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Biotechnological Intervention In The Biosynthesis of Carotenoids

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Abstract *— Carotenoids are a group of naturally occurring pigments found in fruits and other plant parts. Due to their color and nutritional value, they have many industrial applications and are generally synthesized by micro-organisms. Here in this review, we discussed the history, sources, biosynthetic pathways in various microorganisms, and biotechnology interventions in the production of these industrially important molecules. Carotenoids are synthesized in the chloroplast by the action of a series of nuclear-encoded membrane proteins. These gene products are synthesized in the cytoplasm with precursor polypeptides containing amino-terminal extensions that lead them to the chloroplasts. The biosynthesis of carotenoids has been exhaustively reviewed in this article. This review will accentuate various biotechnological approaches adopted to increase carotenoid accumulation in plants.*

Keywords — *Carotenoids, xanthophylls, tetra-terpenoids, lycopene*

I. INTRODUCTION

Carotenoids are a group of plant pigments having a similar structure, mostly containing C40 isoprenoids with a polyene chain of conjugated double bonds. There are more than 600 types of carotenoids [1,2]. Carotenoids have a number of applications in different industries, including food, medicine, nutrition, health, and cosmetics. Two geranylgeranyl precursors condense head-to-head to form a primordial carotene molecule. This primordial carotene molecule has an acyclic structure which is analogous to ζ-carotene. Every carotenoid structure is derived from the acyclic structure as a result of a number of reactions, including hydrogenation, cyclization, oxidation, esterification, dehydrogenation, halogenation, etc. [3].

Carotenoids are natural organic pigments, also known as tetra-terpenoids. Usually, non-systematic names are used for most of the carotenoids, but these names are not recognized by the International Union of Pure and Applied Chemistry (IUPAC). However, IUPAC has published regulations for the nomenclature of carotenoids [4, 5]. Many micro-organisms, including yeasts, bacteria, filamentous fungi [6], and micro-algae [7], also synthesize these tetra-terpenoids. These pigments include various structures with varying colors and biological characteristics [8].

II. PROPERTIES

Carotenoids are generally red, yellow, or orange in color. There is a huge application of these pigments in the food, cosmetic, and feed industries [9]. The staining characteristics of carotenoids are mainly due to the light-absorbing chromophore that is formed due to lipophilic isoprenoids molecules containing double bonds [10]. The presence of this double bond makes carotenoids sensitive to various reactions and multiple factors, including light, heat, acids, oxygen, etc. [11,12]. Carotenoids are further classified into two categories; named as carotenes and xanthophylls. Orange and Red pigments are typically carotenes, while yellow pigments fall in the xanthophyll category. Xanthophylls are derived by the oxygenation of hydrocarbons.

Carotenoids aren't soluble in water, but they do dissolve in oils and organic solvents. They are all lipophilic compounds in nature. Heat, acid, and light can be used to isomerize carotenoids. These compounds are unstable in the air as heat and oxygen present in the atmosphere can oxidize them readily. The carotenoid structure comprises 40 carbon atoms and the molecular formula of $C_{40}H_{56}$ [13]. Conjugated double bonds are found in every carotenoid compound, and the number of bonds presents ranges from 3-13. There are four isoprene units present in the central portion of the molecule in almost all carotenoid compounds. Carotenoids mixed in ether or chloroform give intense blue color along with concentrated sulphuric acid.

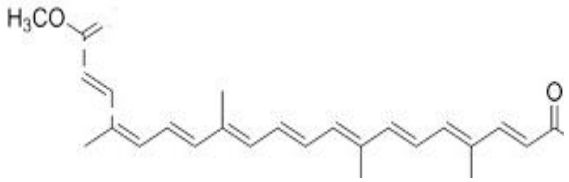

Fig.1 Structure of carotenoids

III. SOURCES

Carotenoids are present in high amounts in a number of food items, including squash, grapefruit, papaya, watermelon, guavas, mangoes, oranges, and apricots. Bright red, yellow, and orange color in plants is mainly due to the presence of carotenoid pigments, so most of the

fruits and vegetables of these colors are edible sources of carotenoids. They also play a vital role in maintaining plant health.

A number of micro-organisms also synthesize carotenoids, including algae, fungi, yeast-type prokaryotes, and non-photosynthetic bacteria, etc. Common micro-organisms producing carotenoid pigments include bacteria *Rhodococcus Maris*, *Micrococcus roseus*, *Gordonia jacobaea*, *Bradyrhizobium* sp., *Haloferax alexandrines*, etc. and the cyanobacteria *Anabaena variabilis*, *Aphanizomenon Flos-aqua* and *Nostoc commune*, etc. [14].

