Chapter 4

Microbiological Synthesis of Carotenoids: Pathways and Regulation

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Abstract

Carotenoids are naturally produced by plants, algae, and some bacteria and fungi, fulfilling functions as accessory photosynthetic pigments and antioxidants. Among carotenoids, the xanthophyll astaxanthin stands out for its antioxidant and nutraceutical properties, which are beneficial to human health, and also for its use in the aquaculture industry as nutritional supplement of salmonid fish. Many studies have focused on the search of natural sources of astaxanthin as an alternative production that guarantees the beneficial properties of this compound. In nature, few astaxanthin-producing organisms are known, being the microalgae *Haematococcus pluvialis* and the yeast *Xanthophyllomyces dendrorhous* the most promising microbiological systems for the biotechnological production of this carotenoid. In this chapter, we describe the carotenogenic pathways in these microorganisms and the proposed carotenogenesis regulation mechanisms. As an example, the influence of the carbon source, the regulation by catabolic repression and by sterols in the carotenogenesis in the yeast *X. dendrorhous* is described.

Keywords: astaxanthin, *Xanthophyllomyces dendrorhous*, *ergosterol*, *catabolite repression*

1. Introduction

Currently, carotenoids are valuable molecules due to their use in different industries such as chemical, pharmaceutical, poultry, food and cosmetics. These pigments have coloring and antioxidant properties that have attracted the attention of researchers and industries as they have useful applications on several fields [1]. Carotenoids are naturally occurring lipid-soluble

pigments, the majority being C_{40} terpenoids, which act as membrane-protective antioxidants scavenging O_2 and peroxyl radicals; their antioxidant ability is apparently attributed to their structure, which in some cases (for example: astaxanthin) contain two terminal rings joined by a double conjugated chain or polyene system. Carotenoids are classified in two major groups: i) carotenes, composed only by carbon and hydrogen, and ii) xanthophylls, which are oxygenated derivatives [2]. Approximately 600 different carotenoid structures have been described to date that can be found in most life forms and have various functions ranging from their original role as photosynthetic pigments to antioxidants, precursors of vitamin A or pigments involved in the visual attraction of pollinating animals [2].

Commercial carotenoids are mainly obtained by extraction from plants and by chemical synthesis. However, some problems regarding seasonal and geographic variability that cannot be controlled arise in the case of production and marketing of several colorants of plant origin. On the other hand, the chemical synthesis may generate hazardous waste that can affect the environment. Unlike these traditional methods, the microbial production of carotenoids turns into a great opportunity of safe use. Microbial production has the advantage to use low-cost substrates, resulting in lower production costs. This explains the increasing interest in the production of microbial carotenoids as substitutes for synthetic carotenoids used as colorants in food and feed industries [1]. Among carotenoids, the xanthophyll astaxanthin stands out for its antioxidant and nutraceutical properties that are beneficial to human health, and for its use in the aquaculture industry as nutritional supplement of salmonid fish [3]. Many studies have pointed to search for a natural source of astaxanthin as an alternative production method that guarantees the beneficial properties of the mentioned compound. In nature, few astaxanthin-producing organisms are known, being the microalgae *Haematococcus pluvialis* and the yeast *Xanthophyllomyces dendrorhous* the most promising microbiological systems for the biotechnological production of this carotenoid. In this context, *X. dendrorhous* produces unmodified astaxanthin as the main carotenoid, does not require light for its growth and pigmentation and can use various carbon sources having a relatively rapid growth [2, 4]. In contrast, the production of astaxanthin by *H. pluvialis* requires specific conditions during its cultivation, because it changes its structure during the growth cycle. This means that the physical properties and nutrient requirements of the alga change during the culture process, altering the optimal conditions for growth and carotenoid production [1].

Despite the potential of *X. dendrorhous*, the specific production of astaxanthin by wild-type strains of this yeast is too low to be a commercially competitive source [5]. Due to the above, many researchers have tried to improve the production of astaxanthin using several methods as the optimization of culture conditions, classical random mutagenesis methods, genetic and metabolic engineering strategies [1, 4, 6–15]. These studies have led to an extensive knowledge about the biology of this yeast and the carotenoid synthesis pathway. However, in recent years, interest has been focused on elucidating the regulatory mechanisms operating on the production of carotenoids in this yeast. The carbon source is the most studied parameter that influences carotenogenesis in *X. dendrorhous*. Glucose and other fermentable sugars are

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initially metabolized by the glycolytic pathway, followed by alcoholic fermentation, even in the presence of oxygen. On the other hand, non-fermentable carbon sources, such as ethanol and succinate, are metabolized through acetyl-CoA oxidation entering directly to the citric acid cycle. In general, non-fermentable carbon sources as ethanol, enhance the synthesis of carotenoid pigments in *X. dendrorhous* [1, 16, 17] and a relation between carotenoid production and ergosterol and fatty acid synthesis have been reported, since these pathways use the same substrates that derive from the mevalonic acid (MVA) pathway [18–20].

This chapter is focused on the microbiological synthesis of astaxanthin, taking as example the microorganisms *X. dendrorhous* and *H. pluvialis* since both are considered among the most promising for biotechnological production of this carotenoid. Also, the potential regulatory mechanisms that influence carotenogenesis is described, particularly, the influence of the carbon source and the regulation of carotenogenesis by catabolic repression in the yeast *X. dendrorhous*.

2. Importance of astaxanthin

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) belongs to the xanthophyll group and its structure consists of two terminal rings joined by a polyene chain (**Figure 1**). In addition to the existence of cis or trans geometric isomers, astaxanthin presents 3 configurational isomers: two enantiomers (*3R, 3′R* and *3S, 3′S*) and one meso form (*3R, 3′S*), due to the existence of two asymmetric carbons located at the 3, 3′ positions of the β-ionone ring with hydroxyl group (–OH) on both ends of the molecule [21, 22]. The stereoisomers (3*S*, *3′S*) and (3*R*, 3′*R*) are the most abundant in nature. *H. pluvialis* biosynthesizes the (3*S*, 3′*S*)-isomer whereas the yeast *X. dendrorhous* produces the (3*R*, 3′*R*)-isomer, while synthetic astaxanthin comprises isomers (3*S*, 3′*S*), (3*R*, 3′*S*) and (3*R*, 3′*R*) [22].

