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Bioactivity and bioavailability of phytoene and strategies to improve its production

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ABSTRACT

Phytoene is a valuable colourless carotenoid, which is used as an ingredient in nutraceuticals as well as in cosmetic products. Its use in formulations has several advantages over other carotenoids due to its stability, photo-insensitivity and long shelf life. In addition, phytoene has beneficial effects on human health because it is an antioxidant which inhibits lipoprotein oxidation and protects against UVB light, while some studies have suggested that it could also have anticancer activity and could decrease cholesterol levels. For these reasons, the demand for phytoene has increased, and new strategies that will allow the production of this bioactive compound in high amounts are needed. The use of *in vitro* cultures of plants, algae and microorganisms has been suggested as a biotechnological strategy to obtain phytoene. In addition, many tools and strategies are available for metabolic engineering that will allow increasing phytoene to be increased using a variety of *in vivo* systems. The source of the phytoene biosynthetic pathway genes used, the design of the construction to express the same and the host strains used, among other factors, can modify the efficiency of the process to obtain phytoene in high amounts.

This review focuses on the strategies used to enhance the production of phytoene, using *in vitro* cultures of plants, algae and microorganisms. Special attention is paid to increasing the production of phytoene using metabolic engineering strategies.

Keywords: Bioactivity, bioproduction, metabolic engineering, phytoene.

Abbreviations: CPTA: 2-(4-chlorophenylthio)-triethylamine, *crtB:* bacterial phytoene synthase, *crtE*: geranylgeranyl diphosphate synthase, *crtI*-MT: *crtI* gene mutated, *crtI*-T: *crtI* gene truncated, CrtM: 4,4'-diapophytoene synthase, *crtW* and *bkt:* bacterial ketolase genes, *crtY*: lycopene β-cyclase, DW: dry weight, ECC-1: endometrial tumoral cells, FPP: C_{15}-farnesyl pyrophosphate, FW: fresh weight, GGPP: C_{20}-geranylgeranyl diphosphate, GPP: C_{10}-geranyl pyrophosphate, *hmgr*: hydroxymethyl-3-glutaryl coenzyme A reductase, MEP: 2-C-methyl-Derythritol-4-phosphate,

MVA: mevalonate pathway, NIH3T3: cell cultures of fibroblasts, PDS: phytoene desaturase, PSY: phytoene synthase, T47D and MCF-7: human breast tumoral cells.

Introduction

Phytoene is a colourless carotenoid often found together with lycopene in fruits such as watermelons, apricot and tomatoes, although its concentration is usually low (Biehler et al. 2012; Khachik et al. 2002; Melendez-Martínez et al. 2015). Phytoene has been described as having several beneficial effects on human health since it may reduce the risk of some types of cancer such as endometrium and breast cancers as well as cardiovascular diseases (Eliassen et al. 2012; Hirsch et al. 2007; Karppi et al. 2013; reviewed in Meléndez-Martínez et al. 2018). Phytoene has 9 double bonds, but the number of conjugated double bonds is much lower as compared to coloured carotenoids (Meléndez-Martínez et al. 2007), so that its maximum absorption is produced in the UV region whereas coloured carotenoids have maximum absorption in the visible region.

Among the possible protection mechanisms of phytoene highlights its ability to absorb in regions of damaging wavelengths (Meléndez-Martínez et al. 2015) or its ability to act as scavenger of singlet oxygen-free radicals since phytoene inhibits lipoprotein oxidation, and it has an important role in protecting against B-UV light, minimizing the degree of UV-induced erythema (Fuller et al., 2006; von Oppen-Bezalel and Shaish, 2009). In fact, recent works showed that the intake of 5 mg of phytoene and phytofluene per day from tomato powder (PhytoflORAL®) for 3 months resulted in a 20% increase in minimal erythemal dose, which increased the resistance of skin to sun and UV damage. In addition to this photoprotective effect, the intake of PhytoflORAL® improved the skin quality parameters (suppleness, elasticity, smoothness, visible skin youthfulness, moisturization, among others) after 6 weeks of treatment (von Oppen-Bezalel et al., 2015). von Oppen-Bezalel (2007) and von Oppen-Bezalel et al. (2014) also showed that the supply of topical products which contained phytoene and phytofluene (IBR-TCLC® and IBR-CLC®) provided benefits in skin lightening and anti-inflammatory or anti-wrinkling effects. Likewise, some studies have suggested that the use of cosmetic products, which contain phytoene and phytofluene, could promote the

overall health by increasing the levels of colourless carotenoids not only in the skin but also in plasma, and probably, in other human tissues (Aust et al. 2005; Moran et al. 2016).

Due to the high value of phytoene, great efforts are being made to develop efficient procedures to obtain it in high amounts because present-day production is insufficient to meet the current market demand (Srinivasan et al. 2017). The most common strategies used to produce phytoene and other carotenoids are their direct extraction from plants and their chemical synthesis. The production by plants is not always satisfactory since it might be induced only under specific stress conditions (Coyago-Cruz et al. 2017) or during a plant specific development stage (Alquezar et al. 2013). In the case of chemical synthesis, the production of carotenoids has certain problems such as the strict conditions of the reactions involved or the high costs of chemical process. However, the use of *in vitro* cultures (Gao et al. 2011), bleaching herbicide treatments (Simkin et al. 2000) or metabolic engineering (Pollmann et al. 2017) is increasing the yield of phytoene production.

In this context, the aim of this review is to describe the strategies used to enhance the production of phytoene using *in vitro* cultures of plants, algae and microorganisms. Special attention is paid to increasing phytoene production using metabolic engineering strategies.

Biosynthesis of phytoene

All carotenoids are synthesized from isopentenyl diphosphate and its double-bond isomer dimethylallyl diphosphate. These C_5 isoprenoid precursors are synthesized via the mevalonate pathway (MVA) in archaebacteria and fungi. In addition, some Streptomyces species produce the isoprenoid precursors using both MVA and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways (Walter and Strack 2011, Moise et al. 2013). Isopentenyl diphosphate is condensed with dimethylallyl diphosphate to produce C_{10}-geranyl diphosphate (GPP) which is elongated to C_{15}-farnesyl diphosphate (FPP) and C_{20}-geranylgeranyl diphosphate (GGPP) (Liang et al. 2017). Then, two molecules of GGPP form phytoene through the action of phytoene synthase (PSY). Moreover, in fungi, Pollmann et al. (2017) described that enzymatic activities of PSY and lycopene cyclase are encoded by one fusion gene (*CrtYB*).

Photosynthetic cyanobacteria, algae, higher plants and bacteria can mainly use the MEP pathway to synthesize isopentenyl diphosphate and dimethylallyl diphosphate (Walter and Strack 2011, Moise et al. 2013). In higher plants and algae, isopentenyl diphosphate and dimethylallyl diphosphate are joined to form GGPP by the action of geranylgeranyl diphosphate synthase, and then two molecules of GGPP form phytoene through the action of PSY, which represents the first limiting step in biosynthesis pathway of carotenoids (Cunningham and Gantt, 1998) (Figure 1). In bacteria, the early step of carotenogenic pathway is the biosynthesis of phytoene which is formed from two GGPP molecules by the action of phytoene synthase (*crtB*) (Liang et al. 2017). Then, phytoene is desaturated by phytoene desaturase (*crtI*) to form lycopene. In some non- phototrophic bacteria, the C_{30}-carotenoid dehydrosqualene is formed by the condensation of two molecules of C_{15}-FPP by the action of 4,4'-diapophytoene synthase (*CrtM*) (Umeno et al. 2002).

