32]. Among these, supercritical CO₂ extraction is a green method that offers both economic and environmental benefits [33]. It has a low critical temperature (31.1 °C) and is non-toxic, making it suitable for extracting heat-resistant compounds. Research has shown that SFE is optimal with a CO₂ content of 90%, 10% methanol (Me), 65 °C, 250 bar, 45 min, 9 mL/min. Under these conditions, the recovery rate of alizarin and its content in *R. tinctorum* extract (RE) were 1.34 g/kg and 6.42%, respectively [34]. Continuous extraction from *Butia capitata* fruits using supercritical CO₂, ethanol and water gave a cumulative yield of 68.72% compared to 49.12% using the traditional methods. Overall, the extracts obtained using the SFE method demonstrated a higher antioxidant capacity for carotenoids, phenolic compounds, and unsaturated fatty acids compared to those obtained by conventional methods [35].

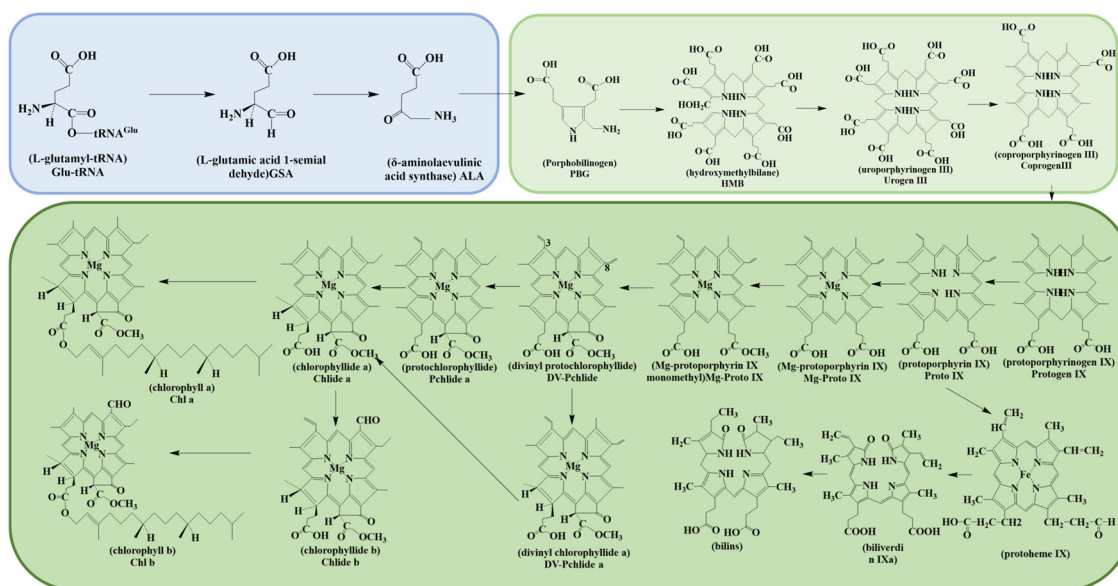


Figure 1. Chlorophyll biosynthesis pathway. In Figure 1, the color of chlorophyll synthesis was re-modified according to the content described. The light blue part represents the synthesis of ALA, and the light green part represents the synthesis of ALA to uroporphyrinogen III (Urogen III) and the final chlorophyll synthesis in the dark green box.

2.3. Correct to Ultrasound-Assisted Extraction, UAE

UAE is applicable for isolating target compounds from complex plant samples [23,36]. In Wang et al.'s study [37], utilizing ultrasonic extraction technology, natural yellow pigments were extracted from *Physalis pubescens* L. with the optimal parameters of 29.21% ultrasonic power, 14.41 min ultrasonic time, and 10.55 s ultrasonic interval, resulting in a yield of 0.193%. Furthermore, the use of corn oil as a green solvent for ultrasound-assisted extraction, in regard to antioxidants with color development results showed UAE > Microwave-Assisted Extraction, MAE > CE compared to MAE and conventional extraction, where UAE had higher TCC, DPPH, and TPC values [38]. Chesnokova et al. [39] reached the conclusion that modifying the ultrasonic-assisted time, temperature, power, and frequency of water and ethanol extraction of anthocyanin pigment resulted in a 22% increase

in the yield of pigment in an aqueous solution. Surmanidze et al. [40] used sunflower seed oil as an alternative solvent to extract lycopene from *Elaeagnus umbellata* fruit by ultrasound-assisted extraction. Compared to the theoretical yield using a hexane–acetone–methanol (2:1:1) mixture as a reagent, the lycopene yield from *Elaeagnus umbellata* fruits can reach up to 85%. Carotenoids were extracted from the peel of the peach palm (*Bactris gasipaes* Kunth) fruit using soybean oil as the solvent. The extraction conditions were optimized to 48 °C for 28 min with a solid–solvent ratio of 0.0037. This process resulted in a carotenoid yield of 151.50 mg/100 g from the desiccated fruit peel [41]. Čulina et al. [42] optimized the UAE of 70% (v/v) ethanol, determining the following optimal conditions: ultrasonic power at 60%, temperature at 50 °C, and extraction time of 20 min. Carotenoids were extracted from *Cucumis melo* L. using a hexane–acetone (80:20) mixture at the optimal ratio, and the operating conditions were 10 min and 100% amplitude. The extraction rate was 124.61 ± 3.82 µg/g [43]. Ordoñez-Santos [41] et al. obtained 4.2 of betacyanin and 2.8 mg/g of betaxanthin by UAE at 52 °C and 37 °C, 90 min, 75% ethanol.

2.4. Microwave-Assisted Extraction, MAE

MAE can improve the quality and quantity of the extracted products due to the use of fewer solvents and shorter high-temperature exposure time [44,45]. The most effective technique for extracting fucoxanthin from cylindrical algae is MAE. The variables affecting MAE include microwave duration, power, temperature, and the type of algae [46]. Pretreated beetroots subjected to MAE using different microwave power levels (200, 400, and 600 W) and solvents (water, ethanol, and ethanol + citric acid) yielded betacyanin contents ranging from 23.77 to 59.28 mg/100 g [44]. Silva et al. [47] initially determined the optimal extraction conditions to be 30 min at 30 °C, with an ultrasonic power of 83 watts and a solid–liquid ratio of 75 mL/g. Compared with orbital shaking extraction (OSE), UAE demonstrated superior outcomes. Table 1 compares the advantages and disadvantages of Soxhlet extraction with the emerging techniques for extracting the three pigments. In conclusion, identifying the most suitable solvent and optimal extraction conditions is crucial. Optimizing the method ensures high extraction rates, functionality of the compounds, and adherence to green and sustainable chemistry principles [42].

Table 1. Comparison of control parameters, advantages, and disadvantages of four primary technologies.

Method	Control Parameter	Advantages	Limitations	Reference
Soxhlet Extraction	Extraction solvent, extraction solvent reflux times (extraction time), ratio of solid to liquid, and extraction temperature	the instrument is low low-cost, simple, and can be used for multiple extractions;; the continuous flow of fresh solvents to the sample is beneficial to the extraction of the sample;; and the final extract does not need to be filtered	the process time is long, glassware is easy to damage, the open system is prone to solvent leakage, high boiling point temperature causes the thermal degradation of compounds, and cosolvent increases the cost	[28,48,49]
Supercritical Fluid [Extraction], SFE	Solvent, temperature, pressure, the addition of modifiers, solid to solvent ratio, and extraction time	high selectivity, it is suitable for highly sensitive applications, heat sensitive compounds are not easy to degrade, CO ₂ as a solvent is non-toxic and easy to obtain, and has a wide range of applications	the equipment is expensive, the operation is complex, CO ₂ is not suitable for some polar compounds, the parameter conditions are difficult, and the processing scale is small	[50–52]

Table 1. Cont.