Being a xanthophyll, astaxanthin possesses chemical properties and physiological characteristics of these compounds: it is highly lipophilic and shows an intense red color based on the light absorbed by its polyene system [22]. Due to its 3-hydroxyl and 4-keto functional groups in the terminal rings, astaxanthin has a higher polarity than other carotenoids and an antioxidant activity greater than that of β-carotene, lutein, zeaxanthin and canthaxanthin [5, 23, 24]. Due to its high antioxidant activity, there is an increasing number of studies referring to the astaxanthin beneficial properties for human health including several properties such as anti-inflammatory, anti-diabetic, antibacterial, immunostimulant, photoprotective, neuroprotective, anticancer and benefits to cardiovascular health, among others [2, 5, 22]. From an economical point of view, astaxanthin is the third most important carotenoid in the carotenoid global market after β-carotene and lutein. Astaxanthin market reached the 29% of total carotenoid sales with a global market size of $225 million, estimating to increase to $253 million by 2018, approximately [1]. Currently, the vast majority of the commercial offer corresponds to synthetic astaxanthin. However, the use of chemical compounds as food additives has been strictly regulated, favoring food free of them from both, the consumers and the

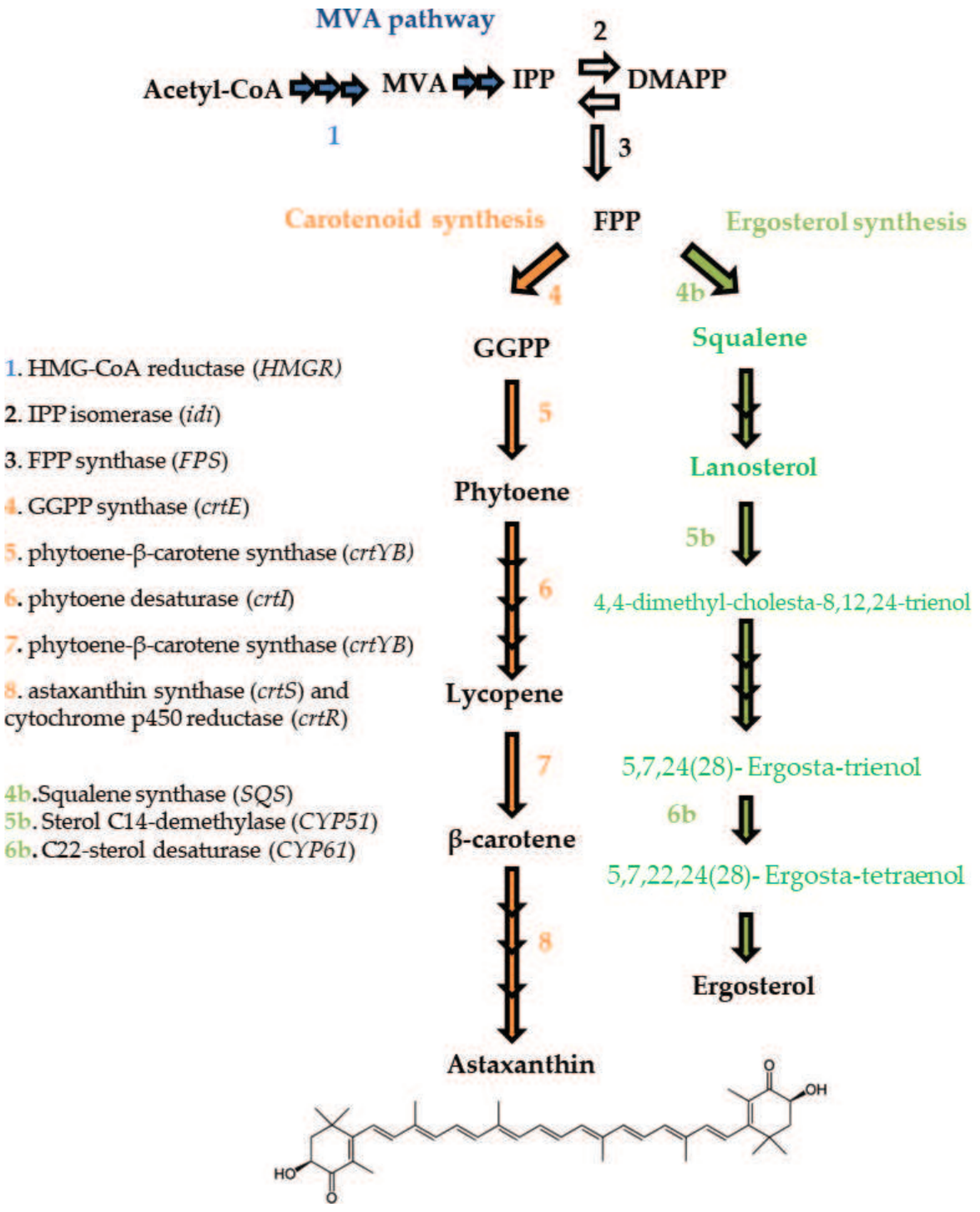

Figure 1. Overview of *X. dendrorhous* carotenogenesis and related pathways. The steps for carotenoid biosynthesis from IPP, generated via the MVA pathway, are shown. Also, an abbreviated ergosterol synthesis pathway based on the route described in *S. cerevisiae* is schematized (in green), highlighting enzymes whose genes have been functionally characterized in *X. dendrorhous* (homologous to those present in *S. cerevisiae)* [19, 66]. MVA: mevalonate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate.

authorities [1, 2, 5]. In this context, the society nowadays is looking toward "green" solutions; thus, astaxanthin from natural sources, as from microorganisms, seems to be more favorable than its synthetic counterpart due to structure, function, application and security [25, 26].