The PSY protein sequences from higher plants, algae and cyanobacteria are similar to the homologous enzymes (CrtB) of bacteria and fungi, but there is more than one PSY isoform in some algae and higher plants (Ampomah-Dwamena et al. 2015; Dibari et al. 2012; Tran et al. 2009). In fact, there are three paralogous genes of *psy* annotated as *psy1*, *psy2* and *psy3* in monocots whereas only *psy1* and *psy2* homologs were reported in eudicots (Dibari et al. 2012). In addition, a single gene encodes PSY has been found in Arabidopsis (Fantini et al. 2013; Li et al. 2008; Chaudhary et al. 2010; Busch et al. 2002), which is expressed in both non-photosynthetic and photosynthetic tissues (Welsch et al. 2003). Some PSY isoforms involved in the biosynthesis of carotenoids have been detected in chloroplast-containing photosynthetic tissues such as the leaf (tomato PSY2) (Bartley and Scolnik 1993), while others PSY isoforms have been found in non-photosynthetic tissues, such as fruits (tomato PSY1) (Fraser et al. 2002), roots (cassava, maize and rice PSY3) (Li et al. 2008a, Welsch et al. 2008, Arango et al. 2010) or seed endosperm (maize PSY1) (Li et al. 2008b). Moreover, PSY isoforms can be involved in different functions in the same plant (Shumskaya et al. 2012; Shumskaya et al. 2013). In fact, in maize, ZmPSY1 is involved in carotenoid accumulation in endosperm, ZmPSY2, which is up-regulated by light, is related with the photosynthesis (Li et al. 2008a), and ZmPSY3 is induced under stress conditions in roots (Li et al.

2008b). Similarly, in rice, OsPSY1 and OsPSY2 are involved in the biosynthesis of carotenoids in green tissues whereas OsPSY3 is up-regulated under stress conditions (Welsch et al. 2008).

On the other hand, PSYs are regulated by light but also by drought and other stress factors such as salt or abscisic acid (Cazzonelli and Pogson, 2010; Welsch et al. 2008; Li et al. 2008). Indeed, salt and drought induced *psy3* transcript accumulation and this accumulation was correlated with increased carotenoid flux in maize roots (Li et al. 2008). *psy3* transcript was also predominantly induced in roots upon drought and salt stress and this was correlated with an increase in the biosynthesis of xanthophyll in rice (Li et al. 2008; Welsch et al. 2008). Light can also regulate the expression of *psy* gene in *Sinapsis alba* and *Arabidopsis thaliana* since the increase in carotenoid levels in light was reflected by the up-regulation of the levels of *psy* transcripts (Lintig et al. 1997). In addition, cis-regulatory elements (box I and box IV) were present in the promoters of Os*psy1* and Os*psy2*, which showed gene expression in the presence of light, but were absent in Os*psy3*, which did not respond to light (Welsch et al. 2008). Other studies showed that phytochrome-interaction factors directly interacted with the *psy* promoter of *Arabidopsis* and repressed *psy* gene expression (Toledo-Ortiz et al. 2010). Under light, phytochrome-interaction factors were degraded and *Atpsy* gene was released from its repressed state. In addition, a putative ABA Response Element–Coupling Element was identified in the promoter region of Os*psy3* and Zm*psy3*. These two genes were induced specifically by salt and drought, which in turn, were related with abscisic acid regulation (Li et al. 2008, 2009; Welsch et al. 2008).

Once phytoene is formed, phytoene desaturase (PDS) can introduce double bonds into this compound to form phytofluene. This enzyme has been intensively used as target site for bleaching herbicides, which in turn, are phytoene desaturase inhibitors (Fig. 1), like norflurazon and diflufenican. These inhibitors block the process of phytoene desaturation, accumulating phytoene at the expense of colored carotenoids (Campbell et al. 2006). Thus, bleaching herbicides prevent the formation of these carotenoids and decrease the efficient photoprotection of the photosynthetic apparatus. In this sense, tomato cell cultures treated with norflurazon have been used to produce phytoene and so gain further insight into the biological actions of this carotenoid (Campbell et al.

2006). In addition, these bleaching herbicides not only increase the accumulation of phytoene but also decrease the production of carotenoids, which are biosynthesized downstream so that their effects can be harmful to plants. In fact, it is well-known that β-carotene and xanthophylls (zeaxanthin, lutein, violaxanthin and neoxanthin) are the photosynthetic pigments involved in protecting plants against high light irradiance since these pigments are responsible for the protective mechanisms which prevent photoinhibition and photooxidation processes (Dall'Osto et al. 2007).

As regards localization of different PSY, these enzymes are generally localized into the chloroplasts where carotenoids are accumulates (DellaPenna and Pogson 2006). However, localization of PSY within the chloroplast can vary among the different plants. In fact, studies using *Pisum sativum* chloroplasts showed that SlPSY1 were peripherally associated with thylakoid membranes (Bonk et al. 1997; Fraser et al. 2000; Lawrence et al. 1993) while pepper PSY was found in the chromoplast stroma (Dogbo et al. 1988). Shumskaya et al. (2012) also investigated localization of PSY isoforms and allelic variants from maize, rice and Arabidopsis. They observed that PSYs of all species studied were found in plastids in both, etiolated and green protoplasts. Studies of transient expression suggested that almost all PSYs were attached to plastoglobuli. However, ZmPSY1 was localized to chloroplasts in two different forms, either bound to the envelope membrane or peripherally bound to thylakoids (Shumskaya et al. 2012). In a similar way, Cai et al. (2014) observed that GhPSY from cotton was highly expressed in leaves, and the protein was localized into plastids where it appeared to be mostly attached to the surface of thylakoid membranes.

Role of phytoene in human health

Several studies have indicated that the high levels of phytoene found in human tissues are due to its involvement in antioxidative processes. Martínez et al. (2014) observed that phytoene showed a high efficiency in scavenging 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical although it was less effective than lycopene. In addition, von Oppen-Bezalel and Shaish (2009) found that a phytoene and phytofluene-rich product obtained from *Dunaliella salina* (IBR-CLC©) decreased the content in hydroxyl radicals (·OH) generated by the Fenton reaction in a dose-dependent manner. Phytoene, phytofluene and lycopene also increased the expression of antioxidant