Some of these micro-organisms are used at an industrial scale for the commercial production of pigments (carotenoids) to be used for mass production. Micro-organisms producing carotenoids pigments at the industrial level include *Pseudomonas putida*; microalgae: *Dunaliella salina*, Spirulina; and the yeast: *Rhodotorula glutinis* [15]. Industrial application of these carotenoids producing microorganisms has a number of advantages. They require a broad range of low-cost substrates, have better cultivation control, and take comparatively less production time. The bacteria isolated from the areas with the highest exposure to solar radiation, including Sahara and Antarctica, have carotenoids that exhibit a photo-protective property in response to exposure.

Table 1: Carotenoids produced by micro-organisms

Micro-organisms	**Category**	**Molecule**
Rhodococcus Maris	Bacterium	β–carotene
Micrococcus roseus	Bacterium	α/ β carotene
Gordonia jacobaea	Bacterium	Canthaxanthin
Aphanizomenon flosaqua)	cyanobacterium	β–carotene
Nostoc commune	cyanobacterium	β–carotene
Dunaliella salina,	Algae	β-carotene
Spirulina	Cyanobacterium	β-carotene
Pseudomonas Putida	Bacterium	Cryptoxanthin and Zeaxanthin
microalga	Algae	Nostoxanthin

IV. CAROTENOID BIOSYNTHESIS IN BACTERIA

In the bacterial cell, carotenids are only present in inner portions of complex cytoplasmic and cell wall membranes [16]. Controlled regulation of environmental factors and provision of optimum conditions for the growth of producing organisms can increase the production of carotenoids at a large scale. There are two pathways involved in carotenoids biosynthesis, via the mevalonate pathway (MVP) in plants [17] and the methylerythritol 4-phosphate pathway (MEP) present in bacteria [18].

Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are two 5C subunits that act as universal precursors for the production of C-40 and C-50 carotenoids in MEP. DMAPP is the isomeric form of IPP. IPP/DMAPP isomerase (IDI) is responsible for carrying the isomerization of IPP into DMAPP. Geranylgeranyl diphosphate (GGPP) synthase enzyme, which acts as a catalyst in the accumulation of one DMAPP molecule and three IPP molecules synthesizing C-20-GGPP molecule that acts as an immediate precursor for the biosynthesis of carotenoids. The first parent molecule, phytoene (C-40), forms by tail-to-tail addition of two GGPP molecules (*Fig. 2*). The synthesis of this phytoene is considered an important step in carotenoid biosynthesis. It is subjected to four consecutive reactions. These reactions involve phytoene desaturase, zeta (ζ)-carotene isomerase, zeta (ζ)-carotene desaturase, and carotene isomerase, respectively. Phytoene desaturase acts as a catalyst in the first desaturation reaction resulting in the synthesis of ζ–carotene. ζ–carotene is then subjected to two more desaturation steps which result in the synthesis of lycopene, linear in structure, and the first colored product in the whole process.

1100 different carotenoid forms are derived from lycopene under subsequent customization reactions; thus, it acts as the backbone of these reactions. α–carotene and β–carotene is formed by cyclization on all sides of lycopene. β-ionone rings are then added by lycopene β cyclase. These β-ionone rings make β-carotene a pro-vitamin competence. Subsequent hydroxylation of α–carotene and β–carotene occur, forming lutein and zeaxanthin. This process occurs via β-ring hydroxylase and α-ring hydroxylase (*Fig. 2*).

The bacterial sources for carotenoids synthesis are a greatly suitable alternative for plant-based carotenoid sources. Bacterial sources have a short life cycle that gives the advantage of quicker production of carotenoids. Carotenoid synthesis can be done for a whole year with the probability of novel carotenoid pigments. Further, it is very easy to regulate controlled environmental parameters during the fermentation process to increase carotenoid production. The bacterial sources are also superior in cost as they convert low-cost substrate, which may be industrial or agriculture waste, into high-valued carotenoids. Moreover, prokaryotic bacteria have an advantage over eukaryotic algae and plant systems in producing genetic manipulations targeting carotenoid biosynthesis. Bacterial species commonly used for biosynthesis of carotenoids include *Flavobacterium*, *Agrobacterium*, *Micrococcus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Chromobacterium*, and *Arthrobacter*.