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3. Microbiological synthesis of astaxanthin

In general, the microbiological synthesis of carotenoids has conservative steps among the different species of carotenogenic microorganisms. The most conserved carotenoid biosynthesis pathways in eukaryotic microorganisms involve three general steps [1]:

1. The synthesis begins with the conversion of acetyl-CoA to 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA), catalyzed by HMG-CoA synthase. Then, HMG-CoA is converted into mevalonic acid (MVA), being the first precursor of the terpenoid biosynthetic pathway. The following steps include two sequential phosphorylation reactions performed by the enzymes mevalonate kinase and phosphomevalonate kinase, respectively, and a final decarboxylation step catalyzed by phosphomevalonate decarboxylase to produce IPP [1, 20].
2. IPP is isomerized to dimethylallyl pyrophosphate (DMAPP). The addition of three IPP molecules to DMAPP, catalyzed by prenyltransferases, produce geranylgeranyl pyrophosphate (GGPP). Then, the condensation of two molecules of GGPP produces phytoene (the first C_{40} carotene of the pathway), which is subsequently desaturated to produce lycopene [1].
3. Lycopene undergoes many modification reactions and several cyclic carotenoids derived from lycopene such as β-carotene, γ-carotene, torulene, torularhodin and astaxanthin [1].

Despite the common steps in carotenogenesis, there are particular differences in the synthesis pathways between different microorganisms. In the following sections, the carotenogenic pathways in the microalgae *H. pluvialis* and in the yeast *X. dendrorhous* are described.

3.1. Astaxanthin biosynthesis in *X. dendrorhous*

It is currently believed that the main function of carotenoids in *X. dendrorhous* is to protect the yeast against damage caused by oxidative stress. This hypothesis is supported by the fact that strains that do not produce astaxanthin are more sensitive and grow less in the presence of ROS [23]. In addition, it has been reported that the presence of certain oxygen species increases the total carotenoid content [23, 24]. Other evidence points to the fact that *X. dendrorhous* has significantly lower catalase activity than other yeasts such as *Saccharomyces cerevisiae*, so carotenoids could compensate this low activity to help to preserve the viability of continuously growing yeasts [23]. On the other hand, *X. dendrorhous* does not have a cytosolic version of a superoxide dismutase, and carotenoids could also be compensating the lack of this enzyme [23]. *X. dendrorhous* is the only known yeast that produces astaxanthin *de novo* [22] and it has been approved by the FDA (Food and Drug Administration) for the commercial production of astaxanthin; therefore, it is a good candidate to allow the natural production of astaxanthin [27]. Knowledge about the biology of this yeast has increased and several methodologies to manipulate it have been developed, but still several aspects need to be improved for an economically competitive production of astaxanthin from *X. dendrorhous* [27].

The synthesis pathway preserves the basic steps of carotenoid synthesis in this yeast; however, some steps involve characteristic enzymes in fungi and other characteristic enzymes in

X. dendrorhous [28–30]. Many studies have attempted to improve astaxanthin production in *X. dendrorhous*, contributing to our current knowledge of the genetic control of carotenogenesis in this yeast (**Figure 1**). As in other eukaryotes, the synthesis of carotenoids in *X. dendrorhous* derives from the MVA pathway. MVA is formed by the condensation of three molecules of acetyl-CoA [31], followed by two phosphorylation reactions by two different kinases and one decarboxylation, giving rise to isopentenyl pyrophosphate (IPP, C_5), which is the building block of all isoprenoids [5, 20, 31]. IPP is isomerized to dimethylallyl pyrophosphate (DMAPP) by the IPP isomerase, encoded by the *idi* gene [32]. In the next steps, a molecule of DMAPP is sequentially condensed with three molecules of IPP to generate geranylgeranyl pyrophosphate (GGPP, C_{20}); these steps involve the prenyl transferase enzymes farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate synthase, encoded by the *FPS* and *crtE* genes, respectively [33]. Subsequently, by means of a bifunctional enzyme phytoene-β-carotene synthase (PBS) with two activities (lycopene cyclase and phytoene synthase), that in fungi are encoded by the same gene (*crtYB* in the case of *X. dendrorhous*), two molecules of GGPP are condensed giving rise to phytoene (C_{40}), the first carotenoid of the pathway, which is colorless [28]. Then, phytoene undergoes four desaturation reactions catalyzed by a single enzyme phytoene desaturase (PDS, product of the *crtI* gene) forming lycopene (a red pigment) [5, 34]. The latter is converted to β-carotene by the lycopene cyclase activity of the bifunctional PBS enzyme. Finally, β-carotene is oxidized by the incorporation of a hydroxyl group in position 3 and a keto group in position 4 of both β-ionone rings of β-carotene generating astaxanthin as the final product. Unlike other organisms that produce astaxanthin, in *X. dendrorhous* a single enzyme catalyzes these last oxidizing steps from β-carotene to astaxanthin named astaxanthin synthase (CrtS, encoded by the *crtS* gene), which is a cytochrome P450 monooxygenase [29]. Astaxanthin synthase requires a redox partner, a cytochrome P450 reductase encoded by the *crtR* gene in *X. dendrorhous* [30, 35, 36], which provides the necessary electrons for the enzyme catalysis. The main pigments produced by *X. dendrorhous* are xanthophylls, of which astaxanthin represents 83–87% of total carotenoids [5].

3.2. Biosynthesis of astaxanthin in the microalgae *H. pluvialis*

Several algae and microalgae are able to produce astaxanthin, but *H. pluvialis* is the dominant specie for commercial astaxanthin [22]. *H. pluvialis* contains 1.5–3% of (3S, 3′S)-astaxanthin by dry weight, mainly as monoesters [37]. The isomer (3S, 3′S)-astaxanthin is the preferred form for human applications, for this reason, *H. pluvialis* is an attractive natural source of this pigment [5].

For the production of pigments, the microalgae *H. pluvialis* is usually grown in a two-stage batch process [5]. The first stage (green stage) is necessary to obtain enough biomass for an efficient carotenoid production. In this stage, microalgae are grown in presence of sufficient nutrient supply, optimum pH and temperature, and low irradiation. Since, the astaxanthin synthesis is induced under stress conditions, the second stage of growth (the reddening stage), consists in exposing the cells to stress conditions such as high light irradiation (sun light), nutrient deprivation (mainly nitrogen and phosphate deprivation), and high temperature and/or high salt concentration [26].