Method	Control Parameter	Advantages	Limitations	Reference
Ultrasound-Assisted Extraction, UAE	Frequency, power intensity, amplitude, extraction time, temperature, type of ultrasonic, and treatment device (probe and bath)	shorten the extraction time, process automation, and reduce the consumption of organic solvents	requires professional equipment, only for laboratory scale (limited application), high energy consumption, extraction equipment conditions and ultrasonic conditions, and complicated operation procedures	[25,52,53]
Microwave-assisted Extraction, MAE)	Ssolvent feed ratio, solvent composition, moisture content, characteristics of plant samples, irradiation time, effect of stirring, microwave energy density, and extraction temperature	high efficiency and productivity, selective extraction of target compounds, high reaction rate, the product and process have high quality, reduce environmental pollution, and can reduce the degradation of heat-resistant components and reduce costs	the uneven heating leads to incomplete extraction, not suitable for reaction monitoring, high cost, complicated operation procedure, and limited application	[53–55]

3. Biosynthesis of Plant Pigments

3.1. Biosynthesis Pathways of Plant Pigments

3.1.1. Chlorophyll

As shown in Table 2 and Figure 1, chlorophyll biosynthesis is a component of tetrapyrrole metabolism [56]. The process of chlorophyll synthesis can be divided into two distinct phases [57]. The initial stage involves the production of δ -aminolevulinic acid (ALA). Glutamic acid (GLU) is first catalyzed by Glu-tRNA Synthetase (GluRS), forming L-glutamyl-tRNA(Glu-tRNA). The intermediate is then reduced to form l-glutamic acid 1-semialdehyde (GSA) [58–60]. Lastly, GSA undergoes an isomerization, catalyzed by glutamate-1-semialdehyde aminotransferase (GSAT), to produce ALA [61]. ALA is a pivotal precursor in the biosynthesis of porphyrins, including chlorophyll and heme, in all organisms [62].

Table 2. The four major pigments exist in plants and their representative structures.

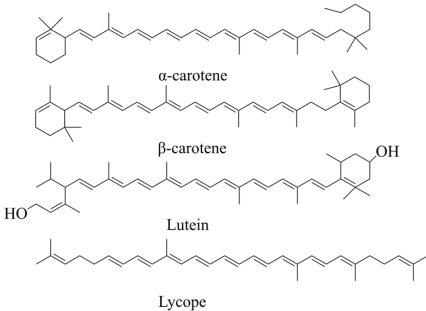
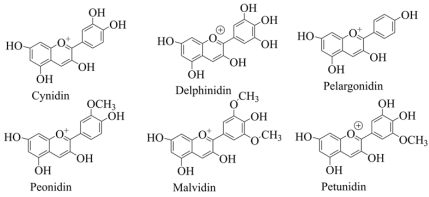
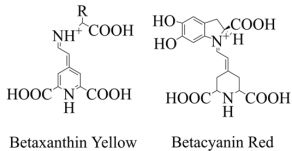
Four Major Plant Pigments	Existence in Plants	The Most Representative Components	Reference
Chlorophyll	Higher plants and other organisms capable of photosynthesis	Chl a, Chl b as shown in Figure 1.	
Carotenoids	Yellow-orange vegetables and fruits, orange fruit, dark green vegetables, and lycopene		[63]

Table 2. Cont.

Four Major Plant Pigments	Existence in Plants	The Most Representative Components	Reference
Anthocyanins	Tissues of purple sweet potato, grape, blood orange, red head cabbage, blueberry, eggplant, cherry, red berry, strawberry, mulberry, hawthorn, morning glory, Elderberries, etc.		[64,65]
Betalaine	Chenopodiaceae, Amaranthaceae, Cactaceae, Mirabilis, and Phytolacca plants and some higher fungi.		[66]

The second phase involves the transformation from protoporphyrin IX (proto IX) to chlorophyll. Tetrapyrroles, such as chlorophyll, heme, and siroheme, share a series of common steps from ALA to Urogen III in the downstream metabolic flux of ALA [60]. First, ALA molecules synthesize proto IX, which is then converted to magnesium-protoporphyrin IX catalyzed by magnesium chelatase (MgCh) [67]. Then, magnesium-protoporphyrin IX methyltransferase (MgPMT) catalyzed the generation of magnesium-protoporphyrin IX methyl ester through the methylation of magnesium-protoporphyrin IX. Following this, through the action of ring cyclase and two rounds of reduction and one round of oxidation reactions, chlorophyllide b (Chlide b) is generated. Finally, in the presence of chlorophyll synthase, chlorophyll a and b, along with the metabolite of protein geranylgeranyl pyrophosphate or phytol pyrophosphate, generate chlorophyll [68].

3.1.2. Carotenoids

Carotenoids are a class of C40 lipophilic isoprene compounds composed of eight isoprene blocks, typically containing 40 carbons in their polyene skeleton [69–72]. As illustrated in Figure 2 and Table 1, in higher plants, carotenoids originate from C5 precursors IPP and DMAPP, which are predominantly derived from the MEP (plastidial methylerythritol 4-phosphate) pathway [73,74]. IPP and DMAPP, catalyzed by enzymes such as isopentenyl-diphosphate δ -isomerase (IDI), farnesyl diphosphate synthase (FDPS), and geranylgeranyl diphosphate synthase (GGPPS) [75] result in the production of the carotenoid precursor geranylgeranyl diphosphate (GGPP) [76]. Subsequently, GGPP is catalyzed by phytoene synthase (PSY) [77] to form the first synthesized colorless C40 carotenoid, phytoene [78,79]. Misawa et al. [80] first demonstrated that only one gene product (*CrtI*) is required to convert octahydrolycopene to lycopene. Lycopene is a major branch point in carotene biosynthesis and serves as a precursor for cyclic carotenoids such as γ -carotene, β -carotene, torularhodin, and astaxanthin [81]. Then, α -carotene is generated through the action of lycopene ϵ -cyclase (LCYE) and lycopene β -cyclase (LCYB). If only LCYB is involved, then only β -carotene is produced [82].

3.1.3. Anthocyanins

During the biosynthesis of anthocyanins, phenylalanine serves as the precursor [83]. As shown in Figure 3A and Table 1, phenylalanine (Phe) is deaminated by phenylalanine ammonia-lyase (PAL), which is then oxidized to 4-coumaric acid by cinnamate 4-hydroxylase (C4H), and finally activated to 4-coumaroyl-CoA (4CL) by the addition of coenzyme A [84,85]. The activity of chalcone synthase (CHS) represents the initiation of a specific flavonoid pathway. The enzyme is responsible for the formation of chalcones by one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, which results in the formation of two phenyl rings (i.e., A and B rings) of the flavonoid skeleton (C6C3-C6).

Chalcone isomerase (CHI) then catalyzes the formation of heterocyclic C, which produces naringenin (flavonoids) as an intermediate compound. Subsequently, naringenin is subjected to hydroxylation by F3H (flavanone 3 β -hydroxylase), resulting in the formation of dihydroflavonol [86]. Finally, the colorless dihydroflavonol is converted into a variety of colored anthocyanins by dihydroflavonol-4-reductase (DFR) and anthocyanidin synthase (ANS). Flavonoid 3-O-glucosyltransferase (UGT) catalyzes the glycosylation of the colored anthocyanins, resulting in the formation of stable, pigmented anthocyanin glycosides after glycosylation, methylation, or acylation modification [13,87].

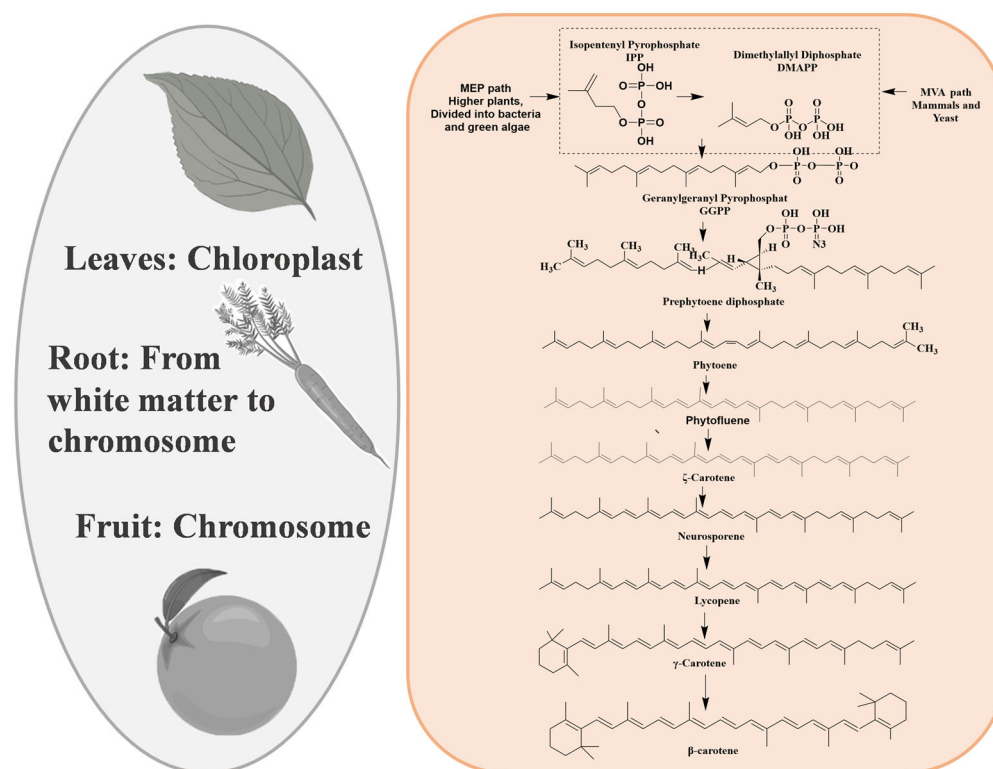


Figure 2. Carotenoid biosynthesis pathway. The pattern of carrots, oranges, and leaves in the figure comes from Figdraw. The imaginary part of the diagram the carotenoid C5 precursor IPP and DMAPP.

