5
Engineering of Ketocarotenoid Biosynthetic Pathway in *Chlamydomonas reinhardtii* through Exogenous Gene Expression

Microalgae have been explored extensively for their role in wastewater treatment, third-generation biofuel production, lipids production, and single-cell protein applications. Pigment production through microalgae has been a relatively less explored domain but has enormous potential. Microalgae produce a wide range of pigments, such as chlorophylls, carotenoids, and xanthophylls. A green microalga, *H. pluvialis* is a rich source of carotenoid astaxanthin with a productivity of up to 4% DCW (g) [Sun *et al*., 2023]. However, a major limitation of this strain is its slow growth. Hence, various strategies have been explored to produce high carotenoids in microalgae [Cao *et al*., 2023]. On the other hand, the unicellular eukaryotic microalgae *C. reinhardtii* is a single-celled green alga that has been well-studied for transgene expression. Previous research has been led to overexpress the rate-limiting enzymes of the carotenoid biosynthetic pathway to improve carotenoid production [Couso *et al*., 2011; Tokunaga *et al*., 2021; Kang *et al*., 2022; Tsai *et al*., 2022; Huang *et al*., 2023]. In the last few years, several other crucial enzymes have also been identified and overexpressed in different microalgae species such as *Chlamydomonas* and *Dunaliella*, to produce astaxanthin and β-carotene.

Previously, this model microalga has been employed for expressing several carotenoid biosynthetic pathway genes [Jeong *et al*., 2023; Perozeni and Baier, 2023]. In line with the literature studies, *H. pluvialis* astaxanthin pathway is established in *C. reinhardtii* through the simultaneous expression of *bkt* and *crt* genes to synthesize the high-value carotenoids, including astaxanthin and β-carotene. *C. reinhardtii* cells were transformed through nuclear genome random insertion events. Furthermore, the GM *C. reinhardtii* was characterized for gene expression, growth rate, and carotenoid production.

5.1 EXPERIMENTAL

C. reinhardtii CC-125 was maintained in TAP media at 25°C, with light intensity 12000 lx (for 16:8 h light and dark conditions). Hygromycin selection was used to maintain GM microalgae [Ahmad *et al*., 2015]. The expression vector pUC57_*bkt*_*crt*_hyg (8325 bp) has an *aph7* gene to provide hygromycin selection. The *Hpbkt* (Accession no. AY603347.1) and *Hpcrt* (Accession no. KP866868.1) gene sequences from *H. pluvialis* were codon-optimized, and integrated into the nuclear transformation vector, which is shown in Figure 3.3. The synthesized plasmid was transferred to a competent *E. coli* Top10 strain to maintain and extract the plasmid stock.

Genetic transformation in microalgae was carried out using electroporation (800 V, 50 µF, and 800 Ω). After electroporation, cells were allowed to recover on ice and plated on hygromycin B containing selective TAP agar plates. The hygromycin-resistant colonies were picked up and analyzed further using molecular techniques, including PCR, Southern hybridization, and Western blotting. UV-visible spectroscopy and HPLC measured carotenoid production. The microalgae growth rate was evaluated using the cell count and standard methods.

5.2 RESULTS AND DISCUSSION

5.2.1 Genetic Transformation and Transgene Integration Analysis using PCR

After electroporation with the selected vector, the microalgae cultures were transferred to an antibiotic selection plate containing 10 µg/ml hygromycin B. Small green colonies appeared after two weeks of cultivation under 16:8 h light and dark conditions at 25 °C. For secondary selection, the putatively transformed colonies were patched in a grid form on the TAP selection medium (Figure 5.1). The hygromycin-resistant colonies were screened for the presence of *aph7* gene using PCR. The PCR results showed that among the 90 colonies screened, 29 colonies showed PCR-positive results. To validate the PCR cycle, plasmid DNA was also amplified with *aph7* primers as a positive control. No amplification was observed in the WT DNA as shown in Figure 5.2A. In addition, No Template Control (NTC) was clear, which further strengthened the results. These *aph7* harboring transgenic lines were further scaled up to check the elevated carotenoid concentration.