191 response elements, regulatory factors which play a critical role in redox homeostasis, and
192 therefore they are involved in the detoxification of harmful compounds. Thus, a 7:3 mixture of
193 phytoene and phytofluene increased the expression of antioxidant response elements in transfected
194 MCF-7 and HepG2 cell lines and decreased the intracellular concentration of reactive oxygen
195 species (Ben-Dor et al. 2005).
196 As regards the involvement of phytoene in lipid metabolism, in an interesting study Shaish et al.
197 (2008) showed that phytoene together with phytofluene acted as lipoprotein antioxidant. They used
198 a phytoene and phytofluene-rich preparation isolated from *Dunaliella bardawil* in an *in vitro* system
199 of low-density lipoproteins and observed that the preparation inhibited the oxidation of these
200 proteins to a similar extent as the action of β-carotene or α-tocopherol. Moreover, it has also been
201 suggested that phytoene could be involved in decreased plasma cholesterol levels (Melendez-
202 Martinez et al. 2010). In fact, Melendez-Martinez et al. (2010) studied the concentration of
203 phytoene, phytofluene and lipids in rats fed either a control diet (35% fat) or a high fat diet (71%
204 fat) with or without tomato extract supplementation for 6 weeks. They showed that tomato extract
205 supplementation resulted in a significantly higher accumulation of phytoene and phytofluene than
206 lycopene in the livers of the subjects. They also found that tomato extract supplementation in both
207 the control diet and high fat diet groups significantly decreased plasma total cholesterol
208 concentrations. However, data from human studies are lacking, and more clinical trials are needed
209 to verify that phytoene can be used as a potential hypocholesterolemic agent.
210 Phytoene is also a potential skin protector since it is accumulated in human skin in the range of
211 nmol/g fresh tissue (Melendez-Martinez et al. 2015). In this sense, Aust et al. (2005) compared the
212 effect of synthetic lycopene and a tomato extract (Lyc-o-Mato®) in volunteers. These two sources
213 provided the same quantity of lycopene but phytoene and phytofluene were only present in tomato
214 extract. After 4 and 12 weeks, erythema was induced with a solar light simulator and the protective
215 effect was more pronounced in volunteers who had ingested Lyc-o-Mato. Phytofluene and phytoene
216 may have contributed to protection against solar light since both carotenoids exhibit absorption
217 maxima at the wavelengths of UV light. In the same way, Fuller et al. (2006) evaluated the effect of

coenzyme Q10, phytoene and phytofluene on the production of inflammatory mediators in human dermal fibroblasts treated with UV-light and investigated the possible synergistic effect of these antioxidant compounds. The results indicated that coenzyme Q10 suppressed the interleukin-1–induced inflammatory response in dermal fibroblasts irradiated with UV-light. In addition, the combination of phytoene and phytofluene plus coenzyme Q10 resulted in an enhanced suppression of the inflammation process. The results suggested that the combination of these three compounds in topical skin care products enhances protection from the inflammation and premature aging caused by sun exposure.

On the other hand, some works showed that phytoene could be involved in immune defense responses (Porrini et al. 2005; von Oppen-Bezalel and Shaish, 2009). Thus, Porrini et al. (2005) determined that the daily intake of a beverage called Lyc-o-Mato® containing a natural tomato extract was able to modify plasma and lymphocyte carotenoid concentrations, particularly those of lycopene, phytoene, phytofluene and β-carotene. These experiments were made using 26 healthy subjects who consumed daily 250 mL of the drink, which contained about 6 mg lycopene, 4 mg phytoene, 3 mg phytofluene and 1 mg β-carotene. After 26 days of consuming the drink, plasma carotenoid levels and lymphocyte carotenoid concentrations increased significantly. The intake of the tomato drink significantly reduced (by about 42 %) DNA damage in lymphocytes subjected to oxidative stress. Therefore, these authors indicated that a drink containing some of the carotenoids present in tomato products might constitute an alternative source of these bioactive compounds for people who do not intake tomato products. Moreover, a phytoene and phytofluene mixture obtained from Dunaliella was able to reduce inflammation in peripheral blood lymphocytes in humans (von Oppen-Bezalel and Shaish, 2009).

Finally, epidemiological evidence suggests that estrogens are one of the most important risk factors for inducing mammary and endometrial cancers (Filardo, 2017; González et al., 2016). Thus, Hirsch et al. (2007) studied the effect of lycopene, phytoene and phytofluene on human breast (using T47D and MCF-7 tumoral cells) and endometrial (ECC-1) tumoral cells, whose promotion and proliferation were induced by phytoestrogen (genistein) and 17β-estradiol. The study showed that

phytoene and phytofluene inhibited cancer cell proliferation and interfered with the estrogenic signalling induced by both genistein and 17β-estradiol. However, these investigations were carried out using tumoral cell lines, while *in vivo* (animal and human models) studies are needed to demonstrate the putative anticancer activity of phytoene. Likewise, in an interesting study reported by Mathews-Roth (1982) was described that the intake of phytoene for 32 weeks delayed the appearance of skin tumours induced by UV-light in mice. Another recent *in vitro* study showed that the presence of phytoene, phytofluene and lycopene had a synergistic effect on the inhibition of the androgen receptor activity, and therefore, they could be involved in the prevention of prostate cancer (Linnewiel-Hermoni et al. 2015).

Bioavailability and bioaccessibility of phytoene

The health-related effect of phytoene depends not only on the amount consumed but also on its bioavailability. In this way, there are very few studies on the bioavailability of phytoene, however, some works indicated that phytoene is readily bioaccesible from grapefruits, carrots, papaya, watermelon, melon, oranges, mandarins, and from tomatoes, carrot, orange, apricot and tomato juices (Meléndez-Martínez et al. 2018 and references therein). Moreover, there is some evidence concerning the higher bioavailability of phytoene than lycopene. In fact, phytoene was found in human liver, breast and lung in similar concentrations to other major dietary carotenoids, even when its intake was lower. Moran et al. (2015) estimated that the bioavailability of phytoene could be over 2 times more than that of lycopene. In addition, Moran et al. (2013) pointed to the kinetic differences in the bioavailability, tissue uptake and metabolism of lycopene and phytoene. For that, these authors fed *Mongolian gerbils* with tomato powder for 26 days to reach steady state serum and tissue concentrations of the carotenoids. When tomato powder was supplied to *M. gerbils* in a vehicle-dosed manner, lycopene was the major carotenoid (≥55% carotenoids) in liver, spleen, testes and the prostate-seminal vesicle complex, whereas phytoene was the major carotenoid found in serum and adipose tissue (≥37% total carotenoid). As regards vehicle dosing, phytoene dosing increased hepatic, splenic and serum phytoene concentrations, whereas lycopene dosing increased only serum concentrations. Moreover, to study the rate of absorption, ^{14}C-phytoene or ^{14}C-lycopene

was provided to *Mongolian gerbils* fed tomato powder. The bioavailability assessed as ^{14}C-content was 23% for phytoene and 8% for lycopene. In addition, the levels of radioactivity accumulated in extra-hepatic tissue were higher after dosing with ^{14}C-phytoene than with ^{14}C-lycopene. All these differences could explain why phytoene is found in high concentrations in some human tissues even when lycopene intake is greater. The most likely reason is that lycopene contains a long-conjugated polyene chain, making it straight and rigid, so that it tends to crystallize, while 15-*cis*-phytoene is more flexible and less crystallisable to allow for inclusion to the tissues (Campbell et al. 2007; Moran et al. 2015; Cooperstone et al. 2015). Moreover, Werman et al. (2002) analysed the amount of phytoene and phytofluene bioavailable in Sprague–Dawley rats fed with a dry powder (1 g/kg diet) which contained 2% phytoene and 0.1% phytofluene. The highest levels of phytoene accumulation were found in liver and adrenal while the amount accumulated in other tissues (kidney, brain, spleen, lung and heart) was significantly lower. Another study evaluated the effect of a tomato powder with similar concentrations of the phytoene, phytofluene and lycopene (0.015, 0.012, and 0.011 g/kg diet, respectively) on 344 rats. At the end of this study, the major plasma carotenoid was phytoene, followed by phytofluene and lycopene (Campbell et al. 2007).