3.1.4. Betalaine

Beet pigments (Betalaine) can be categorized into betacyanins and betaxanthins [88]. As illustrated in Figure 3B and Table 1, betalaine is generated from tyrosine, which serves as the precursor in this process. Tyrosine undergoes hydroxylation by tyrosine hydroxylase (TOH) to form L-DOPA [89]. CYP76AD1 has been demonstrated to play a pivotal role in both the formation of cyclo-DOPA and the catalytic hydroxylation of tyrosine to L-DOPA [90]. Once L-DOPA is formed, it can be converted into betalamic acid (BA), a key intermediate responsible for the chromophore common to all betalaine [91]. Following the synthesis of L-DOPA, the biosynthesis of betalaine can be divided into two distinct branches. First, L-DOPA is catalyzed by DOPA-OX to generate dopaquinone, which is cyclized to form cyclo-DOPA. Cyclo-DOPA and betalaine then spontaneously form betacyanins. In the second branch, L-DOPA is catalyzed by 4,5-dioxygenase (DODA) into 4,5-seco-dopa [92], which then spontaneously condenses with amino acids or other amino groups to generate betalaine.

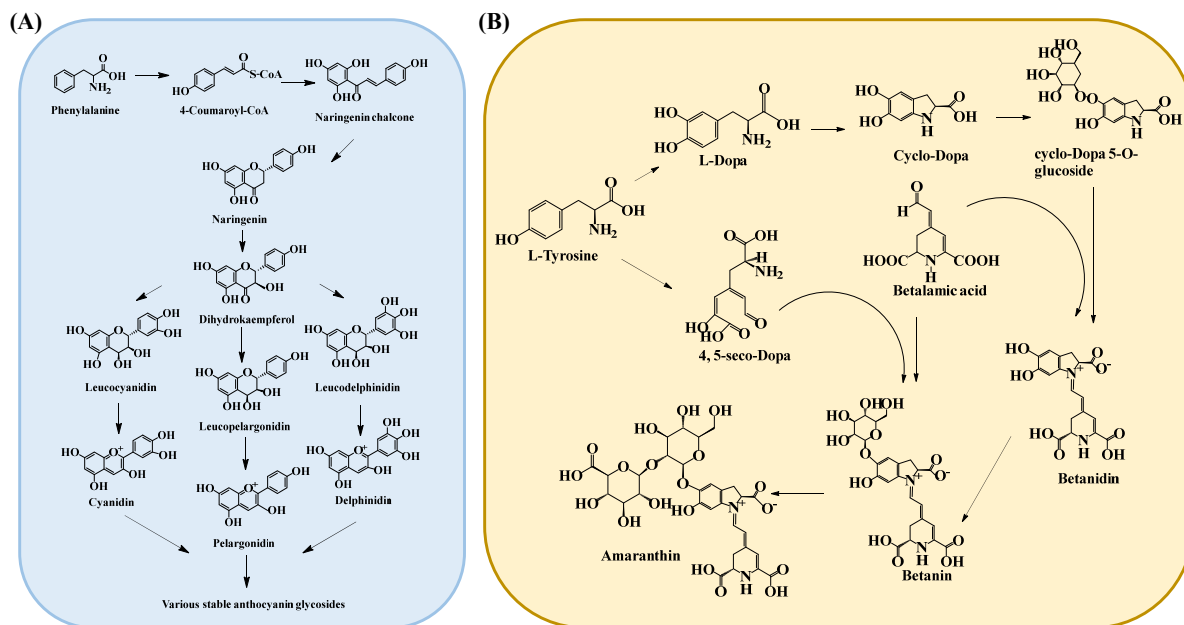


Figure 3. Biosynthesis pathways of anthocyanin (A) and betalaine (B).

3.2. Heterologous Synthesis of Plant Pigments

3.2.1. Chlorophyll Synthesis In Vitro

According to the known pathway of chlorophyll synthesis, Ipekoğlu et al. [93] cloned the chlorophyll a synthase gene *chlG* and successfully transformed it into *R. sphaeroides* to realize the expression of chlorophyll synthesis pathway in bacteria. Chen et al. [79] heterologously expressed genes involved in chlorophyll and carotenoid biosynthesis (*chlI*, *chlD*, *chlH*, *gun4*, *chlM*, *bciB*, *chlP*, *chlG*, *acsF*, *crtE*, *bchN*, *bchB*, *bchL*, *bchC*, *bchX*, *bchY*, *bchZ*, *bchF*, *bchG*, *crtI^{Rs}*, *crtB^{Rs}*, *dxs*, *crtY^{Pa}*, *crtI^{Pa}*, and *crtB^{Pa}*) in *E. coli*, enabling *E. coli* to produce both chlorophyll and carotenoids simultaneously. Moreover, they modified the light-dependent chlorophyll a pathway in *E. coli* to function in the dark. For plants themselves, which naturally contain substantial amounts of chlorophyll, Liu et al. [94] observed that albino tea varieties employ seasonal greening as a survival strategy. They reported that CsRVE1 can directly bind to the promoters of CsLhcb, CsCHLH, and CsPORA, thereby increasing chlorophyll accumulation in tea leaves. The CsCHLI gene has been demonstrated to restore the lethal yellowing phenotype caused by the *atchl1* mutation in different *Arabidopsis thaliana* lines, resulting in increased chlorophyll a and b [95]. In conclusion, mining and analyzing the key genes involved in chlorophyll synthesis across different plants could make significant contributions to enhancing the yield of synthetic chlorophyll in the future.

3.2.2. Carotenoids Synthesis In Vitro

With the continuous progress of biotechnology, the methods for heterologous synthesis of carotenoids have become more and more sophisticated. *Blakeslea trispora* and *Phycomyces blakesleeanus* are natural carotene-producing fungi, and are also considered to be representative pathways for industrial-scale microbial production of carotenoids [96]. In the optimized culture of *B. trispora*, the laboratory-scale β -carotene production reached 78.0 mg/g DCW [97]. Hattan et al. [98] integrated CaCCS and CaZEP into S-tag and designed their interaction on the oligomerization scaffold of the S protein in *E. coli*, synthesizing capsicum yellow, capsanthin, and cucurbitacin A, which are specific carotenoids of red pepper fruits.

Lycopene cyclase has been identified as the rate-limiting enzyme in carotenoid biosynthesis due to its strong substrate inhibition of lycopene. Ma et al. [99] employed protein engineering for lycopene cyclase and established a flow regulator mediated by geranyl-

geranyl pyrophosphate synthase to increase carotenoid production. The Y27R mutant was capable of completely alleviating substrate inhibition without any reduction in enzyme activity. This led to a notable enhancement in β -carotene production with a selectivity of 98%. Using *Schizochytrium* sp., Zhang et al. [100] achieved a new record of 43-fold increase in β -carotene titer in a 5 L bioreactor (653.2 mg/L) by enhancing the precursor supply of geranylgeranyl diphosphate and regulating the carbon sources by limiting sugar fermentation. Sereti et al. [101] studied the fermentation behavior of two marine red yeasts (*R. paludigenum*) (2663 and 2664) cultured on glucose, fructose, sucrose, and glucose mixture (galactose), revealing the dynamic potential of the undeveloped yeast strain *R. paludigenum* 2663 as a robust producer of bioactive components. Fan et al. [102] introduced the NOG pathway to improve the supply of acetyl coenzyme A, and inhibited the ergosterol pathway to reduce the consumption of FPP. They introduced hSapB as a compensation tank for carotenoid storage, and designed the GAL expression system to function in YPD medium, ultimately obtaining the engineered strain JS-BE5-PEST, which produced 166.79 mg/L β -carotene and less than 0.5 mg/L lycopene in YPD medium.