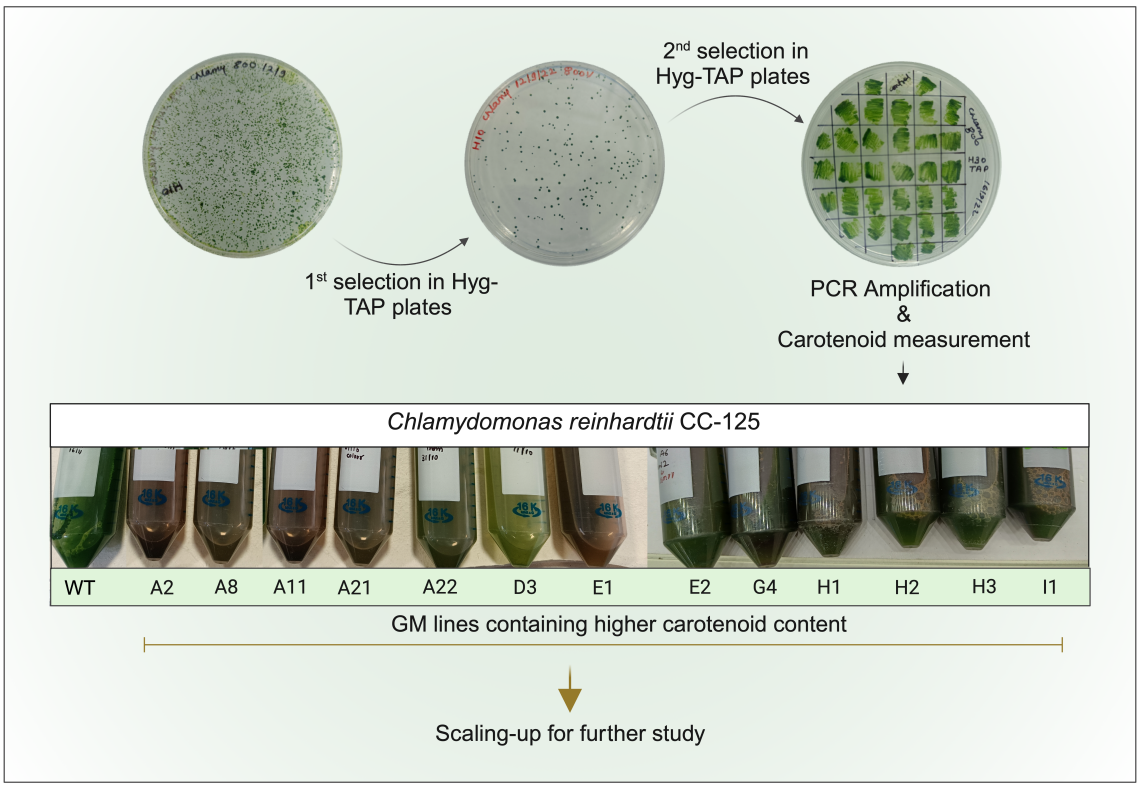

Figure 5.1 : The schematic shows the WT and GM *C. reinhardtii* on antibiotic selection plates containing Hyg B (10 µg/ml), and their scaling up in liquid TAP media for further study. Here, GM microalgal lines are showing yellow-orange pigmentation

5.2.2 Preliminary Screening of Transformed Microalgal Lines

Firstly, the putative transformants were selected on hygromycin-containing solid medium by repeated streaking, ensuring the elimination of any false positive clones. The hygromycin-resistant transformants were confirmed for the presence of gene constructs by *aph7*-specific PCR. Most of the transgenic microalgae strains found to be positive for *aph7* were displaying pale yellowish-light green to reddish green phenotypes, while WT microalgae remained dark green, as shown in Figure 5.1. This change in biomass pigmentation is crucial considering the increase in yellow-orange pigmentations, which may be contributed by β-carotene and astaxanthin in some cases. Initial pigments screening narrowed down the number of putative transgenics to be tested for elevated carotenoid content. Among 29 PCR-positive lines, 13 transgenic lines as shown in Figure 5.1, were found to synthesize higher total carotenoids than the WT cultures as confirmed by UV-visible spectroscopy (Figure 5.3). These results from initial pigment screening were instrumental in narrowing down the sample size, enabling the selection of specific algal lines for subsequent gene integration and expression studies. The transgene

stability was assessed through multi-generational cultivation. The *bkt* and *crt* genes are known to be present in *C. reinhardtii* cells; however, their poor expression in vegetative cells is the limiting factor for the synthesis of these high-value pigments [Tran and Kaldenhoff, 2020]. Therefore, the *aph7* gene was used for the initial screening of transgenic lines. The further confirmation of transgenic microalgae was done by analyzing the copy number of foreign genes and protein expression by performing Southern hybridization and Western blotting, respectively with specific probes and antibodies.