As in *X. dendrorhous*, the biosynthesis of astaxanthin in *H. pluvialis* also begins with the synthesis of IPP, which is a key intermediate of carotenoid synthesis. In general, and depending

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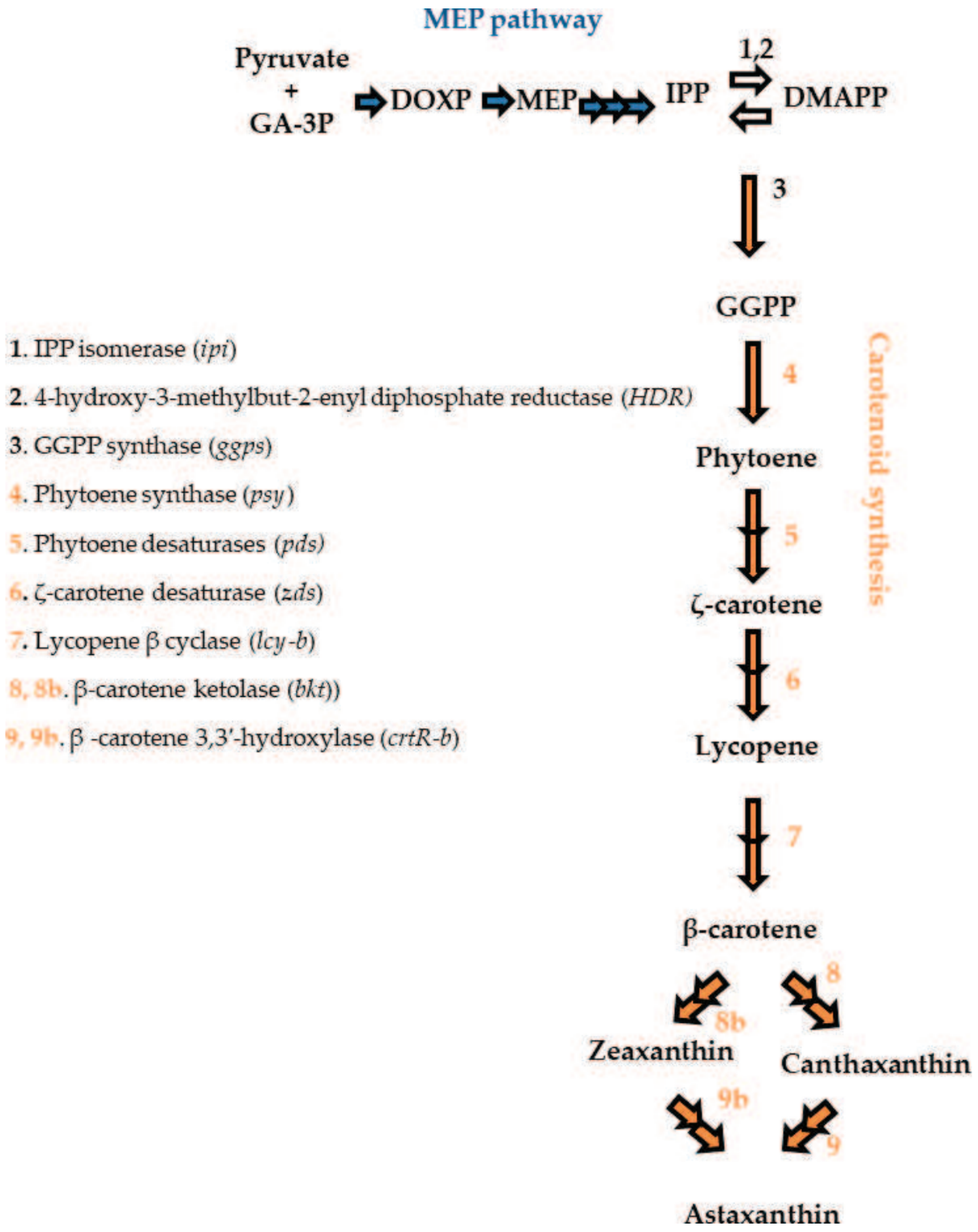

Figure 2. Overview of *H. pluvialis* carotenogenesis. The steps for astaxanthin biosynthesis from IPP, generated via the MEP pathway, in *H. pluvialis* are shown. Enzymes involved in each step are listed at left and the corresponding genes are indicated in parenthesis. GA-3P: glyceraldehyde 3-phosphate; DOXP: 1-deoxy-D-xylulose-5-phosphate; MEP: methylerythritol 4-phosphate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate.

on the organism, IPP may be produced by two different pathways, the i) MVA pathway (cytosolic) and a ii) pathway located in the chloroplast known as the MEP (Methylerythritol 4-phosphate) or as the DOXP (due to the formation of 1-deoxy-D-xylulose-5-phosphate in the

first stage of the pathway) pathway [38–40]. Previous studies have shown that in *H. pluvialis*, the intermediate IPP most probably derives from the MEP pathway as it lacks three key enzymes of the mevalonate pathway involved in the formation of IPP from acetoacetyl-CoA [41]. To date, the enzymes required for the conversion of photosynthesis derived products i.e., pyruvate and glyceraldehyde-3-phosphate into isopentenyl pyrophosphate through the DOXP pathway inside *H. pluvialis* chloroplasts has been extensively studied [41], being this, the most likely source of IPP in *H. pluvialis* cells.