3.2.3. Anthocyanins Synthesis In Vitro

Postma et al. [103] assembled linear and circular chromosomes containing 41 genes in *Saccharomyces cerevisiae*. The resulting strain, IMF48, produced a titer of $0.049 \pm 0.007 \mu\text{M}$ of pelargonidin-3-O-glucoside, establishing the first microbial cell factory for the de novo production of pelargonidin-3-O-glucoside (a type of anthocyanin). Ji et al. [104] screened a transcription factor, ChMYB1 (ChMYB90), which was significantly up-regulated during the fruit development of *C. humilis*. ChMYB1 was found to enhance the expression of *ChCHS* and *ChUFGT* genes by binding MBS (MYB binding element), resulting in a significant increase in the anthocyanin content in fruits. Furthermore, the exogenous application of abscisic acid (ABA) also promoted the expression of *ChMYB1* and the accumulation of anthocyanin.

3.2.4. Betalaine Synthesis In Vitro

Wang et al. [105] assembled the pYB: CDD vector containing three betacyanin biosynthesis genes (*BvCYP76AD1S*, *BvDODA1S*, and *MjcDOPA5GTS*) and introduced it into carrot seedlings. The transgenic seedlings can produce betaine throughout the entire plant without substrate feeding. Metabolic engineering and fermentation optimization by Thomsen et al. [106] achieved the production of $1271 \pm 141 \text{ mg/L}$ beet glycosides and $55 \pm 7 \text{ mg/L}$ isobety glycosides by controlled fed-batch fermentation using glucose as a carbon source. Subsequently, Zhang et al. [107] used *S. cerevisiae* as a host to produce 28.7 mg/L betaine from glucose by screening and modifying the CYP76AD1- α branch protein, and optimizing the fermentation conditions, making the future metabolic engineering of betalaine possible. Betalaine can express color not only in plants themselves but also in other organisms such as yeast. By co-expressing three betalaine synthesis genes, cytochrome P450 enzyme CYP76AD1, DOPA 4,5-dioxygenase, and beet ligand 5-O-glucosyltransferase, it efficiently synthesized betalaine in silkworms. The betalaine in the cocoon layer can be extracted with water at room temperature, reaching a content of 14.4 $\mu\text{g/mg}$ [108].

In total, chlorophyll, carotenoids, anthocyanins, and betalains each have distinct biosynthesis pathways reflecting their unique roles in plants. Chlorophyll biosynthesis begins with 5-aminolevulinic acid and involves complex steps including porphyrin formation and magnesium insertion. Carotenoids are synthesized from geranylgeranyl pyrophosphate through the isoprenoid pathway, with key steps involving lycopene formation and cyclization. Anthocyanins arise from the phenylpropanoid pathway, starting with phenylalanine and progressing through flavonoid intermediates to produce anthocyanidins. Betalains are synthesized from tyrosine via the betalamic acid pathway, involving the formation of betalamic acid and its coupling with cyclo-dopa. For in vitro synthesis, chlorophyll can be produced using chemical methods or through plant cell cultures, carotenoids via microbial fermentation or plant cell cultures, anthocyanins through enzymatic reactions

or chemical synthesis, and betalains via plant cell cultures or microbial fermentation. These variations in synthesis methods reflect the diverse applications and production approaches for each pigment.

4. Improvement in Production of Plant Pigments

The extraction of natural pigments is further complicated by a number of external environmental factors, including light exposure, temperature changes, oxidation, pH, meso-electrode properties, the presence of metal ions, additives, and other variables. This intricate interaction of factors makes the extraction of natural pigments a difficult challenge [109]. Moreover, the direct extraction process often results in pigment loss and reduced purity. Therefore, more and more research prefers biosynthesized pigments as an alternative to plant-derived pigments. As shown in Table 3 the current methods for the biosynthesis of plant pigments. Involve microbial fermentation, microalgae culture, and plant tissue culture. Among these, plant cell culture and tissue culture allow for the direct extraction of pigments from plants.

Table 3. Optimization in acquirement of natural pigments.

Methods of Improvement	Optimization Methods	Biological Excitation Factors	Reference
Plant cell culture and tissue culture	(1) Selection and development of high-yield cell lines; (2) optimizing cell culture process; (3) including the optimization of culture conditions; (4) recovering cells by immobilization; (5) redesign the bioreactor.	(1) Exogenous compounds secreted by microorganisms and insects when attacking plants; (2) exogenous compounds formed by plant enzymes degrading microbial cells, such as fungal and bacterial lysates and polysaccharides of microbial cell walls; (3) plant cell wall fragments degraded by pathogens; (4) intracellular proteins or compounds synthesized by plants in response to pathogen attack or abiotic stress (e.g., plant hormones); (5) non-biological elicitors (such as heavy metals, ultraviolet radiation, inorganic salts, etc.).	[16]
Microbial culture	(1) Medium; (2) process parameters; (3) extraction conditions.	(1) Cell growth; (2) nutritional factors (carbon source, nitrogen source, and C/N ratio); (3) microbial parameters (spore age, seed age, and inoculation age); (4) environmental conditions.	
Heterologous biosynthesis	(1) Engineering of rate-limiting biological components/factors (promoters, protein engineering to optimize enzyme properties, cofactors); (2) metabolic network engineering (supply of precursor substances to target products, enhancement of target product formation ability, inhibition of competitive pathways); (3) the engineering of cell system (external environment and cell expression).	Functional genes and responsible pathways.	
Co-culture system	Modularization	The biosynthetic pathway is divided into different host strains.	

5. Conclusions and Prospects

Plant pigments play a crucial role in a variety of cosmetic, and pharmaceutical products, especially in the food industry, where they are used as food additives. In recent

years, many synthetic color additives have been banned due to their potential health risks resulting from excessive or improper usage. Therefore, enhancing the production of natural pigment and exploring new synthetic methods with improved safety profiles are particularly important. Despite the growing demand for pigments, there are still several challenges that need to be addressed: (1) the pigment extraction process is intricate and susceptible to factors such as pH, temperature, and time during the extraction process; (2) extracted plant pigments may have the taste of the plant itself, causing issues in the subsequent processing and utilization; (3) pigment yields are unstable, resulting in poor product quality and high costs; (4) the extraction process requires a large amount of biomass, and there will be waste generated, the management and reuse of these wastes have yet to be resolved.

The biosynthetic pathways for the four major plant pigments are now relatively well understood, particularly due to advances in omics technology. This has significantly enhanced the heterologous synthesis of pigments *in vitro*. According to their synthetic characteristics, the yield, purity, and stability of the pigments are also improved. Future developments in plant pigments should focus on the following aspects: deepening research into the factors influencing the synthesis of pigments, such as the pH, temperature, extraction agents, and culture media during cultivation and extraction processes; using transgenic or genome editing strategies to create new varieties with high pigment content; and establishing a co-culture system utilizing multiple engineered microbial strains to reconstruct the target biosynthesis. No matter what technology is used, future developments and applications of biosynthetic pigments should aim towards eco-friendly products that are safer, easily degradable, and free from adverse effects.

Author Contributions: X.Y., H.W. and W.X. were responsible for the conceptualization, writing, and revision of the original manuscript; investigation and revision: X.Y. and H.W.; investigation and supervision: X.W., X.X., J.F. and Y.Z.; review and editing: X.Y., W.X. and H.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the earmarked fund (CARS-17), Sugar Beet Germplasm Resource Safe Preservation Project (19240442), the Inner Mongolia Autonomous Region “the open competition mechanism to select the best candidates” project, entitled “Creation of Elite Beet Germplasm and Breeding of Varieties Suitable for Mechanized Operation” (2022JBGS0029), Ministry of Science and Technology and Ministry of Finance National Science and Technology Resources Sharing Service Platform project “Sugar beet sub-bank operation service” (NCGRC-2024-017).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: We appreciate the support from the Tianchi Program and Sugar Beet Breeding Research Project.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Chen, C.X. Overview of Plant Pigments. In *Pigments in Fruits and Vegetables: Genomics and Dietetics*; Chen, C., Ed.; Springer: New York, NY, USA, 2015; pp. 1–7.
2. Kamble, P.N.; Giri, S.P.; Mane, R.S.; Tiwana, A. Estimation of chlorophyll content in young and adult leaves of some selected plants. *Univ. J. Environ. Res. Technol.* **2015**, *5*, 306–310.
3. Ali, K.A.; Noraldein, S.S.; Yaseen, A.A. An Evaluation Study for Chlorophyll Estimation Techniques. *Sarhad J. Agric.* **2021**, *37*, 1458–1465. [[CrossRef](#)]
4. Fernandes, A.S.; do Nascimento, T.C.; Jacob-Lopes, E.; De Rosso, V.V.; Zepka, L.Q. Carotenoids: A brief overview on its structure, biosynthesis, synthesis, and applications. *Prog. Carotenoid Res.* **2018**, *1*, 1–17.
5. Zakar, T.; Laczko-Dobos, H.; Toth, T.N.; Gombos, Z. Carotenoids Assist in Cyanobacterial Photosystem II Assembly and Function. *Front. Plant Sci.* **2016**, *7*, 295. [[CrossRef](#)]
6. Eggersdorfer, M.; Wyss, A. Carotenoids in Human Nutrition and Health. *Arch. Biochem. Biophys.* **2018**, *652*, 18–26. [[CrossRef](#)]
7. Karak, P. Biological Activities of Flavonoids: An Overview. *Int. J. Pharm. Sci. Res.* **2019**, *10*, 1567–1574.
8. Saati, E.A. Antioxidant Power of Rose Anthocyanin Pigment. *J. Eng. Appl. Sci.* **2016**, *11*, 1201–1204.