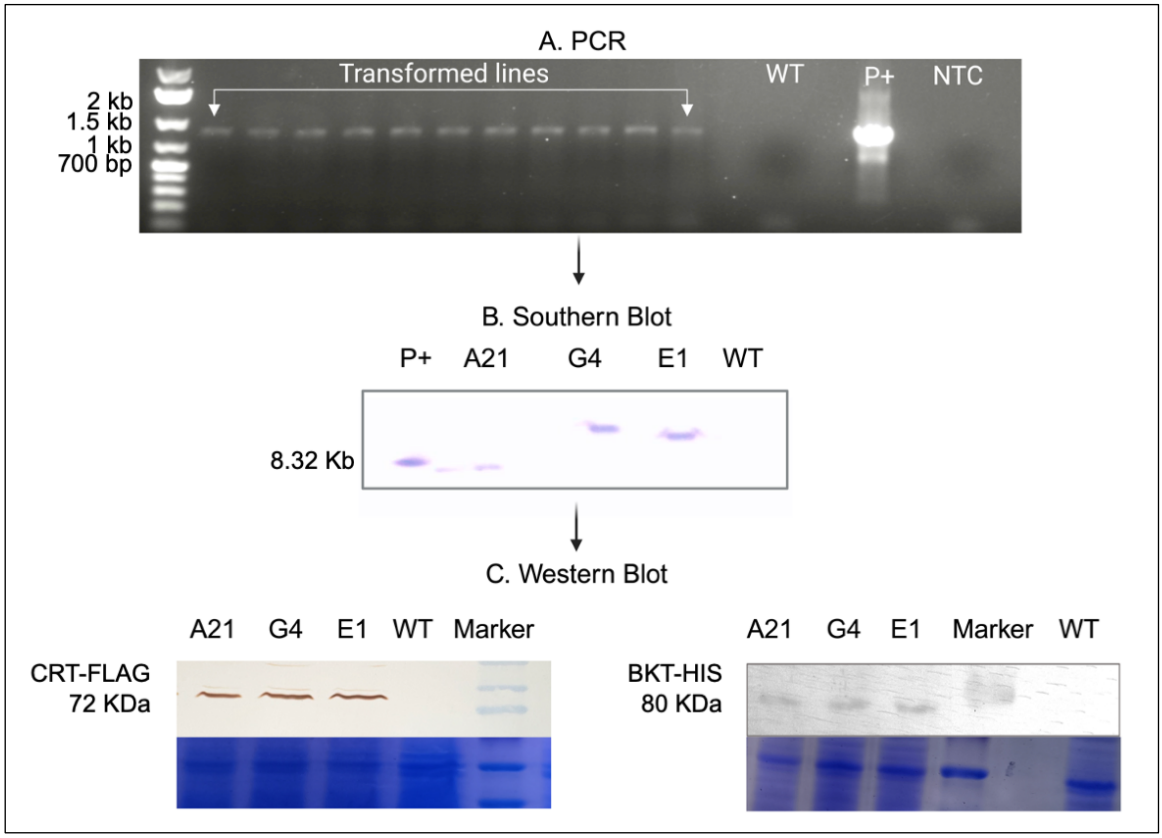

Figure 5.2 : The schematic showing gene integration and expression in GM and WT *C. reinhardtii* using molecular tools. A) PCR result with *aph7*-specific gene primers for GM and WT *C. reinhardtii*, NTC (no template control), P+ is positive control (plasmid); B) Southern blot for GM *C. reinhardtii* showing a single band and WT where no band is observed. P+, which is plasmid (positive control), shows a single band at about 8.3 kb; C) protein expression through Western blotting left panel shows the expression for FLAG-tagged CRT protein at 72 kDa, and the right panel shows the protein expression for HIS-tagged BKT protein at 80 kDa. WT shows no signal in both blots

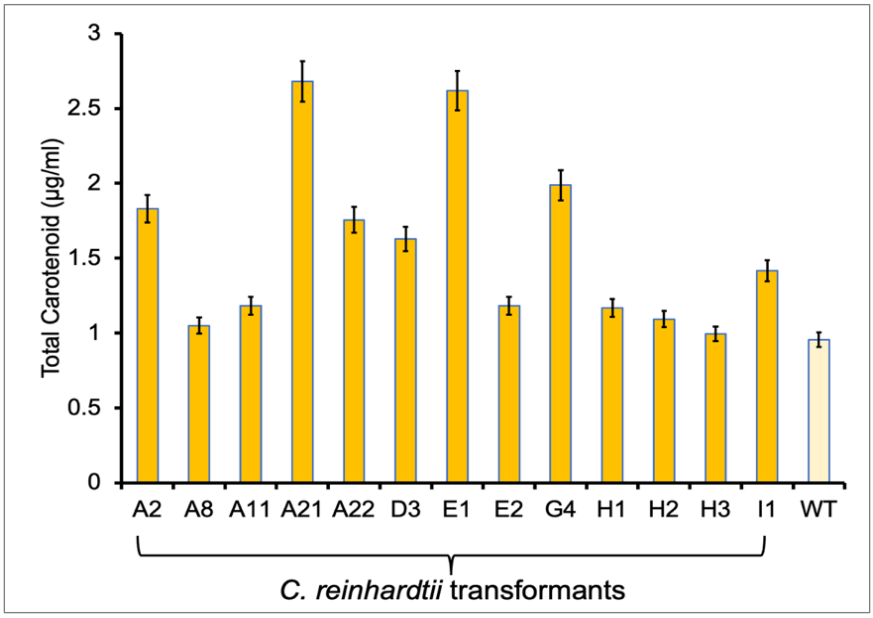

Figure 5.3 : Screening of *C. reinhardtii* transformants for carotenoid production. Here total carotenoids normalized with absorbance at 750 nm are shown for transformants and WT *C. reinhardtii*

5.2.3 Analysis of Copy Number in GM Microalgae using Southern Hybridization

The transformed and WT *C. reinhardtii* were grown for five days, as the total carotenoid concentration was found to be the highest during that period. The result from Southern blots has shown one single prominent band in the algal lines A21, E1, and G4, indicating a single copy insertion. These alphabetic numbers were assigned to GM microalgal lines based on their confirmation. The linearized plasmid was used as a positive control and a molecular weight marker (about 8325 bp) as shown in Figure 5.2B. The position of the bands was found to be different for all the transgenic lines, indicating a random insertion event. No band was observed in the WT *C. reinhardtii*. These results have confirmed the transgenes' integration in the *C. reinhardtii* nuclear genome.