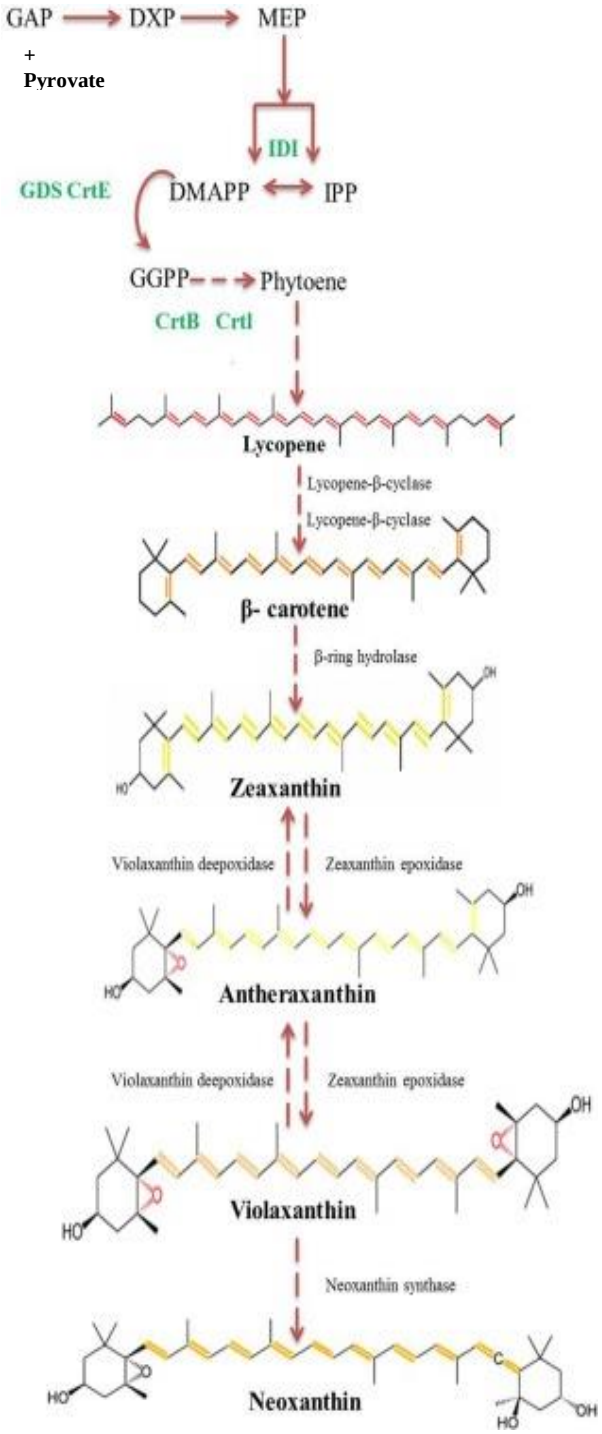

Fig. 2 methylerythritol 4-phosphate pathway (MEP) involved in the biosynthesis of carotenoids in bacteria.

Many bacteria used for the biosynthesis of carotenoids have been obtained from freshwater and marine water. These bacteria possess diverse carotenoid profiles. 30 different bacteria found from King George Island Antarctica were capable of producing ten novel carotenoids. These bacteria belonged to the *Flavobacterium*, *Chryseobacterium*, and *Zobellia* genera. Carotenoids produced by these bacteria include zeaxanthin, β-cryptoxanthin, and β-carotene.

Table 2. Carotenoids are produced by bacterial strains and their utilization.

Carotenoid	Color	Microorganism	Application
Astaxanthin	Red	*Agrobacterium aurantiacum, Paracoccus carotinifaciens*	Used in the feed industry, Pharmaceutical industry, aquaculture [19]
β- carotene	Yellow /orange	*Sphingomonas sp*	Used as antioxidants, as food colorants, also used in preventing night blindness [20, 21]
Canthaxanthin	Reddish-orange	*Monascusroseus, Paracoccus carotinifaciens*	Antioxidants and inhibit lipid oxidation in liposomes, widely used as Food colorants [22]
Lycopene	Red	*Rhodospirillum rubrum*	Helps in preventing Breast and stomach cancer [23]
Zeaxanthin	Golden Yellow	*Staphylococcus aureus, Vibriopsychroerythrus, Flavobacterium sp., Paracoccusxanthinifaciens*	Prevents age-related macular degeneration, accentuates the color of egg yolk.

V. CAROTENOID PRODUCTION IN FUNGI

Various fungi produce carotenoids of our interests. These carotenoids include β-carotene, lycopene, canthaxanthin, cryptoxanthin, and astaxanthin. The fungi are important to biotechnologists as they are easy to cultivate, and there is a number of techniques and possibilities regarding genetic, industrial, and physiological manipulations.

Carotenoids are important industrial components of animal feeds for coloration and as precursors of vitamin A. Fungi produce higher levels of carotenoids than *Escherichia coli*, eubacteria, archaebacteria, and plants. Many fungi have the ability to form isoprenes and assemble them into various products derived by the isoprenoid pathway. Various fungi produce sesquiterpenes, sterols, and carotenoids by the mevalonate pathway [24,25]. In fungi, isoprenoids have many essential functions in membrane structure, primary metabolism, and response to stress.