The carotenogenic pathway described for *H. pluvialis* is presented in **Figure 2**. As mentioned before, the astaxanthin synthesis precursor IPP derives from the DOXP (or MEP) pathway. As in *X dendrorhous*, the first step is the isomerization of IPP to DMAPP. It has been long assumed that this conversion was catalyzed exclusively by isopentenyl pyrophosphate isomerase (IPI, encoded by *ipi* genes in *H. pluvialis*) [39, 42]. However, recent transcriptomic studies suggest that neither of the two *ipi* genes of *H. pluvialis* (*ipi1* and *ipi2*) are upregulated during cellular accumulation of astaxanthin [41]. On the contrary, suggestions have been made that another enzyme of similar activity, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) is more likely to be responsible for catalyzing the interconversion between IPP and DMAPP [41]. However, the contribution of these three enzymes to this step of astaxanthin biosynthesis is still unclear. As in *X. dendrorhous*, the isoprenoid chain is elongated by the addition of a molecule of DMAPP and subsequent additions of three molecules of IPP, being these steps catalyzed by the geranylgeranyl pyrophosphate synthase (GGPS) enzyme giving rise to GGPP [43]. The first committed step of carotenoid synthesis is the formation of phytoene from two molecules of GGPP which are condensed in a head-to-tail manner by the enzyme phytoene synthase (PSY) [43]. It must be noted that the same step in *X. dendrorhous* is catalyzed by the bifunctional enzyme PBS [28]. Then, phytoene is desaturated four times. In *H. pluvialis*, these steps involve two phytoene desaturases (PDS) and a ζ-carotene desaturase (ZDS), and two plastid terminal oxidases (PTOX1, PTOX2) acting as co-factors [44, 45], giving as final product the red colored carotene lycopene [43]. These steps constitute other difference with the synthesis of carotenoids in *X. dendrorhous*, where a single enzyme catalyzes the four desaturations necessary for the synthesis of lycopene from phytoene [34]. Both termini of lycopene are cyclized by lycopene cyclases (LCY-e and LCY-b). In most organisms, cyclization of an extreme of lycopene results in the production of α-carotene (precursor of lutein) and β-carotene (precursor of astaxanthin, among others). In *H. pluvialis,* the carbon flux is directed mainly through the production of β-carotene [41]. The final oxygenation steps that lead to astaxanthin from β-carotene are catalyzed by two different enzymes: a β-carotene ketolase (BKT) and a β-carotene hydroxylase (CrtR-b) [46–48]. This is another difference with the astaxanthin synthesis in *X. dendrorhous*, in which the astaxanthin synthase yields the hydroxylation and ketolation of the β-carotene β-ionone rings [29].

4. Potential mechanisms that regulate the synthesis of astaxanthin

The astaxanthin synthesis pathway has been extensively studied, and most of genes and enzymes involved are currently known. More recent studies have focused on elucidating the possible regulation mechanisms of carotenogenesis. In the case of yeast *X. dendrorhous*, special

emphasis has been placed on understanding the effect of the carbon source on carotenogenesis and how this pathway is affected by other related pathways, such as the synthesis of sterols. In the case of *H. pluvialis*, special interest has been placed on the effect of small molecules on the synthesis of astaxanthin.

4.1. Regulation of carotenogenesis in *X. dendrorhous*

An important function of astaxanthin in *X. dendrorhous* is the inactivation of singlet and oxygen radicals, which is consistent with the fact that astaxanthin production increases in the presence of these reactive oxygen species [5, 23]. In addition, it has been observed that high light intensity inhibits the growth of the yeast and the content of carotenoids. However, at low light intensities, light has a positive regulatory effect on the synthesis of carotenoids [5].

It is known that *X. dendrorhous* is able to grow using various carbon sources, among them: glucose, sucrose, maltose, xylose, starch, succinate, glycerol and ethanol. Several studies have shown that there is a relationship between the carbon source used by the yeast and the synthesis of carotenoids. This effect is observed in both: in the amount of total pigments and in their composition [16, 17].

As in other yeasts, *X. dendrorhous* is capable of carrying out two types of metabolisms depending on the carbon source that is present in the culture medium: i) a fermentative and ii) an aerobic metabolism. In previous studies, it has been shown that astaxanthin production decreases during fermentative metabolism (in presence of fermentable carbon sources as glucose or fructose) and it increases during aerobic metabolism (with non-fermentable carbon sources as succinate or ethanol) [16, 17, 49]. Also, it has been observed that carotenoid content is significantly higher when *X. dendrorhous* is cultivated in complete medium (YM) supplemented with different non-fermentable carbon sources (xylose, succinate, sodium acetate, glycerol and ethanol), compared with the carotenoid content when the yeast is cultured in presence of glucose [17]. In cultures supplemented with glucose as the sole carbon source, carotenogenesis is induced only after the culture medium runs out of glucose. While in cultures using succinate as the sole carbon source, it was observed that the production of carotenoids coincides with the growth of the yeast, increasing steadily until reaching the stationary phase of growth. This shows that the production of carotenoids starts earlier and it is higher when a non-fermentable carbon source is used in cultures [17].

On the other hand, studies of carotenogenic gene transcripts (*crtI*, *crtYB* and *crtS*), show that their levels reach their maximum value at the late exponential phase of growth coinciding with the induction of carotenogenesis, the exhaustion of glucose in the medium and with the beginning of the consumption of ethanol produced as result of sugar fermentation [17, 49]. It has also been observed that the addition of glucose to the culture medium decreases the transcript levels of genes *crtYB*, *crtI* and *crtS*, which correlates with a complete inhibition of pigment synthesis. On the other hand, the addition of ethanol to the culture medium of the yeast causes an induction of the expression of the *crtYB* and *crtS* genes, and promotes the synthesis of carotenoids [16]. Furthermore, the promoter region of the *crtS* gene contains four potential CreA binding motifs [50], which is a negative regulator involved in glucose repression in *Aspergillus nidulans* [51]. According to this background, it is clear that glucose causes suppression of carotenogenesis in *X. dendrorhous.*

It is well known that glucose has a global effect on cellular metabolism; generally, when this sugar is present in the culture medium, the expression of genes involved in the metabolism of alternative to glucose carbon sources and secondary metabolism is repressed [52]. This phenomenon is known as "catabolic repression" or "repression by glucose" [53] and could be responsible for the repression of carotenogenic genes in *X. dendrorhous* during fermentative metabolism.

4.1.1. Catabolic repression: mechanism and components

In microorganisms of free life, the availability of nutrients is in constant change and is the main factor regulating their growth and development. For yeasts, as for many other microorganisms, glucose is the preferred source of carbon and energy. Therefore, it is not surprising that glucose, in addition to its function as a nutrient, plays an important regulatory role in the metabolism of microorganisms. Thus, a high concentration of glucose in the medium resembles optimal growth conditions to the cellular machinery, causes the induction of various signal transduction pathways, and the activation or inactivation of various proteins. The regulatory role of glucose is more prominent at the transcription level and the general mechanism of catabolic repression involves a parallel decrease in the transcript levels of the target genes and, consequently, of the proteins they encode [52, 54].