5.2.4 Analysis of *bkt* and *crt* Expression in GM Microalgae using Western Blotting

The Southern blot-positive transgenic strains, A21, E1, and G4, were further scaled up in batch cultivation systems for total protein extraction. These were checked for expression of 10X-His-tagged *Hpbkt* and FLAG-tagged *Hpcrt* genes using Western blot. The blot has shown the single bands at 80 kDa using anti-His Ab and 72 kDa using anti-FLAG Ab, as shown in Figure 5.2C, confirming the presence of *Hpbkt* and *Hpcrt* gene proteins in the transgenic algal lines, respectively. No signal was observed in the WT *C. reinhardtii* in either of the blots. These results have showcased that both *Hpbkt* and *Hpcrt* genes were functionally expressed in the transgenic *C. reinhardtii*. The molecular tools such as PCR and Southern blot confirmed the presence of the heterogeneous construct in the genome of transgenic *C. reinhardtii,* whereas Western blot with 10X HIS and FLAG ensured the presence of both the gene proteins.

5.2.5 Comparison of GM and WT Microalgae Growth in Batch Cultivation

The comparative growth curves have shown the retardation in microalgae growth for all GM microalgae compared to WT, which displayed faster growth in TAP media (Figure 5.4). The specific growth rates for A21, E1, G4, and WT *C. reinhardtii* are 0.287 ± 0.007 d^{-1}, 0.256 ± 0.009 d^{-1}, 0.342 ± 0.017 d^{-1}, and 0.422 ± 0.014 d^{-1}, respectively. The doubling time and divisions per day were also calculated, and the data is shown in Table 5.1.

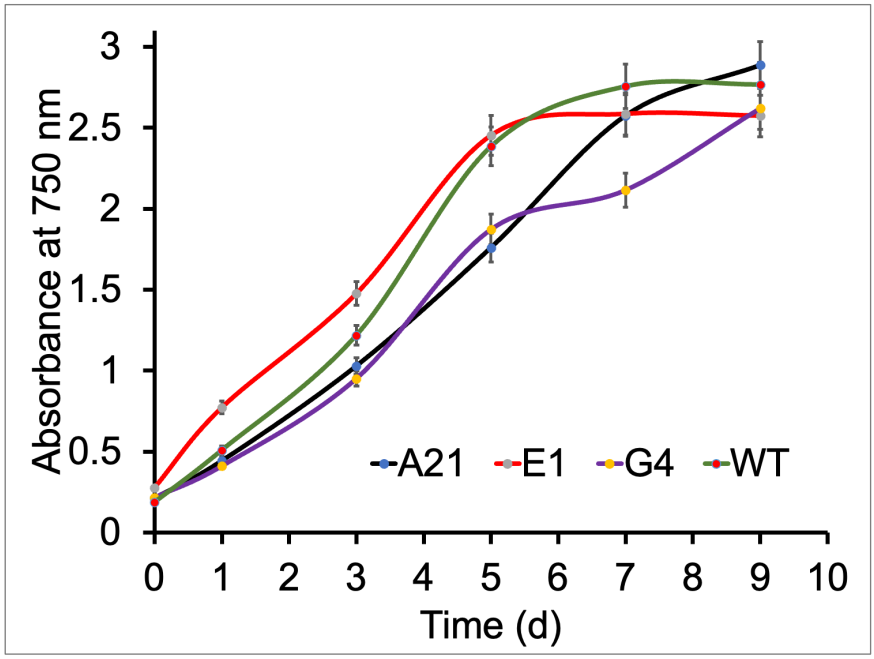

Figure 5.4 : The schematic shows the microalgae growth curve as absorbance at 750 nm vs time (d)

Table 5.1 : Microalgae Growth Comparison for GM and WT *C. reinhardtii*

S. No.	Algae strain	Specific growth rate (d^{-1})	Doubling time (d)	Divisions per day (K)
1	A21	0.287 ± 0.007	2.410 ± 0.0.041	0.415 ± 0.010
2	E1	0.256 ± 0.009	2.709 ± 0.052	0.369 ± 0.013
3	G4	0.342 ± 0.017	2.023 ± 0.089	0.494 ± 0.025
4	WT	0.422 ± 0.014	1.642 ± 0.060	0.609 ± 0.020

It was observed that transgenic microalgae growth was hindered (18-39%) compared to WT microalgae. Microalgae growth generally decreases when the cell is burdened with the extra genetic load, as reported in earlier transgenic microalgae investigations [Chen *et al.*, 2023; Sun *et al.*, 2023]. In response to the insertion of extra genetic load, the cellular activity is affected in transgenic cells, resulting in slower growth than the WT. However, β-carotene production was found to be very high in the transgenic microalgae, which increases the overall pigment production. One of the transgenic strains also displays astaxanthin production, which is not observed in the WT *C. reinhardtii* cells. The slow growth, thus, may be offset by the high productivity of the VAPs. Along with β-carotene and astaxanthin, some unidentified pigments were found elevated in transgenic *C. reinhardtii* compared to WT; that needs further identification. These results suggest the augmentation of carotenoid biosynthesis in the transgenic *C. reinhardtii* through the expression of heterogeneous genes via nuclear genome transformation.