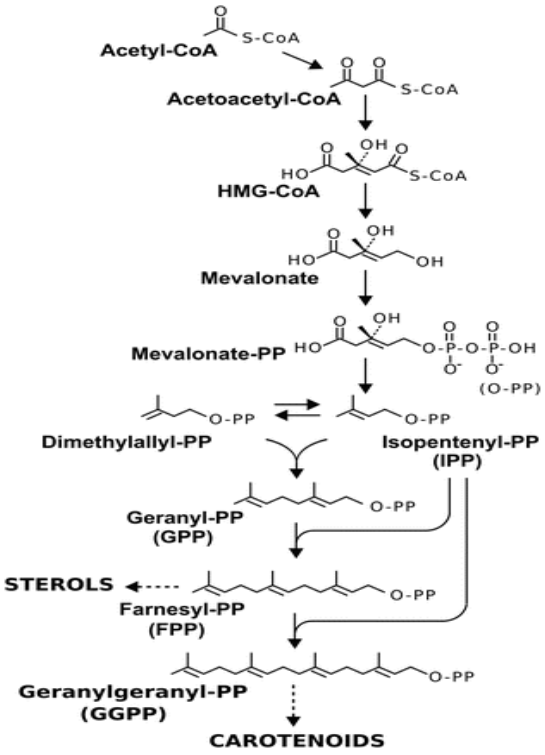

Fig. 3 carotenoid biosynthesis in fungi by the mevalonate pathway.

VI. CAROTENOIDS PRODUCTION IN MICROALGAE

Our current understanding of carotenoid metabolism and its regulation in microalgae is still somewhat limited and mainly inferred from the knowledge acquired from higher plants. Microalgae are capable of converting carbon dioxide into different pigments, proteins, lipids, polysaccharides, and vitamins. This whole process is driven by the electron transport chain induced by sunlight. Microalgae-based carotenoid production is garnering interest due to a number of advantages at the industrial level, including easy cultivation at a large scale, swift and active growth, a shorter life cycle, and greater production in less time. Microalgae have an active isoprenoid metabolism precursor for the carotenogenic pathway. They also have better storage capacity, thus considered a better alternative for carotenoids synthesis at the industrial level. *Dunaliella salina, Haematococcus pluvialis*, and *Chlorella zofingiensis* (astaxanthin) are among microalgae species that exhibited greater carotenoid productivity. In severe stress conditions, i.e., high salt, high light, or lower availability of nutrients, microalgae are capable of producing carotenoids. Phytohormones working as metabolic enhancers have great potential of increasing carotenoid biosynthesis and productivity and thus can be utilized for enhancing the production of carotenoids. A number of species of microalgae are exposed to higher light stress conditions to increase the production of carotenoids. The biosynthesis of carotenoids by microalgae is also greatly influenced by temperature through the modulation of enzymes in the carotenoid biosynthesis process. Carotenoids also have an important role in light-harvesting and energy transfer during the photosynthesis process. They also play a vital role in preventing photo-oxidative damage to photosynthetic apparatus.

IPP and DMAPP are used for the production of isopernoids by microalgae. In the cytosolic acetate/mevalonate pathway [26, 27], Mevalonic acid was considered as the only natural precursor for IPP and DMAPP, and the bioproduction of sterols, sesquiterpenes, triterpenoids, and carotenoids is done via cytosolic acetate/mevalonate pathway for a long time. However, the methylerythritol 4-phosphate or 1-deoxy-D -xylulose-5-phosphate is considered the pathway for bioproduction of plastidic isopernoids now. The biosynthesis of carotenoids, phytol, cytosolic iospernoids, mono and di-terpenes, isoprene, and plastoquinone-9 is carried out via the MEP pathway in algae (Chlorella, Chlamydomonas, and Dunaliella). Microalgae from diverse environments are screened for the production of various carotenoids.

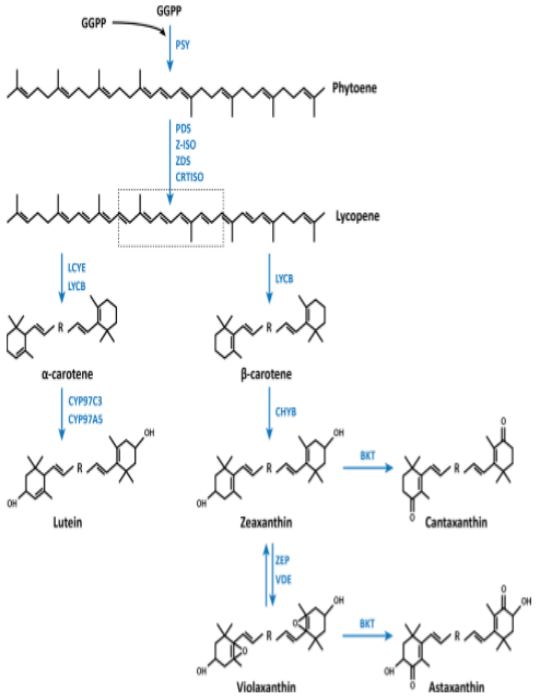

Fig. 3 Carotenoid biosynthesis pathway in chlorophytes.