On the other hand, there is extremely scarce information about the concentration of phytoene in human organs. In this sense, Paetau et al. (1998) analyzed the effect of the ingestion of four different treatments with carotenoid-enriched extracts in 9 women and 7 men for 28 days. The four treatments consisted of a lycopene-rich tomato juice (476 mL/day; 74.9 mg of lycopene, 5.76 mg of phytoene, and 5.11 mg of phytofluene/day), lycopene beadlets (15 capsules/day; 70.2 mg of lycopene, 2.46 mg of phytoene and 3.67 mg of phytofluene/day), oleoresin soft-gel capsules (4 capsules/day; 75.4 mg of lycopene, 4.40 mg of phytoene and 4.91 mg of phytofluene/day), and placebo. At the end of these experiments the highest proportion of the three carotenes (ranging from 65% in phytofluene to 76% in phytoene and lycopene) was detected in LDL proteins (Paetau et al. 1998). These results agree with those reported by Biehler et al. (2012) who observed that carotenes were mainly found in lipoproteins.

In another study performed by Aust et al. (2005), 36 healthy volunteers ingested a commercial tomato extract in soft gel capsules (2 capsules per day; 4.9 mg lycopene, 0.4 mg phytofluene, 0.5 mg phytoene, and 0.2 mg β-carotene/capsule), or a commercial tomato-based drink (250 mL two times/day; 4.1 mg lycopene, 1.6 mg phytofluene, 2.3 mg phytoene, and 0.2 mg β-carotene/250 mL), or a synthetic lycopene in beadlets (two hard shell capsules per day; 5.1 mg/capsule) for 3 months. At the beginning of the experiment, the basal plasma levels of lycopene, phytofluene, and phytoene ranged between 0.28 and 0.37 nmol/mL, 0.27 and 0.44 nmol/mL and 0.06 and 0.07 nmol/mL, respectively. At the end of the experiment, the levels of lycopene ranged between 0.55 and 0.88 nmol/mL while the plasma levels of phytofluene enhanced from 0.27 to 0.87 nmol/mL in patient group which ingested tomato-based drinks, and from 0.44 nmol/mL to 0.94 nmol/mL in patient group which ingested tomato extract in soft gel capsules. However, the intake of the three products only increased 2-fold the serum phytoene levels (Aust et al. 2005). Likewise, 23 male volunteers were exposed to a 2-week carotenoid depletion period and then, complemented their diet with carotenoid rich products (330 mL carrot juice, 330 mL tomato juice and 10 g spinach powder for 2 weeks). The average levels of phytoene were 0.14 and 0.09 nmol/mL at the beginning and the end of depletion period, respectively. After tomato and carrot supplementation period, the phytoene plasma levels were 0.44 nmol/mL whereas after 2 weeks of supplementation with spinach the phytoene plasma levels decreased markedly (0.29 nmol/mL) (Müller et al. 1999).

On the other hand, phytoene bioavailable has also been studied using *in vitro* models (Jeffery et al. 2012; Rodrigo et al. 2015; Mapelli-Brahm et al. 2017). These *in vitro* models can be useful to analyse the impact of the food matrix on carotenoid release and potential availability during the digestion process despite that they do not account for host factors influencing carotenoid bioavailability (Bohn et al. 2017). In this way, Jeffery et al. (2012) studied carotenoid bioaccessibility from fruits and vegetables using an *in vitro* digestion model. These authors showed that lycopene was the most bioaccesible from 100 g of grapefruit or watermelon (69 and 64 µg respectively); α-carotene (559 µg), β-carotene (1078 µg), lutein (91 µg) and phytoene (23000 µg) from 100 g of carrot, and violaxanthin (177 µg) from mango. In another *in vitro* digestion model

applied to obtain the micellar fraction for the determination of carotenoid bioaccessibility, Rodrigo et al. (2015) showed that the relative bioaccessibility of phytoene was higher in freshly hand-squeezed juice of the sweet orange Washington Navel (over 22%) than in the pulp obtained from this fruit (over 8%). However, the relative bioaccessibility of phytoene was higher in the pulp obtained from Clementine mandarins (over 20%) than in the freshly hand-squeezed juice of this fruit (over 16%).

Moreover, using a simulated gastro-intestinal digestion model, the bioaccessibility of phytoene and phytofluene from tomato, carrot, blood orange, and apricot juices was also analysed (Mapelli-Brahm et al. 2017), and this bioaccessibility decreased in the following order: blood orange>apricot>tomato>carrot. In addition, phytoene had highest bioaccessibility (up to 97%) than other carotenoids (β-cryptoxanthin, β-carotene, α-carotene, and lycopene), it could mainly be due to its marked difference in chemical structure and matrix distribution. The dietary source that provided highest levels of absorbable phytoene/phytofluene was tomato juice (5 mg/250 mL juice). In the same way, Mapelli-Brahm et al. (2018) also analysed the effect of thermal treatments on the bioaccessibility of phytoene and phytofluene in relation to changes in the microstructure and size of orange juice particles. For these studies ultrafrozen juices which were thawed at room temperature, in microwave oven, or in fridge were used. The results showed that phytoene and phytofluene suffered less degradation because of ultrafreezing than other carotenoids. In addition, phytoene was the carotenoid with highest bioaccessibility in the fresh juice, and the samples with lower particle size had a higher bioaccessibility. These authors concluded that thermal treatments could enhance the release of carotenoids from the matrix due to the degradation of cells which could explain the higher bioaccessibility.

Phytoene production

At present, carotenoids could be obtained using biotechnological production systems, including yeasts, algae and plant *in vitro* cultures (Gao et al. 2011; Engelmann et al. 2010a; Wozniak et al. 2011); however, commercially, carotenoids are obtained by chemical synthesis (Coulson et al. 1980) or direct extraction from vegetables (Dufossé et al. 2005). In fact, direct extraction of

352 phytoene content in white Marsh and the red-fleshed Star Ruby grapefruits (*C. paradise*) at eight
353 developmental stages, ranging from immature-green to full coloured fruits was studied by Alquezar
354 et al. (2013). These authors observed that the production of phytoene in the flavedo of Star Ruby
355 (11 µg/ g FW) increased substantially during maturation, whereas the content of phytoene in the
356 flavedo of Marsh remained low in all the stages studied (2.5 µg/ g FW). In addition, the maximal
357 levels of phytoene in pulp were found in Star Ruby grapefruit in the full coloured stage (3 µg/ g
358 FW, Table 1). Moreover, the content of phytoene in *C. sinensis* Osbeck cv. Cara Cara was
359 exclusively accumulated in peel and pulp, although the content in pulp (176.8 µg/ g DW) was 10
360 times higher than in peel (14.09 µg/ g DW) 209 days after flowering (Table 1) (Tao et al. 2007). On
361 the other hand, deficit irrigation was used to increase the production of phytoene in cherry and
362 common tomatoes (Coyago-Cruz et al. 2017). These authors showed the maximal levels of
363 phytoene were found in *S. lycopersicum* var. Tigerella under a water stress of -1 MPa of leaf water
364 potential (55.1 mm of applied water) reaching levels of 167 µg/ g DW (Table 1). Other authors
365 (Fraser et al. 2007) analysed the concentration of phytoene during the development and ripening of
366 tomato fruits, observing a direct relationship between the levels of ripening and the concentration of
367 phytoene; thus, the phytoene content was higher in the turning and ripe stages (102.3 µg/ g DW),
368 whereas the lowest levels were found in immature, mature green and breaker stages (<6 µg/ g DW)
369 (Table 1). All these results indicate that it is important to consider not only the genotype and
370 cultivar as well as their developmental stages but also the environmental factors on phytoene
371 content.
372 On the other hand, the use of plant *in vitro* cultures has been proposed as a sustainable alternative to
373 produce plant natural products (Rao and Ravishankar, 2002). Such in *vitro* cultures have a series of
374 advantages compared to the use of whole plants: i) pathogens are removed; ii); cultured plant cells
375 require only simple nutrients to grow, and therefore the operational costs for growing them in
376 bioreactors are lower than for mammalian cell growth in bioreactors; iii) the purification and
377 processing of the natural products obtained using plant *in vitro* cultures is less complicated than in
378 whole plants, which also decreases production costs (Ramirez-Estrada et al. 2016). For these