Catabolic repression has been widely studied in *S. cerevisiae* and in general, genes that are under regulation by glucose repression encode enzymes that are involved in gluconeogenesis, Krebs cycle, glyoxylate cycle, respiration, mitochondrial development, uptake and metabolization of carbon sources alternative to glucose (such as the genes *GAL*, *SUC* and *MAL*) and high affinity glucose transporters [54, 55]. On the other hand, genes that encode a variety of transcriptional activators are also repressed by glucose. Finally, a large group of genes encoding proteins that are involved in the response to various types of stress highlights, as they have STRE elements (stress response element) in their promoter regions, which are also repressed in the presence of glucose [54].

One of the ways in which glucose influences gene expression is by facilitating the action of negative regulators [52], among them, the Mig1 factor (homologous to CreA in *A. nidulans*) is a DNA binding protein that recognizes and binds to specific sequences called "Mig1 boxes" (with the consensus sequence (G/C)(C/T)GGGG)) in the promoters of the target genes. Several studies have identified genes potentially encoding Mig1 in yeasts like *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe*, *Candida albicans* [52] and recently in *X. dendrorhous* [56].

In general, glucose repression in *S. cerevisiae* (**Figure 3**) mainly depends on the subcellular localization of the Mig1 regulator. At high glucose levels, the repressor Mig1 is dephosphorylated and localizes at the nucleus where it recognizes and binds to "Mig1 boxes" in the promoter region of the target genes. Then, Mig1 recruits a co-repressor complex formed by the Cyc8 and Tup1 proteins that represses the transcription of the target genes. In the absence of glucose, Mig1 is phosphorylated by the Snf1 kinase complex, loses its interaction with the Cyc8–Tup1 complex, and it is exported to the cytoplasm [57–59]. In *S. cerevisiae*,

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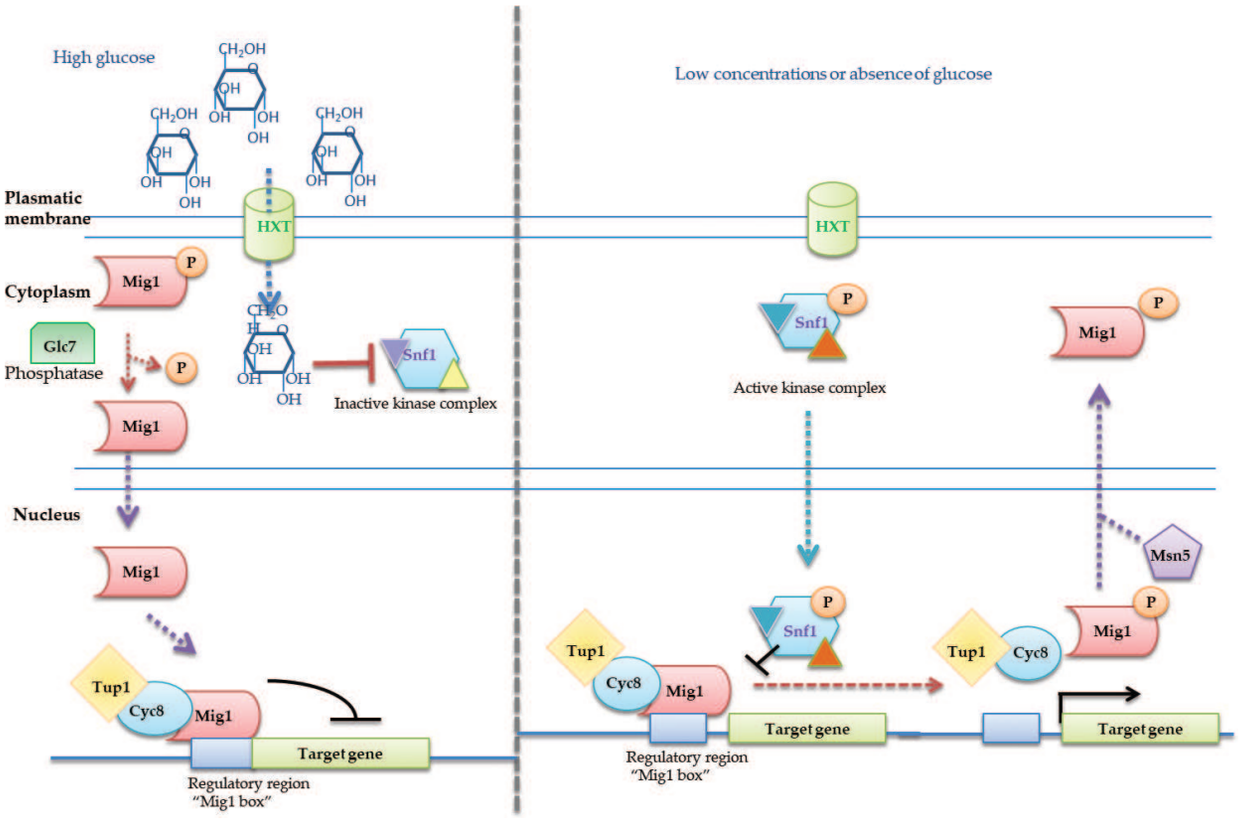

Figure 3. Catabolite repression mechanism in yeasts. Scheme of the principal components of the general catabolite repression mechanism. At high levels of glucose, Mig1 is dephosphorylated by phosphatase Glc7 and enters the nucleus where it recognizes regulatory sequences in the target gene promoters. Then, Mig1 recruits a co-repressor complex formed by Cyc8 and Tup1 that represses the expression of the target genes at transcriptional level. In absence of glucose, Mig1 is phosphorylated by the kinase complex Snf1, and loses its interaction with Cyc8-Tup1 complex, being exported to the cytoplasm [52].

the co-repressor complex Cyc8–Tup1 is considered as a global transcriptional co-repressor, because it regulates the expression of more than 180 genes, including those regulated by glucose. The proteins Cyc8 and Tup1 belong to evolutionarily highly conserved protein families, and similar repressors have been described in yeasts, worms, flies and mammals [60]. In *S. cerevisiae*, the *CYC8* and/or *TUP1* genes knock-out mutations are not lethal, but have pleiotropic effects causing slow growth, flocculation, sporulation and loss of certain aspects of glucose repression, among others [52, 60, 61].