5.2.6 β-Carotene and Astaxanthin Production

β-carotene is extensively utilized as a precursor for vitamin A and antioxidants. β-carotene is a primary carotenoid in microalgae that transfers the light energy to chlorophylls in the photosynthetic reactions. Microalgal β-carotene is an FDA-approved product for direct human consumption, which became the first-ever commercialized product from microalgae [Gong and Bassi, 2016]. It also serves as the precursor for high-value-added xanthophylls such as zeaxanthin and astaxanthin in the carotenoid biosynthesis pathway. Therefore, various strategies were undertaken to produce these high-value carotenoids in different microalgae strains including *Chlamydomonas* and *Dunaliella* (Table 5.2). The *bkt* and *crt* genes are pivotal in synthesizing ketocarotenoids, notably astaxanthin, in both *H. pluvialis* and *Chlorella zofingiensis*. Nevertheless, *C. reinhardtii* fails to produce astaxanthin due to the low expression of its *Crbkt* gene, potentially attributable to an atypical C-terminal extension in the CrBKT enzyme, speculated to be a

pseudogene [Perozeni *et al*., 2020]. Extensive research has been conducted on enhancing the production of high-value pigments in *C. reinhardtii*, owing to its ease of genetic transformability and product retrieval.

Expression of the *bkt* and/or *crt* genes individually in other organisms has led to an increase in β-carotene content. For example, Rathod *et al*. have shown a 30% increase in β-carotene when the *crtYB* gene from red yeast was expressed in *C. reinhardtii* [Rathod *et al*., 2020]. These two genes/enzymes were found to be appealing in enhancing the carotenoid biosynthesis in other microalgae species as well. *H. pluvialis* is known for higher astaxanthin accumulation and shows characteristic red color biomass in the stationary growth phase. When β-carotene hydroxylase from *H. pluvialis* was expressed in *C. reinhardtii*, the β-carotene productivity was not enhanced significantly [Tan *et al*., 2007; Zheng *et al*., 2014]. Moreover, Tan *et al*. produced transgenic lines through chloroplast transformation, which required high-light induction to produce these carotenoid pigments [Tan *et al*., 2007]. Recently, Sun *et al*. have shown the role of lipid accumulation in carotenoid synthesis where they have co-expressed *bkt* and *crt* along with *mecR* (mitochondrial trans-2-enoyl-coa reductase gene) in *C. reinhardtii,* which has resulted in 50% higher astaxanthin production [Sun *et al*., 2023]. The β-carotene synthesis pathway in *C. reinhardtii* bifurcates into two branches: the β-cyclization branch, facilitated by *lcy*E, which converts β-carotene into β-cryptoxanthin and astaxanthin, and the ε-cyclization branch, catalyzed by *crt*, transforming β-carotene into echinenone and canthaxanthin. The *bkt* gene contributes to the monocyclic pathway, where β-carotene converts into astaxanthin through ketolation and carboxylation by *bkt* and *chy*B/*crt*, while zeaxanthin production follows a bicyclic pathway. However, the regulatory interplay between these pathways remains unclear.

In the present study, we have successfully expressed *bkt* and *crt* genes from *H. pluvialis* into *C. reinhardtii* cells to boost ketocarotenoid production. The GM lines exhibited heightened total carotenoid production compared to the WT *C. reinhardtii*. Nonetheless, due to the poor expression levels and limited activity of native *lcy*E and *chy*B in *C. reinhardtii*, β-carotene predominated among observed carotenoids [Chen *et al*., 2023]. Therefore, the overexpression of *bkt* and *crt* can redirect the carotenoid biosynthetic pathway towards the production of β-carotene. While *bkt* could potentially lead to the synthesis of ketolated forms of β-carotene, the overall effect might be an increase in β-carotene levels due to the enhanced flux through the pathway. While *bkt* catalyzes the conversion of β-carotene into astaxanthin, it's important to note that astaxanthin itself can be converted back into β-carotene under certain conditions. Therefore, the increased production of astaxanthin, combined with potential isomerization reactions facilitated by *crt*, may contribute to an increased pool of β-carotene within the cells.

In a previous study, *Chlamydomonas* cell line-C18, overexpressing *Crbkt*, demonstrated transcriptional up-regulation of *bkt* and *lcy*E genes [Chen *et al*., 2023]. Transcriptomics data unveiled that *lcy*E up-regulation facilitated a greater flux of β-carotene into the bicyclic pathway, thereby diminishing the monocyclic pathway intermediate canthaxanthin. The heightened carotenoid content spurred the expression of chlorophyll synthase genes and led to metabolic competition for IPP and DMAPP, that are universal precursors for terpenoid compounds, carotenoids, and chlorophylls in *C. reinhardtii*. Transcriptomics and metabolomics data also suggested that elevated carotenoid content redirected more carbon towards sugar metabolism, with most glycolysis and pentose phosphate pathway genes up-regulated, resulting in higher soluble sugar content in *Crbkt* overexpression mutants compared to the WT [Chen *et al*., 2023].