VII. CAROTENOID BIOSYNTHETIC PATHWAY IN PLANTS

In higher plants, the synthesis and localization of carotenoids occur in the plastids. The bio-synthesis of plastid-derived isoprenoids is achieved through the production of IPP. IPP synthesis occurs via the mevalonic aid pathway (MVP) and MVA independent pathway. The synthesis of phytoene from IPP is catalyzed by three enzymes. IPP isomerase catalyzes the reversible isomerization of IPP and its allylic isomer DMAPP at first. After that, Geranylgeranyl diphosphate (GGPP) synthase covert IPP/DMAPP formed in the first step into geranylgeranyl pyrophosphate. Lastly, phytoene synthase (PSY) converts GGPP into phytoene. The formation of

phytoene is the first step held towards the biosynthesis of carotenoids [28]. Conjugated carbon-carbon double bonds are introduced by a number of desaturation reactions that give color to it. In the next step, zeta carotene is synthesized from phytoene with the help of the phytoene desaturase enzyme via phytofluene. The desaturation of zeta carotene into lycopene is carried by the zeta carotene desaturase enzyme. The cyclization of lycopene is Mediated by two enzymes. They are lycopene β-cyclase (βLCY) and lycopene ε-cyclase (εLCY). Lycopene is converted into β carotene by βLCY introducing beta rings to an acyclic precursor, whereas α carotene is formed from lycopene via εLCY. α carotene undergoes hydroxylation to form lutein via cryptoxanthin. The enzyme epsilon ring hydroxylase is responsible for this reaction. Likewise, beta carotene also undergoes hydroxylation to synthesize zeaxanthin via beta-cryptoxanthin, which is catalyzed by beta ring hydroxylase. Epoxidation of zeax-anthin by zeaxanthin epoxidase produces violaxanthin via antheraxanthin. This reaction is reversible as de-epoxidation by violaxanthin de-epoxidase can lead to the formation of zeaxanthin. Neoxanthin synthase is responsible for the conversion of violaxanthin to neoxanthin. Capsanthin synthase and capsorubin synthase produce the ketolated carotenoids such as capsanthin and capsorubin from anther-axanthin and violaxanthin, respectively [28].

VIII. EXTRACTION OF CAROTENOIDS

There are various methods for the extraction of carotenoids from tissues and for the preparation of extracts for pigment purification. The choice of a solvent system depends on the biological material, its pre-treatment, and carotenoid composition. Carotenoids are usually extracted from biological samples with water-miscible organic solvents such as acetone. Because biological sources usually contain a mixture of carotenoid pathway intermediates and a major carotenoid in the mixture so the method must be able to extract a variety of carotenoids of diverse polarity. Extraction should be repeated until the residue, and the filtrate is colorless or no more pigment is released. Pooled samples in organic solvent solution are then concentrated and dried by rotary evaporation or for small samples in a nitrogen gas stream. Carotenoids extracted in this manner are contaminated with various nonpolar substances including colorless lipids. Such compounds may interfere in later methodologies such as mass spectrometry. Saponification can be performed to remove contaminants. Saponification is not recommended when it can alter the structure of the carotenoid, e.g. hydrolysis of carotenoid esters or conversion of astaxanthin to astacene by oxidation under the alkaline conditions of saponification. In such cases, an anaerobic saponification procedure is recommended. Colorless bio-elements are identified by using a special reagent that results in colored bio-compounds.

Two–dimensional chromatography technique is used to separate the desired bio-elements from the mixture. Silica gel or alumina is used as adsorbent in the stationary layer in thin layer chromatography. The colored pigments can also be visually identified. Spraying the plates with solution of paraffin in petroleum and ether can prevent the discoloration of carotenoids on chromatographic plate. Rodamine 1% solution in acetone and spraying a $SbCl_3$ solution in chloroform can be utilized to separate and identify some carbonylic carotenoids or products of oxidative compositions. One of the technique for the identification of carotenoids is using iodine vapors. Brown spot formation identifies the eventual impurities. Another method of identification of colorless bio-compounds includes the visualization of the fluorescence characteristics to absorption in UV.

IX. IDENTIFICATION OF CAROTENOIDS

The absorption spectrum is the key character of carotenoids which is the function of chromophore of carotenoids. The determination of absorption spectrum of carotenoids in various solvents is the key feature of identification process of carotenoids. Column chromatography techniques are used to study the absorption characteristics. Carotenoids can also be identified by using chemical tests and techniques. Functional groups in molecule of the pigments can also be identified by using chemical tests. Color reactions are responsible for identification in these techniques [33].

In Infrared spectroscopy, absorption spectrum is considered advantageous in determining the structure due to its richer informative material and presentation of a large number of bands. The presence and position of various functional groups in carotenoid molecules and kind of polyenic system can be determined by using IR spectra [34]. Carotenoids are tableted in solid phase in KBr or in solution with different solvents (CCl4, CHCl3, CS2 etc.) to prevent any interferences.