4.1.2. Catabolic repression and carotenogenesis regulation in X. dendrorhous

In view of the diverse background that suggests that carotenogenesis is regulated by catabolic repression, recent studies have advanced characterizing the catabolic repression mechanism in *X. dendrorhous*, including its components and role in the regulation of carotenogenesis. Several evidences suggest a functional catabolic repression mechanism in *X. dendrorhous* [16, 56, 59]. For example, extracellular α-glucosidase and invertase activities were not detected in *X. dendrorhous* cultures when glucose was used as a carbon source, suggesting

catabolic repression of these enzymes [62, 63]. This indicates that this regulatory mechanism is operative in *X. dendrorhous*, as genes encoding glycosyl hydrolases are well known targets of catabolic repression.

Moreover, according to descriptions of other yeasts, one consequence of catabolic repression is the preferred use of glucose over other alternative carbon sources, deferring their use until glucose has been completely consumed. The preferential use of glucose over an alternative carbon source can be evidenced by a characteristic growth rate change of the microbial culture, known as diauxic growth [59, 64]. This aspect has been evaluated in *X. dendrorhous* through experiments in which the yeast was grown in the presence of glucose and a non-preferred carbon source such as glycerol or sucrose. In both cases, a diauxic-type growth curve was obtained when the yeast was cultivated with both carbon sources simultaneously, indicating the change in the used carbon source and supporting a functional catabolic repression mechanism in *X. dendrorhous* [56, 59]. On the other hand, genes encoding the principal components of the catabolic repression mechanism has been identified and characterized in *X. dendrorhous.* Among them, a gene (*MIG1*) encoding the Mig1 transcriptional factor was identified [56]. The functionality of this gene was assessed by heterologous complementation in *S. cerevisiae*, showing that the protein encoded by the *MIG1* gene is functional and capable of mediating glucose repression [56]. Also, the function of Mig1 in *X. dendrorhous* was determined by evaluating the effect of a *mig1*$^{-}$ mutation on carotenoid production, gene expression and extracellular invertase activity (a known glucose repression target). To evaluate whether carotenoid production is affected in the *mig1*$^{-}$ mutant strain, samples were taken from cultures of the wild-type and mutant strains grown in presence of glucose at 5 different time-points representatives of different phases of growth. It was observed that the carotenoid content was higher in the *mig1*$^{-}$ mutant strain during almost all phases evaluated and the total carotenoid content at the final phase of growth that was evaluated (stationary phase of growth), was approximately 20% higher in the mutant strain compared to the wild-type [56]. The higher carotenoid production in the *X. dendrorhous mig1*$^{-}$ mutant strain strongly suggests a role of Mig1 in the regulation of carotenogenesis in this yeast. Also, in a complementary approach it was demonstrated that when glucose was added to a culture that was previously deprived of this sugar, the carotenoid synthesis stopped in the wild-type strain showing no carotenoid synthesis until 24 h later, while in the case of the *mig1*$^{-}$ mutant strain, carotenogenesis did not stop, suggesting that the *mig1*$^{-}$ mutation alleviate the glucose mediated repression of carotenogenesis [56]. Also, Marcoleta et al. showed that the addition of glucose decreased the transcript levels of the carotenogenic genes in the wild-type strain [16]. Meanwhile, the *mig1*$^{-}$ mutation reverts this repression at the transcriptional level [56]. Additionally, by bioinformatic analysis, possible "Mig1 boxes" were identified in the promoter regions of the carotenogenic genes *crtI*, *crtYB* and *crtS*, precisely those in which a repressing effect of glucose at the transcriptional level was also observed [16]. To confirm whether the *X. dendrorhous MIG1* gene product binds to DNA containing Mig1 boxes, Electrophoretic Mobility Shift Assays (EMSAs) were performed using biotin-labeled DNA fragments of the promoter regions of the *crtI*, *crtYB* and *crtS* containing the potential Mig1 boxes. The results indicated that the Mig1 factor of *X. dendrorhous* is capable of binding specifically to the "Mig1 boxes" present in the

analyzed promoter regions [56]. To further address the functional consequences of the *mig1*⁻ mutation in *X. dendrorhous*, extracellular invertase activity was evaluated in the wild-type and mutant strains. Invertase (encoded by the *INV* gene) catalyzes the utilization of sucrose as carbon source and it is under catabolic repression in presence of glucose [52]. When invertase activity was determined in the wild-type and *mig1*⁻ mutant strain of *X. dendrorhous* cultured in the presence of glucose, it was observed that the *mig1*⁻ mutation caused derepression of the *INV* gene which was evidenced by a higher invertase activity in the mutant strain compared to the wild-type under these conditions [56].

As mentioned before, the Mig1 factor recruits a co-repressor complex formed by the Cyc8 and Tup1 proteins to perform repression at transcriptional level. Thus, in other study, the role of these co-repressors in catabolic repression and carotenogenesis was assessed in *X. dendrorhous* [59]. The *CYC8* and *TUP1* genes were identified in *X. dendrorhous* and similar analyses to those described for *MIG1*, were performed. These analyses showed that the *cyc8*⁻ and *tup1*⁻ mutations increased the specific carotenoid production, reaching production levels at the stationary phase of growth that were approximately 90 and 40% higher in the mutant strains *cyc8*⁻ and *tup1*⁻, respectively, compared to the wild-type when glucose was used as the sole carbon source [59]. Also, the participation of these genes (*CYC8* and *TUP1*) in the repression of invertase activity was demonstrated, showing that *cyc8*⁻ and *tup1*⁻ mutations led to a derepression of the *INV* gene and to a higher invertase activity in presence of glucose, compared to the wild-type strain [59]. Furthermore, it was demonstrated that similarly to the *mig1*⁻ mutation, the *cyc8*⁻ and *tup1*⁻ mutations alleviated the glucose mediated repression of carotenogenesis, since these mutant strains continuously produced carotenoids, even when glucose was added to the culture medium [59].