reasons, plant *in vitro* cultures have been used to increase the production of bioactive compounds. In fact, taxol, berberine or podophyllotoxin are produced from *Taxus* sp., *Coptis japonica* and *Podophyllum hexandrum* cell cultures, respectively (Exposito et al. 2009; Matsubara et al. 1989; Chattopadhyay et al. 2002).

As regards phytoene production using plant *in vitro* cultures, Gao et al. (2011) observed that calli of Tarocco blood orange (*C. sinensis* L.) were able to produce this compound. In addition, these calli accumulated more phytoene when grown under white light (5.8 µg/g DW) compared to darkness (1.3 µg/g DW). However, Red Marsh calli (*C. paradise*) produced higher levels of phytoene after 20 days of darkness (3.7 µg/g DW) than the treatment under white light (over 3 µg/g DW) (Table 1). Moreover, the results showed that the expression profile of *psy* gene was up-regulated in these calli. The authors concluded that light regulated the expression of several carotenogenesis genes in *Citrus* callus, although no significant changes were observed in carotenoid production. For their part, Azadi et al. (2010) observed that *Lilium* x *formolongi* calli were able to produce 0.3 µg phytoene /g FW.

On the other hand, *Xanthophyllomyces dendrorhous* yeast cultures have been used to increase the production of phytoene using a 12-l batch culture fermentor containing 9 l of minimal medium supplemented with 2 % glucose or 2% succinate as carbon source (Wozniak et al. 2011). The highest levels of phytoene (6.7 µg/g DW) were found in yeast cultures grown in the presence of glucose during the early stationary growth phase, which is 6.7-fold increase over the levels of phytoene in cultures grown in the presence of succinate.

Effect of bleaching herbicides on phytoene production

Biotechnological production systems have been optimized by treating different biological materials with bleaching herbicides such as 2-(4-chlorophenylthio)-triethylamine (CPTA), norflurazon, and diflufenican (Engelmann et al. 2009). Thus, Simkin et al. (2000) analysed the effect of two bleaching herbicides, norflurazon and J852 (which work as inhibitors of phytoene desaturase and ζ-carotene desaturase, respectively), on phytoene production in *Capsicum annuum* leaves treated for 3 days. In both cases, there was a significant increase in phytoene levels, which reached values of

406 1480 and 550 µg/ g dry weight (DW) in the presence of 30.3 µg/l norflurazon and J852,
407 respectively (Table 1) whereas no phytoene was found in the corresponding controls. Similar results
408 were found by Campisi et al. (2006), who observed an increase in phytoene levels (27.9 µg/ g fresh
409 weight (FW)) in the presence of fluridone herbicide, which is a phytoene desaturase inhibitor, in *H.*
410 *annuus* leaves grown for 3 weeks, representing a 27.9-fold increase over control levels (Table 1).
411 These results clearly indicated that the addition of bleaching herbicides increased the production of
412 phytoene.
413 In another approach, when tomato was used to obtain calli from leaves and buds, the levels of
414 phytoene were 1.41 and 5.59 µg/g FW for leaf and bud calli, respectively (Engelmann et al. 2010a).
415 The authors also analysed the content of phytoene in different varieties of tomato calli and observed
416 that the variety Alisa Craig hp-1 produced maximal levels of phytoene (2.67 µg/g FW) in the
417 presence of 74.5 µg/l CPTA (acts as a lycopene cyclase inhibitor, Table 1). Moreover, Engelmann
418 et al. (2010b) showed that Alisa Craig hp-1 tomato cell suspensions produced high levels of
419 phytoene in the presence of norflurazon (4960 µg/l, Table 1), while VFNT cherry cell suspensions
420 treated with CPTA at the same concentration produced 5.22 µg/g FW (Engelmann et al. 2010a)
421 using a cell density of 50 g FW/l. The production of phytoene was also increased using VFNT
422 cherry cell suspensions (cell density of 50 g FW/l) treated with 750 µg/l norflurazon for 7 days
423 (633.7 µg/l), which is 124-fold higher than for control cells (5.1 µg/L, Table 1) (Campbell et al.
424 2006). Using microalgaes, 39.43 mg/l diflufenican and 30 mg/l norflurazon for 14 days increased
425 the production of phytoene (69.4 and 59.9 %, respectively) in *Haematococcus pluvialis* cultures
426 (Harker and Young, 1995), whereas in control treatments neither was detected.
427 All these results indicate that the use of inhibitors of the carotenogenic pathway is an interesting
428 approach to increasing the production of phytoene. In fact, plant cell cultures or algae treated with
429 norflurazon were able to produce high levels of this compound, suggesting that these systems could
430 represent an alternative strategy for producing phytoene.
431 ***Metabolic engineering of the phytoene biosynthetic pathway***
432 *Increasing phytoene content by metabolic engineering in plants and plant in vitro cultures*

Metabolic engineering can be a valuable tool in plants to increase the production of metabolites as a result of varying the endogenous biosynthetic pathways. Through metabolic engineering, three types of approach are possible to: i) enhance the production of novel compounds, ii) decrease the production of unwanted metabolites, and iii) enhance the production of a metabolite of interest (Kumar et al. 2015). Metabolic engineering uses different strategies to attain these goals, such as the modification of single steps in a biosynthetic pathway to increase or decrease the production of metabolites or blocking some metabolic steps to increase the flow of carbon towards the biosynthesis of a given metabolite. However, all these strategies are of limited value, and new strategies using the metabolic engineering are necessary. Thus, if several steps of the same pathway are controlled using master regulator or transcription factors, greater control of metabolic flux might be obtained. For example, metabolic engineering has been used in plants to increase the ectopic and heterologous production of phytoene (Table 2). Thus, Pierce et al. (2015) studied the effect of overexpressing different genes involved in the carotenoid biosynthetic pathway, specifically the bacterial phytoene synthase (*crtB*) from *Erwinia uredovora* and two ketolase genes (*crtW* from *Brevundimonas* sp. and *bkt* from *H. pluvialis*), on carotenoid biosynthesis in soybean seeds. These authors observed that the transformation with these genes increased phytoene production to 83, 44 and 39 µg/ g DW in the transgenic soybean lines transformed with *crtB* + *crtW*, *crtB* + *bkt* and *crtB*+*crtW*+*bkt*, respectively (Table 2), although phytoene was not detected in the non-transgenic control seeds. Transgenic seeds also accumulated other carotenoids such as lutein, β-carotene, α-carotene, lycopene and β-cryptoxanthin. Similarly, Burkhardt et al. (1997) increased the levels of phytoene in immature embryos of *Oryza sativa* transformed by microprojectile bombardment with a cDNA coding *psy* gene from *Narcissus pseudonarcissus* (0.74 µg/ g dry seeds, Table 2) whereas in the control extracts, phytoene was not detectable or was present in trace amounts. Ravanello et al. (2003) also analyzed the effect of the overexpression of bacterial genes, such as geranylgeranyl diphosphate synthase (*crtE*), *crtB* and lycopene β-cyclase (*crtY*) from *E. uredovora*, and a combination of *crtB* with a lycopene β-cyclase from *Brassica napus*, on carotenoid production in *B. napus* seeds. The transgenic seeds that overexpressing the bacterial *crtB* and a plant lycopene β-