X. BIOTECHNOLOGY FOR PRODUCTION OF CAROTENOIDS

Biotechnological processes are high yielding processes at extremely low cost and decreased waste disposals, thus more liked and considered the most suitable for industrial scale production of carotenoids. These advantages depend on availability of nutrients, the culture medium conditions including inoculum size, pH, aeration, agitation etc. There are a number of factors that should be considered while designing a biotechnological process [29]. Some of these factors include bioreactor design, raw materials required for process, the microorganism or enzyme used, type of fermentation selected. Suitable substrate selection and its treatment. depends on fermentative capacity and the type of enzyme produced in micro-organisms. While the configuration and operational variables of bioreactor are key important to harvest maximum yields. Downstream processing limits the biosynthesis of intracellular pigments [30]. Cell disruption affects recovery yield of desired compound and carotenoids properties [31, 32].

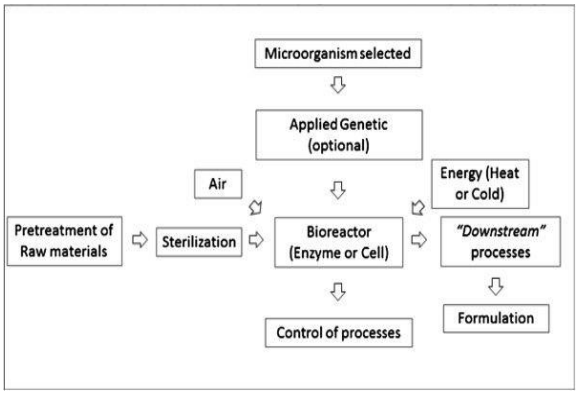

Fig. 4 Metabolic engineering pathway of carotenoids

XI. GENETIC MANIPULATION OF PLANTS FOR CAROTENOID PRODUCTION

Animals cannot synthesize carotenoids on their own. They have to take diet rich in fruits and vegetables that are rich in carotenoid content. Health benefits and commercial utilization of carotenoids have drawn attention towards the genetic modification of carotenoid biosynthetic pathway in order to increase its content in crops. Carotenoid biosynthetic pathway is a highly branched pathway with a complex regulatory network. Experimental data indicates that both transcriptional and post transcriptional events account for the regulation of carotenoid biosynthesis. Xanthophylls and carotenes are key composition of photosynthetic apparatus in plants thus they are important for plant viability. In higher plants, four types of carotenoids i.e. lutein, b-carotene, violaxanthin and neoxanthin are present abundantly [35, 36].

Genetic manipulation of the e-cyclase and b-hydroxylase exhibit the possibilities of altering the carotenoids pool size. The variation in pool size is achieved by manipulating mRNA abundance of one gene. Manipulating gene expressions cause variation in carotenoid accumulation to certain limit. That is, there is a maximal increase of carotenoids 180% for lutein in the sense e-cyclase lines and a maximal decrease of caroteinoids 60% for violaxanthin in the antisense b-hydroxylase lines [37].

Manipulation of genes encoding enzymes in the isoprenoid pathway to increase the metabolic flux is one of the approaches. The sense and the antisense constructs of endogenous 1-deoxy-D-xylulose-5-phosphate synthase were introduced into *Arabidopsis thaliana* under the constitutive CaMV 35S promoter [38]. An increase in the plastidic isoprenoids such as chlorophylls, tocopherols, carotenoids, abscisic acid (ABA) and gibberellin levels was attributed to the accumulation of GGPP, which acts as a common precursor for these isoprenoids [38]. Thus deoxy-D-xylulose 5-phosphate synthase (DXS) was shown to be rate limiting in plastidic 2-C-methyl-D-erythritol 4-phosphate pathway that produces isopentenyl pyrophosphate. Carotenoid levels were enhanced up to 1.6-fold by introduction of bacterial DXS in tomato under DXS transit sequence. There was an increase in phytoene and β-carotene [39]. Extra-plastidic isoprenoids were unaltered in these transgenic lines [40]. Overexpression of DXS gene from *E. coli* in *Solanum tuberosum* under patatin promoter resulted in increase of mean tuber carotenoid in which phytoene occupied a prominent position i.e. up to 7-fold increase in transgenic lines. Besides the elevation in carotenoid level, plant vigor was also improved.

Phytoene synthase enzyme has the highest flux control coefficient among other enzymes in the carotenoid biosynthetic pathway. However, dwarfism resulted as a consequence of the channeling of majority of GGPP into carotenoid formation than the synthesis of gibberellic acid (GA) which has an impact on plant height [41]. The transformants were having reduced chlorophyll and GA levels as well as abnormal pigment accumulation in several organs. This overexpressing phytoene synthase enzyme also resulted in significant rise in carotenoid content in canola seeds.

Even though manipulation of phytoene synthase gene (*crtB*) in canola seeds for carotenoid production was a success, expression of the same gene in other plant sources was not pleasing. Taking into account the nutritional value of flaxseeds, an idea to elevate carotenoid levels in them was also materialized by introduction of *crtB* gene [42] into *Linum usitatissimum* L. obtained from soil bacterium *Pantoea ananatis* under the control of CaMV 35S and *A. thaliana* fatty acid elongase (AtFAE1) seed specific promoter resulted in the rise in yield of total carotenoids. These were the first to increase carotenoid amounts in plants using CaMV 35S promoter successfully without a threat to growth and survival.