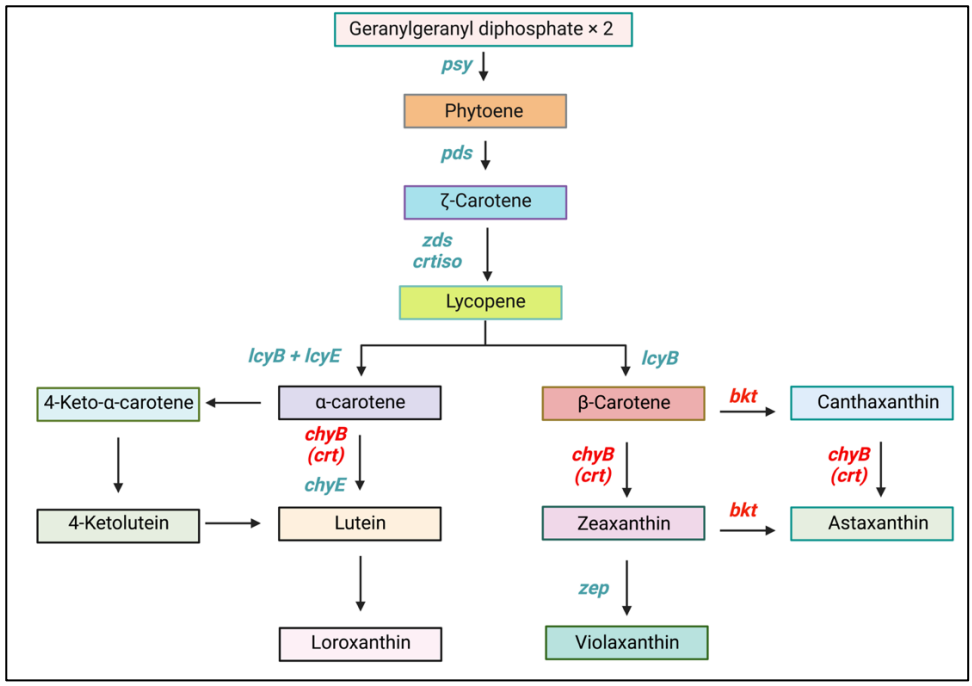

Figure 5.5 : Carotenoid biosynthetic pathway in *C. reinhardtii*. Here, the genes/enzymes marked in red color font were overexpressed

According to the literature studies, carotenoid synthesis is more prominent during the stationary phase of microalgae growth and under stress conditions, such as high light or salinity [Kim *et al*., 2020; Sun *et al*., 2023]. In general, *C. reinhardtii* does not synthesize high-value ketocarotenoids like canthaxanthin and astaxanthin, known for their therapeutic and nutraceutical properties. Hence, several studies have discussed engineering the carotenoid biosynthetic pathway in *C. reinhardtii* by redirecting the carbon flux to the downstream MEP pathway [Kang *et al*., 2022]. To enhance the carotenoid production, its biosynthesis pathway was studied extensively (Figure 5.5). Two genes, namely *bkt* and *crt*, play critical roles in different steps. The *bkt* catalyzes the conversion of β-carotene to canthaxanthin and zeaxanthin to astaxanthin. It was previously reported that *bkt* is present in the *C. reinhardtii* genome; however, it is poorly expressed [Vila *et al*., 2012]. The *chyB* or *crt* catalyzes the conversion of β-carotene to zeaxanthin. In addition, the *crt* regulates the formation of astaxanthin from canthaxanthin. The *crt* gene is also involved in the conversion of α-carotene to lutein. It was also evident from the study by Chen *et al*. that exogenous *crt* expression could result in more up-regulation of the carotenoid pathway and higher carotenoid production than the endogenous *bkt* expression [Chen *et al*., 2023]. Therefore, the present study has successfully expressed *bkt* and *crt* from *H. pluvialis* through the nuclear genome of *C. reinhardtii*. Moreover, the qPCR, an advanced molecular biology tool, can help in correlating the relative gene expression of *bkt* and *crt* with the pigment production.

The pigment characterization was done using HPLC, and a linear regression equation was derived using the standard curve between peak area and pigment concentration. The retention time for the β-carotene and astaxanthin standards were observed at 4.15 min and 1.3 min, respectively, as shown in the HPLC chromatogram (Figures 5.6A and 5.7A). The equations for β-carotene and astaxanthin are given as y=31895x–48263 (R^2=0.9973) and y=92358x-5791.8 (R^2=0.9992), respectively, where y is peak area (µV*s) and x is pigment concentration (µg/ml) (Figures 5.6B and 5.7B), which was used to calculate the β-carotene concentration in different samples (Figures 5.8 - 5.11).