cyclase showed an increase in the levels of total carotenoids. In addition, by combining the expression of *crtE* with *crtB* gene or *crtB* and *lycopene β-cyclase*, the level of phytoene produced was 220 µg/ g fresh seeds, whereas the combination of *crtB* + *crtY*-expressing transgenic lines produced 207 µg/ g fresh seeds (Table 2).

In another assay, *Daucus carota* roots transformed with *crtB* gene from *Dioscorea* sp. were cultivated for 8 and 16 weeks, and the production of phytoene increased from 90 µg/ g DW at 8 weeks to 170 µg/ g DW at 16 weeks of cultivation, representing a 90 and 170-fold increase compared with wild-type cultures (0 µg/ g DW) (Table 2) (Maass et al. 2009). In a similar way, the effect of overexpressing *crtB* gene from *E. uredovora* on carotenoid production in *S. lycopersicum* plants was studied by Fraser et al. (2002). These authors showed that the overexpression of *crtB* enhanced the amount of phytoene, reaching a maximal level of 168 µg/ g DW, which is 3.2-fold higher than that obtained in non-transgenic plants (52 µg/ g DW, Table 2). In addition, lycopene and β-carotene also enhanced in transgenic lines, and lutein levels in these lines were increased 1.6-fold. Also, Fraser et al. (2007) analyzed the carotenoid content of *S. lycopersicum* fruits expressing *psy* gene in a constitutive manner during fruit development and ripening. The levels of phytoene increased from the immature stage (6.5 µg/ g DW) of fruit development to the ripe stage (240.2 µg/ g DW), that is 10 days after breaker (Table 2). Although phytoene was detected in the ripening stage in non-transgenic fruits, the levels were lower (102.3 µg/ g DW) than those found in *psy*-transgenic fruits.

Alternatively, transgenic calli obtained from plants are a valuable cell biomass for producing carotenoids (Maass et al. 2009). For example, *Arabidopsis thaliana* transgenic calli overexpressing *psy* gene from *A. thaliana* were highly effective for producing phytoene (400 µg/g DW) and α/β-carotene (over 650 µg/g DW) after 12 days in darkness (Maass et al. 2009). In addition, the transformation of *Lilium* x *formolongi* calli with the bacterial β-carotene hydroxylase (*crtZ*), isopentenyl diphosphate isomerase (*idi*), *crtW, crtE, crtB,* phytoene desaturase (*crtI*) and *crtY* genes provoked an increase in the levels of total carotenoids (Azadi et al. 2010). In fact, the total amount of carotenoids (133.3 µg/g FW) in the transgenic calli increased 26.1-fold compared to wild-type

calli (5.1 µg/g FW). More specifically, the production of phytoene (50.5 µg/ g FW) enhanced compared to wild-type cultures (0.3 µg/ g FW) (Azadi et al. 2010). The accumulation of phytoene in *D. salina* following the down regulation of its *pds* gene using RNA interference (RNAi) and antisense technology was studied by Srinivasan et al. (2017). Both the RNAi and antisense transgenic lines showed a significant decrease in the expression of *pds* gene and an increase in phytoene levels (1080 µg/ g DW) compared to wild-type cultures (in which phytoene was not detected).

Increasing phytoene content by incorporating genes from the phytoene biosynthetic pathway in microbial organisms

Another promising production system is based on the metabolic engineering of microorganisms to synthesize plant metabolites. In fact, synthetic biological approaches have made it possible to reconstruct entire plant biosynthetic processes in microbial systems. In all these processes, it is important to express the specific biosynthetic pathway enzymes in an active form in microbial systems. However, efficient production of the active forms of the recombinant enzymes depends on different factors and culture conditions. Thus, microorganisms have been widely used for this purpose since they have fast growth rates, are easy to transform, grow in simple culture media and a large variety of strains, mutants and plasmid-based expression systems can be used. They also show high expression rates in the production of foreign proteins. These are the main reasons why microorganisms have been frequently used to produce plant secondary metabolites (Da Silva and Srikrishnan, 2012).

Tools and techniques for genetic manipulation of microorganisms, including several integrative transformation plasmids as well as a wide variety of carotenogenic mutants such as bacteria and yeasts, are available (Gassel et al. 2014; Schmidt et al. 2011). These transformed strains have been used to generate phytoene-producing strains. In this regard, much more information is available concerning metabolic engineering used to transform *Escherichia coli*. Thus, Lu et al. (2011a) studied the accumulation of carotenoids using the engineered *E. coli* strain harboring *crtE* and *crtB* genes from *E. herbicola*, when this strain was cultured for 24 hours. These authors observed that

engineered *E. coli* strains produced higher levels of phytoene (35 µg/g FW) and lycopene (100 µg/g FW) than wild-type cultures. Similarly, when *crtB* and *crtE* genes from *Enterobacter agglomerans* were overexpressed in *E. coli* strain and *crtI* gene was mutated (*crtI*-MT) or truncated (*crtI*-T) phytoene production increased (Lu et al. 2011b). Interestingly, transgenic *E. coli* harboring *crtB* and *crtI*-MT genes accumulated more phytoene (73 µg/g wet cells) than those harboring *crtB* and *crtI*-T (53 µg/g wet cells) (Table 2). Yamano et al. (1994) increased the production of lycopene and β-carotene as well as phytoene by using *Saccharomyces cerevisiae* yeast transformed with different key enzymes of the carotenoid biosynthetic pathway, specifically *crtE, crtB* and *crtI* from *E. uredova* using Y514 plasmid (15 µg phytoene/ g DW and 113 µg lycopene/ g DW) or *crtE, crtB, crtI* and *crtY* using Y5143 plasmid (14 µg phytoene/ g DW, 14 µg lycopene/ g DW and 103 µg β-carotene/ g DW) (Table 2). Furthermore, when hydroxymethyl-3-glutaryl coenzyme A reductase (*hmgr*), *crtE* and *crtYB* (a fusion gene encoding a phytoene synthase together with a lycopene cyclase) genes were overexpressed in the red yeast *X. dendrorhous* and *crtI* gene was inactivated, an increase in phytoene production (over 7500 µg/g DW) was observed in shaking cultures (Table 2) (Pollmann et al. 2017). These authors also carried out the scaling-up to 2l and obtained the highest phytoene levels recorded for any organism, producing over 10000 µg/g DW. In a similar way, a squalene/phytoene synthase homolog from an acidothermophilic archaeon *Sulfolobus acidocaldarius* was introduced into a hyperthermophilic archaeon *Thermococcus kodakarensis*, and were able to produce phytoene ranged between 80–750 µg/l (Fuke et al. 2018).