9. Sokolova, D.V.; Shvachko, N.A.; Mikhailova, A.S.; Popov, V.S.; Solovyeva, A.E.; Khlestkina, E.K. Characterization of Betalain Content and Antioxidant Activity Variation Dynamics in Table Beets (*Beta vulgaris* L.) with Differently Colored Roots. *Agronomy* **2024**, *14*, 999. [\[CrossRef\]](#)
10. Sakuta, M. Diversity in plant red pigments: Anthocyanins and betacyanins. *Plant Biotechnol. Rep.* **2014**, *8*, 37–48. [\[CrossRef\]](#)
11. Nabi, B.G.; Mukhtar, K.; Ahmed, W.; Manzoor, M.F.; Ranjha, M.M.A.N.; Kieliszek, M.; Bhat, Z.F.; Aadil, R.M. Natural pigments: Anthocyanins, carotenoids, chlorophylls, and betalains as colorants in food products. *Food Biosci.* **2023**, *52*. [\[CrossRef\]](#)
12. Shen, N.; Wang, T.; Gan, Q.; Liu, S.; Wang, L.; Jin, B. Plant Flavonoids: Classification, Distribution, Biosynthesis, and Antioxidant Activity. *Food Chem.* **2022**, *383*, 132531. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Li, Z.; Ahammed, G.J. Plant Stress Response and Adaptation via Anthocyanins: A Review. *Plant Stress.* **2023**, *10*. [\[CrossRef\]](#)
14. Lu, X.; Li, W.; Wang, Q.; Wang, J.; Qin, S. Progress on the Extraction, Separation, Biological Activity, and Delivery of Natural Plant Pigments. *Molecules* **2023**, *28*, 5364. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Azmin, S.N.H.M.; Sulaiman, N.S.; Nor, M.S.M.; Abdullah, P.S.; Kari, Z.A.; Pati, S. A Review on Recent Advances on Natural Plant Pigments in Foods: Functions, Extraction, Importance and Challenges. *Appl. Biochem. Biotechnol.* **2022**, *194*, 4655–4672. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Lyu, X.; Lyu, Y.; Yu, H.; Chen, W.; Ye, L.; Yang, R. Biotechnological Advances for Improving Natural Pigment Production: A State-of-the-Art Review. *Bioresour. Bioprocess.* **2022**, *9*, 8. [\[CrossRef\]](#)
17. Rodríguez-Mena, A.; Ochoa-Martínez, L.A.; González-Herrera, S.M.; Rutiaga-Quíñones, O.M.; González-Laredo, R.F.; Olmedilla-Alonso, B. Natural Pigments of Plant Origin: Classification, Extraction and Application in Foods. *Food Chem.* **2023**, *398*, 133908. [\[CrossRef\]](#)
18. Kumar, G.; Upadhyay, S.; Yadav, D.K.; Malakar, S.; Dhurve, P.; Suri, S. Application of Ultrasound Technology for Extraction of Color Pigments from Plant Sources and their Potential Bio-Functional Properties: A review. *J. Food Process. Eng.* **2023**, *46*. [\[CrossRef\]](#)
19. Garcia-Vaquero, M.; Rajauria, G.; Tiwari, B. Conventional Extraction Techniques: Solvent Extraction. In *Sustainable Seaweed Technologies*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 171–189.
20. Ngamwonglumlert, L.; Devahastin, S.; Chiewchan, N. Molecular Structure, Stability and Cytotoxicity of Natural Green Colorants Produced from *Centella asiatica* L. Leaves Treated by Steaming and Metal Complexations. *Food Chem.* **2017**, *232*, 387–394. [\[CrossRef\]](#)
21. López-Cruz, R.; Sandoval-Contreras, T.; Iñiguez-Moreno, M. Plant Pigments: Classification, Extraction, and Challenge of Their Application in the Food Industry. *Food Bioprocess. Technol.* **2023**, *16*, 2725–2741. [\[CrossRef\]](#)
22. Ngamwonglumlert, L.; Devahastin, S.; Chiewchan, N. Natural Colorants: Pigment Stability and Extraction Yield Enhancement Via Utilization of Appropriate Pretreatment and Extraction Methods. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3243–3259. [\[CrossRef\]](#)
23. Linares, G.; Rojas, M.L. Ultrasound-Assisted Extraction of Natural Pigments from Food Processing by-Products: A Review. *Front. Nutr.* **2022**, *9*, 891462. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Novais, C.; Molina, A.K.; Abreu, R.M.V.; Santo-Buelga, C.; Ferreira, I.C.F.R.; Pereira, C.; Barros, L. Natural Food Colorants and Preservatives: A Review, a Demand, and a Challenge. *J. Agric. Food Chem.* **2022**, *70*, 2789–2805. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Didion, Y.P.; Tjalsma, T.G.; Su, Z.; Malankowska, M.; Pinelo, M. What Is Next? The Greener Future of Solid Liquid Extraction of Biobased Compounds: Novel Techniques and Solvents Overpower Traditional Ones. *Sep. Purif. Technol.* **2023**, *320*. [\[CrossRef\]](#)
26. Joseph, J.A.; Denisova, N.A.; Arendash, G.; Gordon, M.; Diamond, D.; Shukitt-Hale, B.; Morgan, D. Blueberry Supplementation Enhances Signaling and Prevents Behavioral Deficits in an Alzheimer Disease Model. *Nutr. Neurosci.* **2003**, *3*, 153–162. [\[CrossRef\]](#)
27. Butnariu, M. Methods of Analysis (Extraction, Separation, Identification and Quantification) of Carotenoids from Natural Products. *J. Ecosyst. Ecography* **2016**, *6*, 193. [\[CrossRef\]](#)
28. Liberal, Â.; Molina, A.K.; Pereira, C.; Dias, M.I.; Ferreira, I.C.; Barros, L. Solid-Liquid Extraction of Polyphenols. In *Technologies to Recover Polyphenols from AgroFood By-Products and Wastes*; Academic Press: Cambridge, MA, USA, 2022; pp. 73–112.
29. Tzanova, M.T.; Yaneva, Z.; Ivanova, D.; Toneva, M.; Grozeva, N.; Memdueva, N. Green Solvents for Extraction of Natural Food Colorants from Plants: Selectivity and Stability Issues. *Foods* **2024**, *13*, 605. [\[CrossRef\]](#)
30. Ashenafi, E.L.; Nyman, M.C.; Shelley, J.T.; Mattson, N.S. Spectral Properties and Stability of Selected Carotenoid and Chlorophyll Compounds in Different Solvent Systems. *Food Chem. Advances.* **2023**, *2*, 100178. [\[CrossRef\]](#)
31. Morón-Ortiz, A.; Mapelli-Brahm, P.; Meléndez-Martínez, A.J. Sustainable Green Extraction of Carotenoid Pigments: Innovative Technologies and Bio-Based Solvents. *Antioxidants.* **2024**, *13*, 239. [\[CrossRef\]](#)
32. Yadav, S.; Tiwari, K.S.; Gupta, C.; Tiwari, M.K.; Khan, A.; Sonkar, S.P. A Brief Review on Natural Dyes, Pigments: Recent Advances and Future Perspectives. *Results Chem.* **2023**, *5*, 100733. [\[CrossRef\]](#)
33. Tzima, S.; Georgiopolou, I.; Louli, V.; Magoulas, K. Recent Advances in Supercritical CO₂ Extraction of Pigments, Lipids and Bioactive Compounds from Microalgae. *Molecules.* **2023**, *28*, 1410. [\[CrossRef\]](#)
34. Elmaaty, T.A.; Sayed-Ahmed, K.; Magdi, M.; Elsis, H. An eco-friendly method of extracting alizarin from *Rubia tinctorum* roots under supercritical carbon dioxide and its application to wool dyeing. *Sci. Rep.* **2023**, *13*, 30. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Pereira, G.S.L.; Magalhães, R.D.; Fraga, S.; de Souza, P.T.; de Lima, J.P.; Meirelles, A.J.D.A.; Sampaio, K.A. Extraction of Bioactive Compounds from *Butia capitata* Fruits Using Supercritical Carbon Dioxide and Pressurized Fluids. *J. Supercrit. Fluids* **2023**, *199*, 105959. [\[CrossRef\]](#)