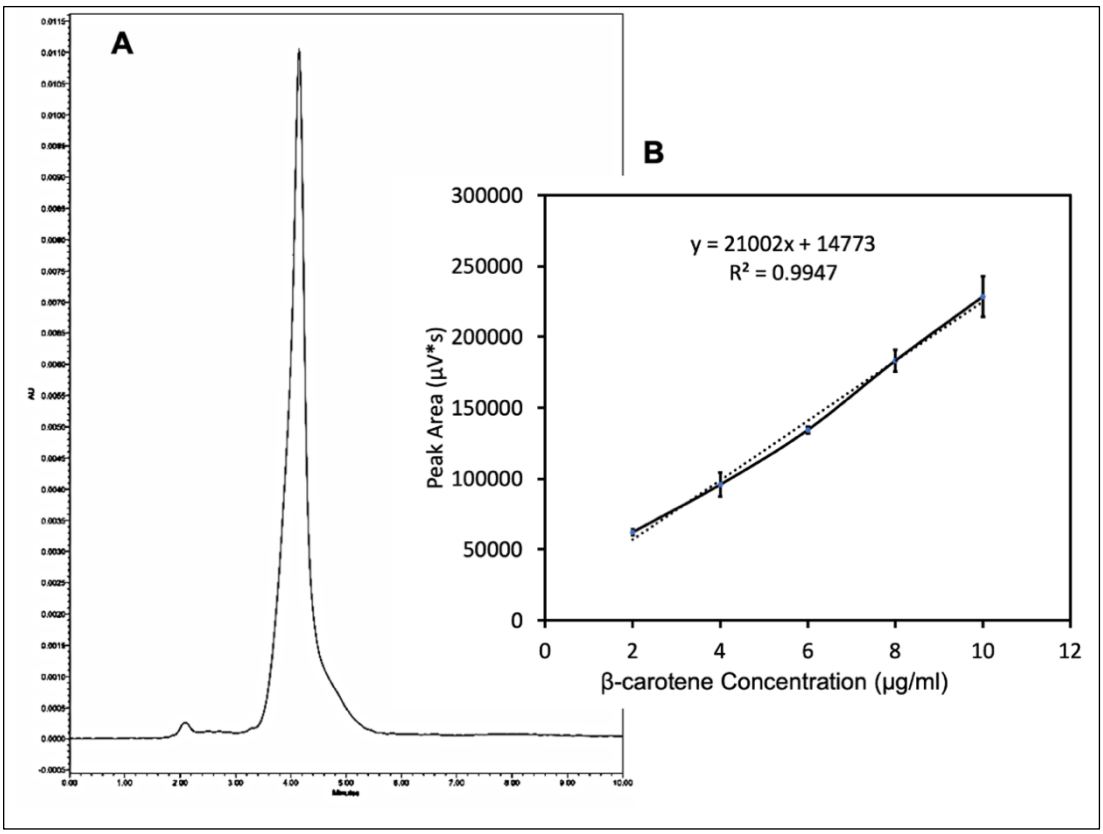

Figure 5.6 : The schematic shows A) HPLC chromatogram for β-carotene (10 µg/ml) showing peak area and retention time (min), and B) standard curve for β-carotene concentration (2-10 µg/ml) vs peak area (µV*s)

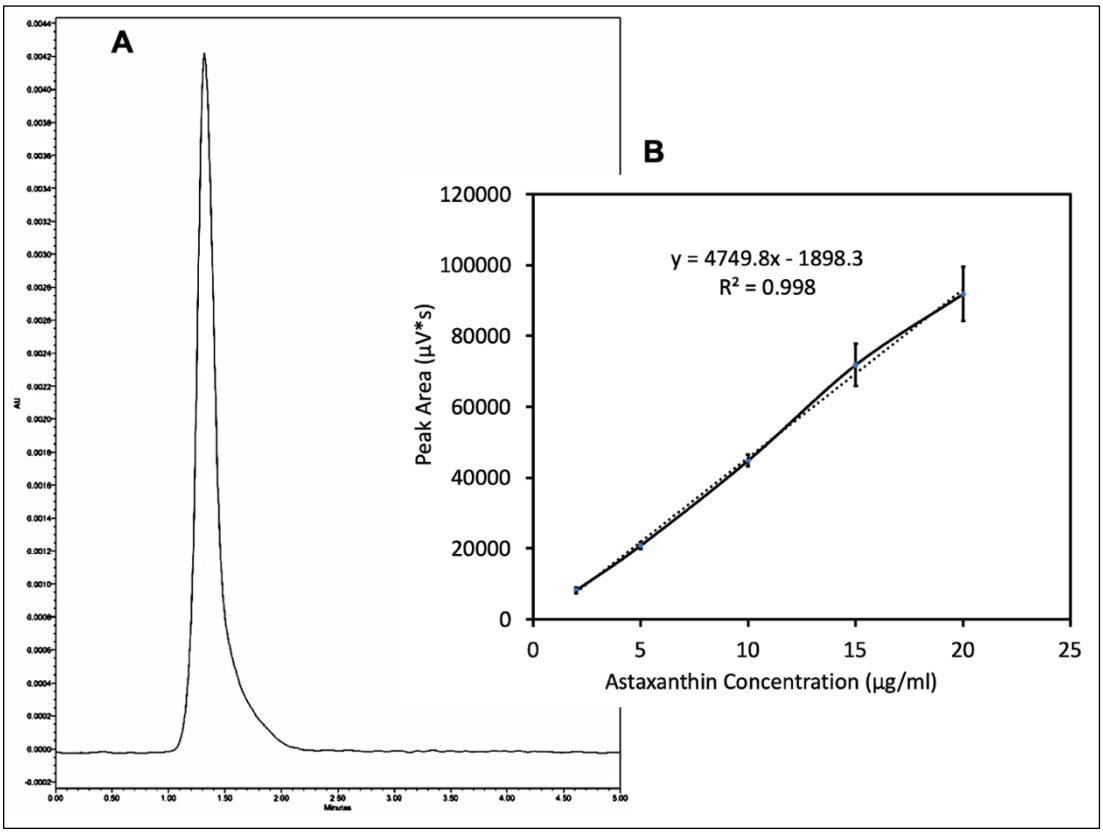

Figure 5.7 : The schematic shows A) HPLC chromatogram for astaxanthin (10 µg/ml) showing peak area and retention time (min), and B) standard curve for astaxanthin concentration (2-20 µg/ml) vs peak area (µV*s)