Sub-Saharan Africans use cassava roots (*Manihot esculenta*) as a primary source of carbohydrates but the carotenoid content is far less. Two allelic variants of cassava roots i.e yellow colored roots possessing colored pro-vitamin A carotenoids and white colored which has low levels of carotenoids differ by a single amino acid change in the phytoene synthase gene. Commercial cassava varieties with increased carotenoids and vitamin A content were developed by replacing this single amino acid gene [43].

Phytoene desaturase (PDS) plays a major role in carotenoid biosynthesis pathway. Perturbation of PDS gene in *A. thaliana* resulted in an increase in phytoene levels along with a negative impact on chlorophyll, carotenoid and gibberellic acid biosynthesis. It eventually resulted in albino and dwarf phenotypes [44]. The phytoene desaturase gene from *E. uredovora* introduced under CaMV 35S promoter fused with pea ribulose bisphosphate carboxylase small subunit transit sequence in tomato increased beta carotene levels along with a reduction in the total carotenoid content due to feedback inhibition on phytoene synthesis stage of the pathway [45].

The carotenoid lycopene in ripe fruits serves as a large pool of precursor for beta carotene. Lycopene cyclase encoding gene was also subjected to manipulation in a view to enhance carotenoids. Lycopene cyclase gene was expressed in *A. thaliana* under CaMV 35S promoter. This resulted in an elevation in total carotenoid and lutein content in comparison with the wild type [46]. Over expression of *A. thaliana* βLCY gene in a fruit specific

manner under tomato PDS promoter elevated β-carotene in tomato. Total carotenoids were increased up to 112 µg/g FW in tomato fruit [47]. Down regulation of βLCY gene in tomato fruit showed 50% inhibition of cyclase expression with a slight increase in lycopene. Almost the entire pool of lycopene was converted into β-carotene when βLCY from tomato was expressed under CaMV 35S promoter in tomato [48]. When εLCY was silenced using antisense technology under patatin promoter, a significant elevation of β-carotenoid levels was observed with preferential accumulation of β-carotene up to 14 fold [49]. The lycopene content in tomato was improved by suppressing the expression of LCY through RNA interference (RNAi) technology in a fruit specific manner. When εLCY was silenced through RNAi mechanism in seeds, β-carotene, zeaxanthin, violaxanthin and unexpectedly lutein also was increased. A relation between εLCY activity and fatty acid biosynthesis was observed as transgenic seeds produced low levels of fatty acids. β-carotene levels up to 1 mg/g DW were observed in tomato fruits when βLCY gene from eubacterium *Erwinia herbicola* and higher plant daffodil (*Narcissus pseudo Narcissus*) was introduced into tomato plastid genome [50]. These tomatoes also presented a >50% increase in total carotenoid, showing an enhanced flux through the pathway in chromoplasts.

Apart from focusing on experiments with a single gene a combinatorial approach was also adopted to increase the carotenoid levels. PSY from *Narcissus pseudo narcissus* and CrtI from *Erwinia uredovora* inoculated in rice endosperm under endosperm glutellin promoter along constitutive CaMV 35S promotor respectively. As a result, endosperm produced 1.6µg/g of carotenoids. Maize PSY-1 and CrtI produced carotenoids up to 37 µg/g, 31 µg/g were β carotene giving golden rice 2. This may be due to the induction of lycopene cyclase gene following the lycopene accumulation in rice endosperm.

Brassica napus was seen to be a suitable crop to enrich carotenoid levels successfully. The CrtB gene along with bacterial geranyl geranyl pyrophosphate synthase (CrtE) gene behind napin promoter had not reported any change in the total carotenoid levels, ratio of β carotenoids to α carotenoids or even to phytoene levels [66]. As GGPP serves as a common precursor for many other metabolites like tocopherol, phylloquinone and plastoquinone, the GGPP formed might have been used up for their synthesis. But when CrtB gene was cloned in conjunction with CrtI, there was a decrease in phytoene levels. But in the seeds from these transgenic plants, higher lycopene levels and higher ratio of β to α carotenoids were observed. When bacterial lycopene β cyclase (CrtY) or endogenous canola βLCY was introduced along with CrtB, even though total carotenoid level and amount of phytoene remained high, it did not observe a change in ratio of β to α carotenoids. This reflects the fact that combination of CrtB and CrtY favored a significant increment in the levels of β-carotene. An interesting elevation was witnessed when 3 genes CrtE, CrtY and CrtI was overexpressed simultaneously. There was an increase of 50% in β to α carotenoid [51].

Expression of CrtB, CrtI and CrtY under tuber specific promoter exhibited tubers with yellow color which showed 20fold increases in carotenoid levels to 114 mcg/g DW and β-carotene 3600 fold to 47 mcg/g DW [52]. This was the highest carotenoid and β-carotene content reported for bio fortified potato as well as for any of 4 major staple foods. Endosperm specific expression of two bacterial genes CrtB and CrtI under influence of super zein promoter triggered pro-vitamin A level of maize to 10 folds. Carotenoids levels were increased by 34fold [53].