36. Peng, L.-Q.; Cao, J.; Du, L.-J.; Zhang, Q.-D.; Xu, J.-J.; Chen, Y.-B.; Shi, Y.-T.; Li, R.-R. Rapid Ultrasonic and Microwave-Assisted Micellar Extraction Of Zingiberone, Shogaol and Gingerols from Gingers Using Biosurfactants. *J. Chromatogr.* **2017**, *1515*, 37–44. [\[CrossRef\]](#)
37. Wang, H.T.; Shi, L.; Yang, X.Y.; Hong, R.; Li, L. Ultrasonic-Assisted Extraction of Natural Yellow Pigment from *Physalis pubescens* L. and Its Antioxidant Activities. *J. Chem.* **2018**, *1*, 7861639. [\[CrossRef\]](#)
38. Sharma, M.; Bhat, R. Extraction of Carotenoids from Pumpkin Peel and Pulp: Comparison between Innovative Green Extraction Technologies (Ultrasonic and Microwave-Assisted Extractions Using Corn Oil). *Foods* **2021**, *10*, 787. [\[CrossRef\]](#)
39. Chesnokova, N.Y.; Levochkina, L.V.; Prikhod'ko, Y.V.; A Kuznetsova, A.; A Chebukin, P. Influence of Ultrasound and Extraction Conditions on the Intensity of Extraction of Anthocyanin Pigment from Berry Raw Materials. *IOP Conf. Ser. Earth Environ. Sci.* **2021**, *848*, 012211. [\[CrossRef\]](#)
40. Surmanidze, N.; Vanidze, M.; Djafaridze, I.; Davitadze, R.; Qarcivadze, I.; Khakhutaishvili, M.; Kalandia, A. Optimization of the Method of Ultrasonic Extraction of Lycopene with a Green Extract from the Fruit of *Elaeagnus Umbellata*, Common in Western Georgia. *Food Sci. Nutr.* **2024**, *12*, 3593–3601. [\[CrossRef\]](#)
41. Ordoñez-Santos, L.E.; Martínez-Girón, J.; Rodríguez-Rodríguez, D.X. Extraction of Total Carotenoids from Peach Palm Fruit (*Bactris gasipaes*) Peel by Means of Ultrasound Application and Vegetable Oil. *Dyna* **2019**, *86*, 91–96.
42. Čulina, P.; Balbino, S.; Jokić, S.; Dragović-Uzelac, V.; Pedisić, S. Efficiency of Supercritical CO₂ and Ultrasound-Assisted Extraction Techniques for Isolation of Bioactive Molecules from Sea Buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) Berry Oils and Cakes. *Processes* **2024**, *12*, 698. [\[CrossRef\]](#)
43. Molina, A.K.; Corrêa, R.C.G.; Prieto, M.A.; Pereira, C.; Barros, L. Bioactive Natural Pigments' Extraction, Isolation, and Stability in Food Applications. *Molecules* **2023**, *28*, 1200. [\[CrossRef\]](#)
44. Pramudika, S.; Paramita, V. Efficiency of Betacyanin and Betaxanthin Extraction from Red Beets (*Beta vulgaris* L.) using Microwave-Assisted Extraction (Mae). *J. Vocat. Stud. Appl. Res.* **2024**, *5*, 68–72. [\[CrossRef\]](#)
45. Yuan, Y.; Zhang, J.; Fan, J.; Clark, J.; Shen, P.; Li, Y.; Zhang, C. Microwave Assisted Extraction of Phenolic Compounds from Four Economic Brown Macroalgae Species and Evaluation of Their Antioxidant Activities and Inhibitory Effects on A-Amylase, A-Glucosidase, Pancreatic Lipase and Tyrosinase. *Food Res. Int.* **2018**, *113*, 288–297. [\[CrossRef\]](#)
46. Kiruthika, M.; Shivaswamy, M.S. Extraction of Provitamin and Non-Provitamin Carotenoid Using Conventional and Modern Extraction Methods—A Review. *Food Humanit.* **2024**, *2*, 100241. [\[CrossRef\]](#)
47. Silva, J.P.P.; Bolanho, B.C.; Stevanato, N.; Massa, T.B.; da Silva, C. Ultrasound-Assisted Extraction of Red Beet Pigments (*Beta vulgaris* L.): Influence of Operational Parameters and Kinetic Modeling. *J. Food Process. Preserv.* **2020**, *44*, e14762. [\[CrossRef\]](#)
48. López-Bascón, M.A.; De Castro, M.D.L. Soxhlet Extraction. In *Liquid-Phase Extraction*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 327–354.
49. Mathews, A.; Arbal, A.V.; Kaarunya, A.; Jha, P.K.; Le-Bail, A.; Rawson, A. Conventional Vs Modern Extraction Techniques in the Food Industry. In *Extraction Processes in the Food Industry*; Elsevier: Amsterdam, The Netherlands, 2024; pp. 97–146.
50. Wani, F.A.; Rashid, R.; Jabeen, A.; Brochier, B.; Yadav, S.; Aijaz, T.; Makroo, H.A.; Dar, B.N. Valorisation of Food Wastes to Produce Natural Pigments Using Non-Thermal Novel Extraction Methods: A Review. *Int. J. Food Sci. Technol.* **2021**, *56*, 4823–4833. [\[CrossRef\]](#)
51. Goti, D.; Dasgupta, S. A Comprehensive Review of Conventional and Non-Conventional Solvent Extraction Techniques. *J. Pharmacogn. Phytochem.* **2023**, *12*, 202–211. [\[CrossRef\]](#)
52. Bhadange, Y.A.; Carpenter, J.; Saharan, V.K. A Comprehensive Review on Advanced Extraction Techniques for Retrieving Bioactive Components from Natural Sources. *ACS Omega* **2024**, *9*, 31274–31297. [\[CrossRef\]](#)
53. Da Silva, D.V.T.; Baião, D.D.S.; Magalhães, A.; Almeida, N.F.; Conte, C.A.; Paschoalin, V.M.F. Combining Conventional Organic Solvent Extraction, Ultrasound-Assisted Extraction, and Chromatographic Techniques to Obtain Pure Betanin from Beetroot for Clinical Purposes. *Antioxidants* **2023**, *12*, 1823. [\[CrossRef\]](#)
54. Sooch, B.S.; Sandhu, N.; Mann, M.K.; Ray, R.C. Valorization of Beetroot Waste for Extraction of Natural Dye for Textile and Food Applications. In *Roots, Tubers, and Bulb Crop Wastes: Management by Biorefinery Approaches*; Springer: Singapore, 2024; pp. 237–260.
55. Lozano Pérez, A.S.; Lozada Castro, J.J.; Guerrero Fajardo, C.A. Application of Microwave Energy to Biomass: A Comprehensive Review of Microwave-Assisted Technologies, Optimization Parameters, and the Strengths and Weaknesses. *J. Manuf. Mater. Process.* **2024**, *8*, 121. [\[CrossRef\]](#)
56. Wang, P.; Grimm, B. Organization of chlorophyll biosynthesis and insertion of chlorophyll into the chlorophyll-binding proteins in chloroplasts. *Photosynth. Res.* **2015**, *126*, 189–202. [\[CrossRef\]](#)
57. Wang, H.; Li, Z.; Yuan, L.; Zhou, H.; Hou, X.; Liu, T. Cold Acclimation can Specifically Inhibit Chlorophyll Biosynthesis in Young Leaves of Pakchoi. *BMC Plant Biol.* **2021**, *21*, 172. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Qiu, N.; Jiang, D.; Wang, X.; Wang, B.; Zhou, F. Advances in the Members and Biosynthesis of Chlorophyll Family. *Photosynthetica* **2019**, *57*, 974–984. [\[CrossRef\]](#)
59. Fang, Y.; Zhao, S.; Zhang, F.L.; Zhao, A.G.; Zhang, W.X.; Zhang, M.; Liu, L. The Arabidopsis Glutamyl-Trna Reductase (Glutr) Forms a Ternary Complex with Flu and Glutr-Binding Protein. *Sci. Rep.* **2016**, *6*, 19756. [\[CrossRef\]](#)
60. Wu, Y.; Liao, W.; Dawuda, M.M.; Hu, L.; Yu, J. 5-Aminolevulinic Acid (Ala) Biosynthetic and Metabolic Pathways and Its Role in Higher Plants: A Review. *Plant Growth Regul.* **2019**, *87*, 357–374. [\[CrossRef\]](#)