Therefore, it seems possible to consider microorganisms as factories to produce plant-derived compounds, as seen from their successful production as described above (Fowler et al. 2011; Pickens et al. 2011), and their possible advantages compared to the use of plant *in vitro* cultures would include shorter process cycles and easier scale-up. Among microorganisms, yeasts, which are unicellular, devoid of endotoxins, amenable to genetic improvement and suitable for large-scale fermentation, are particularly attractive for the development of these biotechnological approaches. Furthermore, the emergence of new processing techniques suggests an optimistic future for the commercial production of yeasts with a high phytoene content in the future.

Conclusions and perspectives

Phytoene is a carotenoid with beneficial effects on human health. It has antioxidant activity, inhibits lipoprotein oxidation and protects against B-UV light; also, some studies have suggested that phytoene could have anticancer activity and could decrease cholesterol levels. However, the lack of *in vivo* human studies on the effect of phytoene restricts its use as a drug component or nutraceutical. There is therefore an urgent need to perform *in vivo* trials in human with phytoene to verify its biological activities.

Whatever the case, the beneficial effects of phytoene have led to many new strategies being developed to increase its production, using *in vitro* cultures as an alternative system to the extraction from raw material. Phytoene production using *in vitro* cultures has been improved by optimizing culture conditions, assaying several media, the use of inhibitors of the carotenogenic pathway, sugar supplements, etc. Nevertheless, these empirical methods have not been able to satisfy the growing demand for this product.

On the other hand, results have shown that metabolic engineering is a feasible strategy to increase phytoene production in different biological systems. The results also suggested that genes from the phytoene biosynthetic pathway, the construction designed to express them, the host strains used, among other factors, can modify the efficiency of the process to obtain phytoene using metabolic engineering. It is important to gain new insight into how the phytoene biosynthetic pathway is regulated as well as to identify new transcription factors and key genes that would permit higher phytoene yields.

As this review has shown, the best results in terms of phytoene production were obtained overexpressing *hmgr*, *crtE* and *crtY* genes and inactivating *crtI* in *X. dendrorhous* yeast cultivated in a small-scale fermenter (10000 µg/ g DW). It is clear, then, that the metabolic engineering of microorganisms constitutes an innovative approach to phytoene production. More precisely, yeasts seem to be the best candidates for this purpose. However, it is important to state, that only high-value bioactive compounds like phytoene have any chance of being produced by such

biotechnological means as it is currently unrealistic to use these microorganisms to produce bioactive compounds as cheaply as is possible with plant sources.

Future perspectives should focus on the simultaneous use of empirical and rational approaches using *in vitro* cultures to obtain biotechnological systems for producing high phytoene biosynthesis levels.

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850
851 **Figure captions**
852 **Figure 1.** Biosynthetic pathway of phytoene. GGPS: geranylgeranyl-diphosphate synthase, PSY:
853 phytoene synthase, PDS: phytoene desaturase, ZISO: ζ-carotene isomerase, ZDS: ζ-carotene

desaturase, CRTISO: carotenoid isomerase, CrtE: bacterial geranylgeranyl diphosphate synthase, CrtB: bacterial phytoene synthase, CrtI: bacterial phytoene desaturase/carotene isomerase.

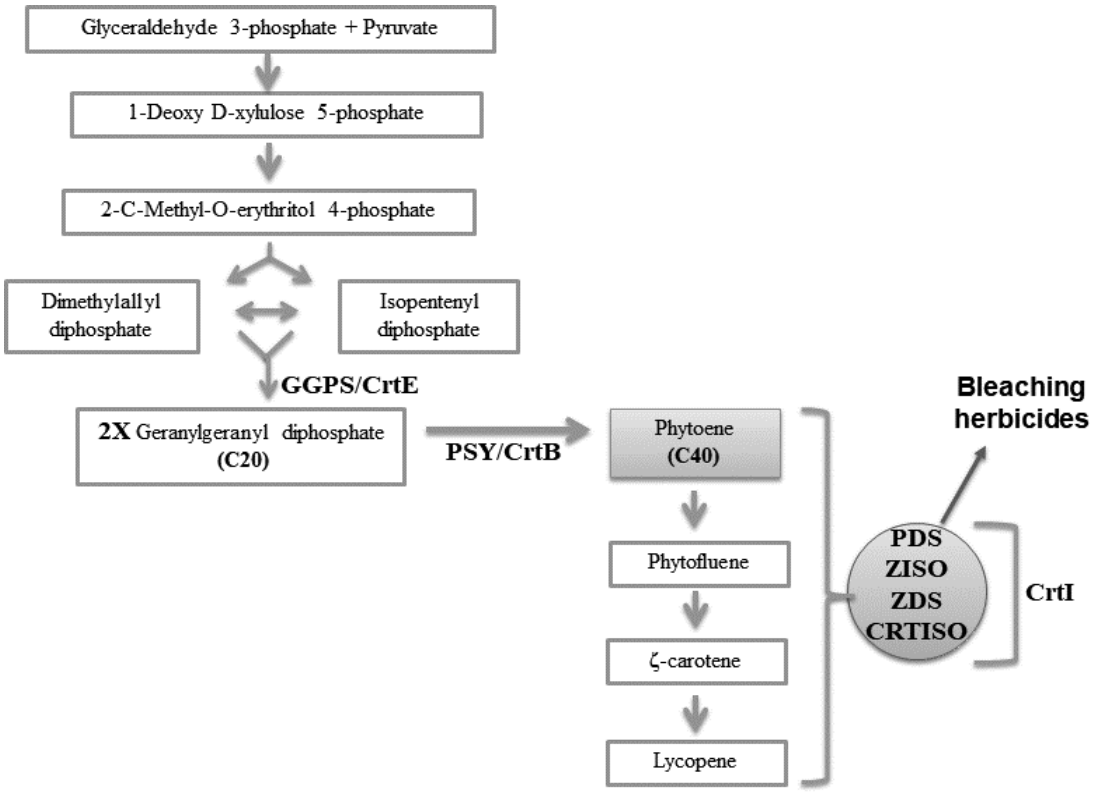

Table 1. Strategies to enhance the production of phytoene.

Material	Species	Strategy	Phytoene production	References
Plant	*Citrus paradise* Marsh fruits	Direct extraction from different tissue: Flavedo (Mature-green stage) Pulp (Full-coloured stage)	 2.5 µg/ g FW 0.2 µg/ g FW	Alquezar et al. (2013)
Plant	*Citrus paradise* Star Ruby fruits	Direct extraction from different tissue: Flavedo (Full-coloured stage) Pulp (Mature-green stage)	 11 µg/ g FW 3 µg/ g FW	Alquezar et al. (2013)
Plant	*Citrus sinensis* Osbeck cv Cara Cara	Direct extraction from different tissue: Peel after 209 days flowering Pulp after 209 days flowering	 14.09 µg/ g DW 176.8 µg/ g DW	Tao et al. (2007)
Plant	*Solanum lycopersicum* fruit	Water stress	167 µg/ g DW	Coyago-Cruz et al. (2017)
Plant	*Solanum lycopersicum* fruit	Direct extraction from different tissue: Turning: 3 days after breaker Ripe: 10 days after breaker	 102.3 µg/ g DW 102.3 µg/ g DW	Fraser et al. (2007)
Calli	*Citrus paradise*	20 days under darkness	3.7 µg/ g DW	Gao et al. (2011)