Wheat became another host for genetic engineering experiments to enhance carotenoid levels. When maize PSY with CrtI gene was introduced into wheat culivar-EM12, driven by endosperm specific 1Dx5 promoter and constitutive CaMV 35S promoter, the carotenoids content was increased up to 10.8folds. A different approach to simultaneously engineer 3 different pathways was carried out in maize. It produced increased carotenoid level by 112 fold comprising 60 µg/g β-carotene, 23 µg/g lycopene and 36 µg/g zeaxanthin. To elevate ascorbate levels and folate levels, rice dehydro-ascorbate reductase cDNA and *E.coli* folE gene encoding GTP cyclohydrolase were introduced respectively, both under the influence of the barley D-hordein promoter [54].

Various experiments aimed at enzymes downstream to the enzyme lycopene cyclase also became a subject of interest for genetic manipulation. As xanthophylls are formed by the oxidation of carotenes, the suppression of those carotene hydroxylases resulted in an increase of the cyclic carotenes such as α carotene and β carotene. Antisense inhibition of beta carotene hydroxylase gene reduced violaxanthin, neoxanthin and lutein. Dramatic change was observed when antisense constructs were used to silence non heme beta carotene hydroxylase 1 and 2 under patatin promoter [55]. The β-carotene increased up to 38 fold and total carotenoids up to 4.5 fold with a decrease in zeaxanthin. Another approach towards enhancing the beta carotene content was silencing the genes downstream to β-carotene in the pathway. Through RNAi mechanism, the silencing construct of BCH gene was introduced into potato via Agrobacterium mediated transformation. The construct has shown the highest stability and more levels of β-carotene when construct was under the influence of tuber specific granule bound starch synthase (GBSS) promoter [56].

ketocarotenoids possess a vast number of health advantages and a number of industrial applications, thus acquires a great interest from the scientific community. This results in high demand of ketocarotenoids like astaxanthin increasing the synthesis. When β carotene ketolase (CrtO) gene from alga *Haematococcus pluvialis* was expressed under PDS promoter in *Nicotiana tabacum*, the chromoplasts in the nectary tissue of tobacco produced around 17% of carotenoid [50]. This proved the flexibility of the pathway showing that plants can also be engineered to produce novel carotenoids like astaxanthin in them. As nectary tissue was the site of production, the levels of astaxanthin

produced were extremely low. Seed specific expression of beta carotene oxygenase cDNA from *H. pluvialis* under 2S, seed storage protein promoter napA, produced ketocarotenoids. These ketocarotenoids included 4 keto-lutein, adonirubin and canthaxanthin in *A. thaliana* [57].

As an attempt to enhance the availability of substrate for the enzyme action, plants were crossed with transgenic plants over expressing endogenous PSY. This visualized an increase in carotenoids content. There was also 11 % increase in above mentioned ketocarotenoids in *A. thaliana*. Based on the health benefits and commercial applications attributed to astaxanthin, algal β ketolase 1 (BKT1) from unicellular algae *H. pluvialis* linked with PDS transit peptide sequence was expressed under the influence of patatin promoter which produced astaxanthin and keto-lutein in *S. tuberosum*. In a view to increase the production, co transformation approach was adopted to co-express CrtB and BKT1 simultaneously. But ketocarotenoids were not elevated due to the lack of proper sequestering structures [58].

In order to enhance the beauty and the economic importance of ornamental crops, carotenoid biosynthetic pathway was manipulated in order to change the flower color [59]. The β carotene ketolase (CrtW) gene from marine *Agrobacterium auramtiacum* under CaMV 35S promoter was targeted to the plastid of *Lotus japonicus*. This was done using transit peptide sequence of pea Rubisco small subunit. Its color was changed from light yellow to deep yellow and then finally to orange due to production of ketocarotenoids including astaxanthin, adonixanthin. As β-carotene is accumulated in high amounts by nature in carrot (*Daucus carrota*), β carotene ketolase (CrtO) from H. pluvialis was introduced under the influence of 3 promoters such as double CaMV 35S, Arabidopsis ubiquitin and RolD from *Agrobacterium rhizogene* [60]. CaMV 35S and ubiquitin promoters exhibited the highest mRNA expression in callus, leaf and root tissues. This work reported that 70% of total carotenoid content was converted into novel carotenoids possessing 2400 µg/g root DW.

As zeaxanthin played a vital role in prevention of retinal damage carotenoid biosynthesis pathway was engineered resulting in more production of zeaxanthin through antisense and co suppression strategies of zeaxanthin epoxidase (ZEP). ABA level was not altered and the transformants revealed 2 to 3 fold elevation of α-tocopherol. No impact on plant growth was seen [61].

XII. REFERENCES

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