61. Tan, S.; Cao, J.; Xia, X.; Li, Z. Advances in 5-Aminolevulinic Acid Priming to Enhance Plant Tolerance to Abiotic Stress. *Int. J. Mol. Sci.* **2022**, *23*, 702. [[CrossRef](#)]
62. Wang, X.; Zhang, J.; Wang, L. Study on the Mechanism of Exogenous 5-Aminolevulinic Acid (Ala) in Regulating the Photosynthetic Efficiency of Pear Leaves. *Forests* **2024**, *15*, 769. [[CrossRef](#)]
63. Qaisar, U.; Afzal, M.; Tayyeb, A. Commercial Applications of Plant Pigments. *Int. J. Biotech. Trends Technol.* **2019**, *9*, 18–22. [[CrossRef](#)]
64. Yuniati, Y.; Handarini, K.; Mahfud, M. Pigment Extraction Method for Anthocyanin Natural Resources in Indonesia: A Review. *ASEAN J. Chem. Eng.* **2024**, *24*, 66–78. [[CrossRef](#)]
65. Khoo, H.E.; Azlan, A.; Tang, S.T.; Lim, S.M. Anthocyanidins and Anthocyanins: Colored Pigments as Food, Pharmaceutical Ingredients, and the Potential Health Benefits. *Food Nutr. Res.* **2017**, *61*, 1361779. [[CrossRef](#)]
66. Zuluaga, M.Y.A.; Monterisi, S.; Roupahel, Y.; Colla, G.; Lucini, L.; Cesco, S.; Pii, Y. Different vegetal protein hydrolysates distinctively alleviate salinity stress in vegetable crops: A case study on tomato and lettuce. *Front. Plant Sci.* **2023**, *14*, 1077140. [[CrossRef](#)]
67. Zhang, C.; Zhang, B.; Mu, B.; Zheng, X.; Zhao, F.; Lan, W.; Fu, A.; Luan, S. A Thylakoid Membrane Protein Functions Synergistically with GUN5 in Chlorophyll Biosynthesis. *Plant Commun.* **2020**, *1*, 100094. [[CrossRef](#)] [[PubMed](#)]
68. Garnett, J.M.; Wellner, N.; Mayes, A.G.; Downey, G.; Kemsley, E.K. Using Induced Chlorophyll Production to Monitor the Physiological State of Stored Potatoes (*Solanum tuberosum* L.). *Postharvest Biol. Technol.* **2018**, *145*, 222–229. [[CrossRef](#)]
69. Nisar, N.; Li, L.; Lu, S.; Khin, N.C.; Pogson, B.J. Carotenoid Metabolism in Plants. *Mol. Plant* **2015**, *8*, 68–82. [[CrossRef](#)] [[PubMed](#)]
70. Liu, L.; Shao, Z.; Zhang, M.; Wang, Q. Regulation of carotenoid metabolism in tomato. *Mol. Plant* **2015**, *8*, 28–39. [[CrossRef](#)] [[PubMed](#)]
71. Yuan, H.; Zhang, J.; Nageswaran, D.; Li, L. Carotenoid metabolism and regulation in horticultural crops. *Hortic. Res.* **2015**, *2*, 15036. [[CrossRef](#)]
72. Zia-Ul-Haq, M. Past, Present and Future of Carotenoids Research. In *Carotenoids: Structure and Function in the Human Body*; Springer: Cham, Switzerland, 2021; pp. 827–854.
73. Swapnil, P.; Meena, M.; Singh, S.K.; Dhuldhaj, U.P.; Harish; Marwal, A. Vital Roles of Carotenoids in Plants and Humans to Deteriorate Stress with Its Structure, Biosynthesis, Metabolic Engineering and Functional Aspects. *Curr. Plant Biol.* **2021**, *26*, 100203. [[CrossRef](#)]
74. Sathasivam, R.; Radhakrishnan, R.; Kim, J.K.; Park, S.U. An Update on Biosynthesis and Regulation of Carotenoids in Plants. *S. Afr. J. Bot.* **2021**, *140*, 290–302. [[CrossRef](#)]
75. Quian-Ulloa, R.; Stange, C. Carotenoid Biosynthesis and Plastid Development in Plants: The Role of Light. *Int. J. Mol. Sci.* **2021**, *22*, 1184. [[CrossRef](#)]
76. Sun, T.; Rao, S.; Zhou, X.; Li, L. Plant carotenoids: Recent advances and future perspectives. *Mol. Hortic.* **2022**, *2*, 3. [[CrossRef](#)]
77. Zhou, X.; Rao, S.; Wrightstone, E.; Sun, T.; Lui, A.C.W.; Welsch, R.; Li, L. Phytoene Synthase: The Key Rate-Limiting Enzyme of Carotenoid Biosynthesis in Plants. *Front. Plant Sci.* **2022**, *13*, 884720. [[CrossRef](#)]
78. Rodriguez-Concepcion, M.; Avalos, J.; Bonet, M.L.; Boronat, A.; Gomez-Gomez, L.; Hornero-Mendez, D.; Limon, M.C.; Meléndez-Martínez, A.J.; Olmedilla-Alonso, B.; Palou, A.; et al. A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Prog. Lipid Res.* **2018**, *70*, 62–93. [[CrossRef](#)] [[PubMed](#)]
79. Chen, G.Y.E.; Hunter, C.N. Engineering Chlorophyll, Bacteriochlorophyll, and Carotenoid Biosynthetic Pathways in *Escherichia coli*. *ACS Synth. Biol.* **2023**, *12*, 2236–2244. [[CrossRef](#)] [[PubMed](#)]
80. Misawa, N.; Nakagawa, M.; Kobayashi, K.; Yamano, S.; Izawa, Y.; Nakamura, K.; Harashima, K. Elucidation of the *Erwinia uredovora* Carotenoid Biosynthetic Pathway by Functional Analysis of Gene Products Expressed in *Escherichia coli*. *J. Bacteriol.* **1990**, *172*, 6704–6712. [[CrossRef](#)] [[PubMed](#)]
81. Liang, M.-H.; Zhu, J.; Jiang, J.-G. Carotenoids Biosynthesis and Cleavage Related Genes from Bacteria to Plants. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 2314–2333. [[CrossRef](#)]
82. Cazzonelli, C.I. Carotenoids in nature: Insights from plants and beyond. *Funct. Plant Biol.* **2011**, *38*, 833–847. [[CrossRef](#)]
83. Yan, H.; Pei, X.; Zhang, H.; Li, X.; Zhang, X.; Zhao, M.; Chiang, V.L.; Sederoff, R.R.; Zhao, X. MYB-Mediated Regulation of Anthocyanin Biosynthesis. *Int. J. Mol. Sci.* **2021**, *22*, 3103. [[CrossRef](#)]
84. Majumder, J.; Subrahmanyeswari, T.; Gantait, S. Natural Biosynthesis, Pharmacological Applications, and Sustainable Biotechnological Production of Ornamental Plant-Derived Anthocyanin: Beyond Colorants and Aesthetics. *3 Biotech.* **2024**, *14*, 175. [[CrossRef](#)]
85. Ma, Y.; Ma, X.; Gao, X.; Wu, W.; Zhou, B. Light induced regulation pathway of anthocyanin biosynthesis in plants. *Int. J. Mol. Sci.* **2021**, *22*, 11116. [[CrossRef](#)]
86. Nabavi, S.M.; Šamec, D.; Tomczyk, M.; Milella, L.; Russo, D.; Habtemariam, S.; Suntar, I.; Rastrelli, L.; Daglia, M.; Xiao, J.; et al. Flavonoid Biosynthetic Pathways in Plants: Versatile Targets for Metabolic Engineering. *Biotechnol. Adv.* **2020**, *38*, 107316. [[CrossRef](#)]
87. Menconi, J.; Perata, P.; Gonzali, S. In Pursuit of Purple: Anthocyanin Biosynthesis in Fruits of the Tomato Clade. *Trends Plant Sci.* **2024**, *29*, 589–604. [[CrossRef](#)]
88. Bean, A.; Sunnadeniya, R.; Akhavan, N.; Campbell, A.; Brown, M.; Lloyd, A. Gain-of-Function Mutations in Beet DODA2 Identify Key Residues for Betalain Pigment Evolution. *New Phytol.* **2018**, *219*, 287–296. [[CrossRef](#)] [[PubMed](#)]