So, many studies support the hypothesis that carotenogenesis in *X. dendrorhous* is regulated by the catabolic repression mechanism.

4.1.3. Relation between carotenogenesis and sterol biosynthesis

Ergosterol is the main sterol in fungi, fulfilling similar roles as cholesterol in mammalian cells. Since the synthesis of ergosterol and of fatty acids derive from the same precursors as the synthesis of astaxanthin in *X. dendrorhous*, some studies have focused in the interaction between these related pathways. In Ref. [65] was reported that an astaxanthin overproducing strain obtained by random mutagenesis had a decreased production of ergosterol and of fatty acids, which could lead to precursor accumulation favoring the astaxanthin biosynthesis. Moreover, it was also observed higher transcript levels of carotenogenic genes (*crtI*, *crtYB* and *crtS*) in this strain [65]. In the same line, it was shown that the disruption of the C22-sterol desaturase gene (*CYP61*), involved in one of the last steps of ergosterol synthesis, enhanced carotenoid production in *X. dendrorhous* [19].

In *Cryptococcus neoformans* and mammalian cells, the expression of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) encoding gene (*HMGR* gene) that is involved in the synthesis of IPP trough the MVA pathway, is regulated by sterol levels [19]. In accordance,

the transcript level of the *HMGR* gene in the *X. dendrorhous cyp61*⁻ mutant strains was higher than in the wild-type strains. This could explain, at least in part, the increased carotenoid content in these mutants, since the synthesis of carotenoid precursors through the MVA pathway could be favored in these strains, showing an interaction between both biosynthetic pathways and a potential role of sterols in the regulation of carotenogenesis in *X. dendrorhous* [19].

Similar to these results, other studies have demonstrated an increased astaxanthin production in *Phaffia rhodozyma* (anamorphic state of *X. dendrorhous*) when ergosterol levels were reduced by fluconazole treatment [65]. In a similar way, it has been described in *X. dendrorhous* that the mutation of the *CYP51* gene that encodes a cytochrome P450 monooxygenase that catalyzes the C14 demethylation of lanosterol during ergosterol biosynthesis, resulted in a reduced ergosterol production together with an increased carotenoid production compared to the wild-type strain [66]. Moreover, as for the *cyp61*⁻ mutation, the *cyp51*⁻ mutation in *X. dendrorhous* increased the *HMGR* transcript levels. A possible explanation for the increased carotenoid content in the *cyp61*⁻ and *cyp51*⁻ mutants could be the greater availability of carotenoid precursors when sterol biosynthesis is affected.

All together these results suggest that in *X. dendrorhous*, sterol levels, possible by a negative feedback mechanism, regulate at least the *HMGR* gene expression and in this way; it contributes to the regulation of carotenoid biosynthesis [19, 66].

4.2. Regulation of carotenogenesis in *H. pluvialis*

It has been extensively accepted that carotenoid synthesis in *H. pluvialis* is induced under stress conditions such as high light, salinity or carbon to nitrogen ratio [48, 67]. Regulation of the carotenogenic pathway in this microalga can be affected by numerous small molecules like plant hormones or similar compounds. In this context, among the hormones associated with stress response mechanisms and induction of astaxanthin synthesis in *H. pluvialis* are abscisic acid (ABA), jasmonic acid (JA), methyl jasmonate (MJ) or growth regulators like gibberellic acid (GA3), salicylic acid (SA) or brassinosteroid-2,4-epibrassinolide (EBR) [26]. It has been shown that all of these compounds affect the expression of numerous genes involved in astaxanthin synthesis, resulting in an up-regulation from 6- to 10-fold. Among them, SA showed the best results enhancing the astaxanthin production [67].

Studies at the mRNA levels of the carotenogenic genes: *ipi, psy, pds, crtO* and *crtR-b*, encoding the key enzymes of astaxanthin synthesis pathway, and its correlation with algal growth and astaxanthin production, suggested complex and multiple regulatory mechanisms that act at the transcriptional, translational and post-translational levels to regulate carotenogenesis in *H. pluvialis* [44]. Small molecules can exert different and multiple effects on several genes involved in the synthesis of astaxanthin. For example, when *H. pluvialis* was submitted to several nutrient stress conditions, it was observed that expression of carotenogenic genes encoding PSY, PDS, LCY, BKT and CrtR-b enzymes, were up-regulated under all the stress conditions studied. However, the extent of the transcript levels of carotenogenic genes varied among the stress conditions. Some of these genes, as *bkt* and *crtR-b*, were induced only transiently in some conditions. Moreover, studies using various inhibitors indicated that general carotenogenesis genes were regulated at transcriptional and translational levels. The induction of the general carotenoid synthesis genes showed to be independent of cytoplasmic

protein synthesis while *bkt* gene expression was dependent on *de novo* protein synthesis [48]. It is still necessary to elucidate the different mechanisms of response to these molecules and therefore understand carotenogenic gene regulation in *H. pluvialis* and potentially enhance its capacity as a commercial astaxanthin producer.

5. Conclusions

X. dendrorhous and *H. pluvialis* are the most promising natural sources for the biological production of astaxanthin, which is used in several industrial applications. Almost all the genes and enzymes involved in the carotenogenesis pathways in both microorganisms are known. Currently, efforts have been directed in order to elucidate the regulatory mechanisms acting on carotenogenesis in these microorganisms. Studies show that multiple and complex carotenogenesis regulatory mechanisms are involved acting at transcriptional, post-transcriptional and translational level, and that they could be different in these microorganisms. Regarding *X. dendrorhous* carotenogenesis, there is evidence that suggest that it is regulated by catabolic repression and by sterols levels, while in *H. pluvialis*, carotenogenesis is induced under stress conditions and it is affected by numerous small molecules like plant hormones or their analogs.

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Conflict of interest

Authors declare that they have no conflicts of interest.

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