	Citrus sinensis *Citrus reticulata x* *Citrus sinensis*	20 days under white light (16 h photoperiod) 20 days under white light (16 h photoperiod)	5.8 µg/ g DW 0.5 µg/ g DW	
Yeast	*Xanthophyllomyces dendrorhous*	12-l batch culture fermentor containing 9 l minimal medium with 2% glucose + early stationary growth phase	6.7 µg/ g DW	Wozniak et al. (2011)
Plant	*Capsicum annuum* leaves	30.3 µg/l norflurazon for 3 days J852 herbicide for 3 days	1480 µg/ g DW 550 µg/ g DW	Simkin et al. (2000)
Plant	*Helianthus annuus* L. leaves	329 µg/l fluridone for 3 weeks	27.9 µg/ g FW	Campisi et al. (2006)
Calli	*Solanum lycopersicum* cv Mill	calli obtained from leaf calli obtained from bud	1.41 µg/ g FW 5.59 µg/ g FW	Engelmann et al. (2010a)
Calli	*Solanum lycopersicum* cv Ailsa Craig hp-1	Control 74.5 µg/l CPTA	0.44 µg/ g FW 2.67 µg/ g FW	Engelmann et al. (2010a)
Cell suspensions	*Solanum lycopersicum* cv Ailsa Craig hp-1	norflurazon CPTA	4960 µg/l 260 µg/l	Engelmann et al. (2010b)

		CPTA + 30 g/l [$^{13}C_6$] -glucose	300 µg/l	
Cell suspensions	*Solanum lycopersicum*	Control	0.70 µg/ g FW	Engelmann et al. (2010a)
	cv VFNT cherry	CPTA	5.22 µg/ g FW	
Cell suspensions	*Solanum lycopersicum*	750 µg/l norflurazon for 7 days	633.7 µg/l	Campbell et al. (2006)
	cv VFNT cherry			
Algae	*Haematococcus*	39.43 mg/l diflufenican for 14 days	69.4%	Harker and Young (1995)
	pluvialis	30 mg/l norflurazon for 14 days	59.9%	

Table 2. Ectopic and heterologous production of phytoene using metabolic engineering.

Organism	Species/strain	Genes	Origin of genes	Strategy	Production	reference
Plant	Transgenic T1 *Glycine max* seeds	*crtB+crtW*	*crtB/ E. uredovora crtW/Brevundimonas* sp.	Overexpression of *crtB* and *crtW*	83 µg/ g dry seeds	Pierce et al. (2015)
	Transgenic T1 *Glycine max* seeds	*crtB+bkt*	*crtB/ E. uredovora bkt/H. pluvialis* sp.	Overexpression of *crtB* and *bkt*	44 µg/ g dry seeds	Pierce et al. (2015)
	Transgenic T1 *Glycine max* seeds	*crtB+crtW+ bkt*	*crtB/ E. uredovora crtW/Brevundimonas* sp. *bkt/H. pluvialis*	Overexpression of *crtB, crtW* and *bkt*	39 µg/ g dry seeds	Pierce et al. (2015)
	Oriza sativa immature embryos	*psy*	*psy/N. pseudonarcissus*	Overexpression of *crtB*	0.74 µg/ g dry seeds	Burkhardt et al. (1997)
	Brassica napus seeds	*crtE+crtB* *crtB+crtY* *crtB+β-*	*crtE, crtB,crtY/ E. uredovora* *β-cyclase/ B. napus*	Overexpression of *crtE* + *crtB* Overexpression of *crtB* + *crtY* Overexpression of *crtB* +	220 µg/ g fresh seeds 207 µg/ g fresh seeds 220 µg/ g fresh	Ravanello et al. (2003)

		cyclase		*β-cyclase*	seeds	
	Daucus carota roots	*crtB*	*crtB/ Dioscorea* sp.	8-week old plants	90 µg/ g DW	Maass et al. (2009)
				16-week old plants	170 µg/ g DW	
	Solanum lycopersicum fruit	*crtB*	*crtB/E. uredovora*	T_0 generation	168 µg/ g DW	Fraser et al. (2002)
				T_1 generation	71 µg/ g DW	
				T_2 generation	47 µg/ g DW	
	Solanum lycopersicum fruit	*psy*	*psy/S. lycopersicum*	Immature (11 d after anthesis)	6.5 µg/ g DW	Fraser et al. (2007)
					133.1 µg/ g DW	
				Mature green (37 d after anthesis)	161.0 µg/ g DW	
				Breaker (44 d after anthesis)	164.5 µg/ g DW	
				Turning (37 d after breaker)	240.2 µg/ g DW	
				Ripe (10 d after breaker)		
Calli	*Arabidopsis thaliana calli*	*psy*	*psy/ A. thaliana*	12 days after transfer to darkness	400 µg/ g DW	Maass et al. (2009)
	Lilium formolongi	*crtZ, crtW, idi, crtE,*	*crtE, crtB, crtI, crtY/E. uredovora*	Overexpression 7 genes	50.5 µg/ g FW	Azadi et al. (2010)

		crtB, crtI, crtY	*crtZ, crtW/ Brevundimonas sp. idi/Paracoccus sp.*			
Alga	*Dunaliella salina*	*pds*	*pds / D. salina*	Inactivation of *pds* gene	1080 µg/ g DW	Srinivasan et al. (2017)
Yeast	*Saccharomyces cerevisiae*	*crtE, crtB, crtI*	*crtE, crtB, crtI/ E. uredovora*	Transformation with Y514 plasmid	15 µg/ g DW	Yamano et al. (1994)
	Saccharomyces cerevisiae	*crtE, crtB, crtI, crtY*	*crtE, crtB, crtI/ E. uredovora*	Transformation with Y5143 plasmid	14 µg/ g DW	Yamano et al. (1994)
	Xanthophyllomyces dendrorhous	*hmgr, crtE, crtY*	*hmgr, crtE, crtY / X. dendrorhous*	Inactivaction of *crtI* gene Overexpression of *hmgr, crtE, crtY*	7464 µg/ g DW	Pollmann et al. (2017)
	Xanthophyllomyces dendrorhous	*hmgr, crtE, crtY*	*hmgr, crtE, crtY / X. dendrorhous*	Inactivaction of *crtI* gene Overexpression of *hmgr, crtE, crtY.* Scaling-up to 2l	10000 µg/ g DW	Pollmann et al. (2017)

Archaea	*Thermococcus kodakarensis*	squalene/ phytoene synthase	*Sulfolobus acidocaldarius*	Overexpression of squalene/ phytoene synthase	80-750 µg/l	Fuke et al. 2018
Bacteria	*Escherichia coli*	*crtE, crtB*	*crtE, crtB /E. herbicola*	Overexpression of *crtE and crtB*	35 µg/ g FW	Lu et al. (2011a)
	Escherichia coli	*crtB, crtI*	*crtB, crtI* / *E. agglomerans*	Mutations *crtI* gene Overexpression of *crtB*	73 µg/ g wet cells	Lu et al. (2011b)
	Escherichia coli	*crtB, crtI*	*crtB, crtI* / *E. agglomerans*	Truncation *crtI* gene Overexpression of *crtB*	53 µg/ g wet cells	Lu et al. (2011b)