89. Zhao, X.; Zhang, Y.; Long, T.; Wang, S.; Yang, J. Regulation Mechanism of Plant Pigments Biosynthesis: Anthocyanins, Carotenoids, and Betalains. *Metabolites* **2022**, *12*, 871. [\[CrossRef\]](#) [\[PubMed\]](#)
90. DeLoache, W.C.; Russ, Z.N.; Narcross, L.; Gonzales, A.M.; Martin, V.J.J.; Dueber, J.E. An Enzyme-Coupled Biosensor Enables (S)-Reticuline Production in Yeast from Glucose. *Nat. Chem. Biol.* **2015**, *11*, 465–471. [\[CrossRef\]](#) [\[PubMed\]](#)
91. Sunnadaniya, R.; Bean, A.; Brown, M.; Akhavan, N.; Hatlestad, G.; Gonzalez, A.; Symonds, V.V.; Lloyd, A. Tyrosine Hydroxylation in Betalain Pigment Biosynthesis Is Performed by Cytochrome P450 Enzymes in Beets (*Beta vulgaris* L.). *PLoS ONE* **2016**, *11*, e0149417. [\[CrossRef\]](#) [\[PubMed\]](#)
92. Timoneda, A.; Feng, T.; Sheehan, H.; Walker-Hale, N.; Pucker, B.; Lopez-Nieves, S.; Guo, R.; Brockington, S. The Evolution of Betalain Biosynthesis in Caryophyllales. *New Phytol.* **2019**, *224*, 71–85. [\[CrossRef\]](#)
93. Ipekoglu, E.M.; Göçmen, K.; Öz, M.T.; Gürkan, M.; Yücel, M. Cloning and Heterologous Expression of Chlorophyll a Syn-thase in Rhodobacter Sphaeroides. *J. Basic. Microbiol.* **2017**, *57*, 238–244. [\[CrossRef\]](#)
94. Liu, X.Y.; Cao, J.J.; Cheng, X.; Zhu, W.F.; Sun, Y.; Wan, X.C.; Liu, L.L. Csrve1 Promotes Seasonal Greening of Albino Camellia Sinensis Cv. Huangkui by Activating Chlorophyll Biosynthesis. *Tree Physiol.* **2023**, *43*, 1432–1443. [\[CrossRef\]](#)
95. Zhao, Y.; Wang, W.; Zhan, X.; Zhang, M.; Xiao, Y.; Hou, X.; Gao, M.; Xiao, B.; Gao, Y. CsCHLI Plays an Important Role in Chlorophyll Biosynthesis of Tea Plant (*Camellia sinensis*). *Beverage Plant Res.* **2024**, *4*, e004. [\[CrossRef\]](#)
96. Wang, C.; Zhao, S.; Shao, X.; Park, J.-B.; Jeong, S.-H.; Park, H.-J.; Kwak, W.-J.; Wei, G.; Kim, S.-W. Challenges and Tackles in Metabolic Engineering for Microbial Production of Carotenoids. *Microb. Cell Factories* **2019**, *18*, 55. [\[CrossRef\]](#)
97. Naz, T.; Ullah, S.; Nazir, Y.; Li, S.; Iqbal, B.; Liu, Q.; Mohamed, H.; Song, Y. Industrially Important Fungal Carotenoids: Advancements in Biotechnological Production and Extraction. *J. Fungi* **2023**, *9*, 578. [\[CrossRef\]](#)
98. Hattan, J.I.; Furubayashi, M.; Maoka, T.; Takemura, M.; Misawa, N. Reconstruction of the Native Biosynthetic System of Carotenoids in *E. coli*—Biosynthesis of a Series of Carotenoids Specific to Paprika Fruit. *ACS Synth. Biol.* **2023**, *12*, 1072–1080. [\[CrossRef\]](#) [\[PubMed\]](#)
99. Ma, Y.; Liu, N.; Greisen, P.; Li, J.; Qiao, K.; Huang, S.; Stephanopoulos, G. Removal of Lycopene Substrate Inhibition Enables High Carotenoid Productivity in *Yarrowia lipolytica*. *Nat. Commun.* **2022**, *13*, 572. [\[CrossRef\]](#) [\[PubMed\]](#)
100. Zhang, Z.-X.; Xu, L.-W.; Xu, Y.-S.; Li, J.; Ma, W.; Sun, X.-M.; Huang, H. Integration of Genetic Engineering and Multi-Factor Fermentation Optimization for Co-Production of Carotenoid and DHA in *Schizochytrium* sp. *Bioresour. Technol.* **2024**, *394*, 130250. [\[CrossRef\]](#) [\[PubMed\]](#)
101. Sereti, F.; Alexandri, M.; Papadaki, A.; Papapostolou, H.; Kopsahelis, N. Carotenoids Production by Rhodosporidium Paludigenum Yeasts: Characterization of Chemical Composition, Antioxidant and Antimicrobial Properties. *J. Biotechnol.* **2024**, *386*, 52–63. [\[CrossRef\]](#) [\[PubMed\]](#)
102. Fan, J.; Zhang, Y.; Li, W.; Li, Z.; Zhang, D.; Mo, Q.; Cao, M.; Yuan, J. Multidimensional Optimization of *Saccharomyces cerevisiae* for Carotenoid Overproduction. *BioDesign Res.* **2024**, *6*, 0026. [\[CrossRef\]](#)
103. Postma, E.D.; Hassing, E.-J.; Mangkusaputra, V.; Geelhoed, J.; de la Torre, P.; van den Broek, M.; Mooiman, C.; Pabst, M.; Daran, J.-M.; Daran-Lapujade, P. Modular, synthetic chromosomes as new tools for large scale engineering of metabolism. *Metab. Eng.* **2022**, *72*, 1–13. [\[CrossRef\]](#)
104. Ji, X.; Li, Z.; Zhang, M.; Lang, S.; Song, X. ChMYB1-ChbHLH42-ChTTG1 Module Regulates Absciscic Acid-Induced Anthocyanin Biosynthesis in *Cerasus humilis*. *Hortic. Plant J.* **2024**, *10*, 51–65. [\[CrossRef\]](#)
105. Wang, B.; Wang, Y.; Deng, Y.; Yao, Q.; Xiong, A. Synthesis of Betanin by Expression of the Core Betalain Biosynthetic Pathway in Carrot. *Hortic. Plant J.* **2024**, *10*, 732–742. [\[CrossRef\]](#)
106. Thomsen, P.T.; Meramo, S.; Ninivaggi, L.; Pasutto, E.; Babaei, M.; Avila-Neto, P.M.; Pastor, M.C.; Sabri, P.; Rago, D.; Parekh, T.U.; et al. Beet red food colourant can be produced more sustainably with engineered *Yarrowia lipolytica*. *Nat. Microbiol.* **2023**, *8*, 2290–2303. [\[CrossRef\]](#)
107. Zhang, L.; Liu, X.; Li, J.; Meng, Y.; Zhao, G.-R. Improvement of Betanin Biosynthesis in *Saccharomyces cerevisiae* by Metabolic Engineering. *Synth. Syst. Biotechnol.* **2023**, *8*, 54–60. [\[CrossRef\]](#)
108. Chen, K.; Yu, Y.; Zhang, Z.; Hu, B.; Liu, X.; James, A.A.; Tan, A. Engineering a Complex, Multiple Enzyme-Mediated Synthesis of Natural Plant Pigments in the Silkworm, *Bombyx mori*. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2306322120. [\[CrossRef\]](#)
109. Zhang, Z.H.; Huang, X.; Chen, J.L.; Qin, Y.Y.; Gao, X.L. Recent Research and Prospects of Non-Thermal Physical Technologies in Green and High-Efficient Extraction of Natural Pigments: A Review. *Innov. Food Sci. Emerg. Technol.* **2024**, *92*, 103593. [\[CrossRef\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.