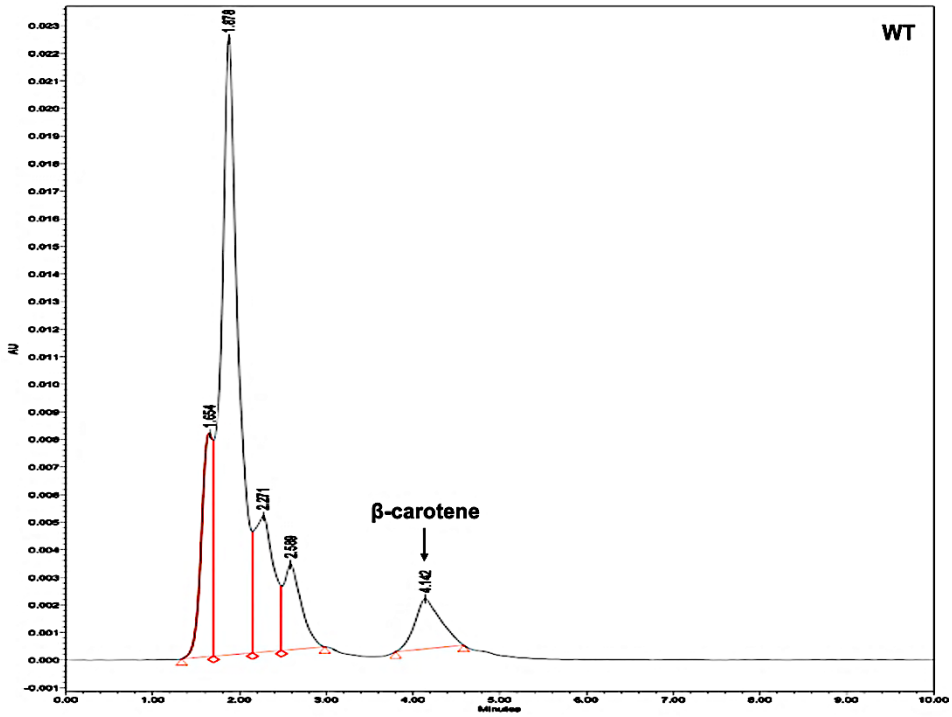

Figure 5.8 : HPLC chromatogram for WT *C. reinhardtii* showing peak arbitrary values and retention time (min)

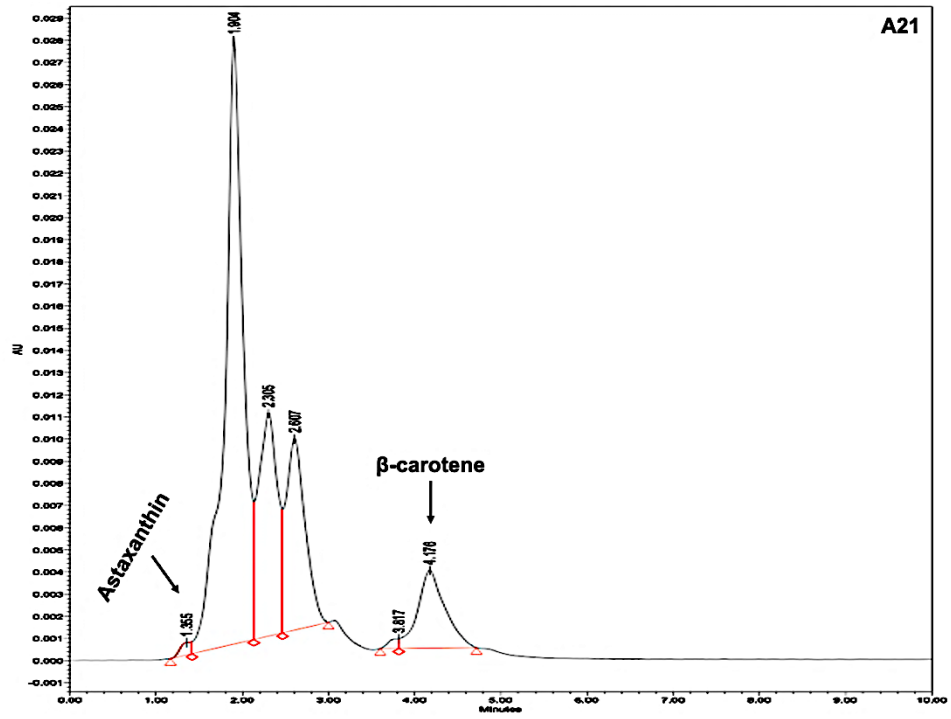

Figure 5.9 : HPLC chromatogram for GM *C. reinhardtii* A21 showing peak arbitrary values and retention time (min)

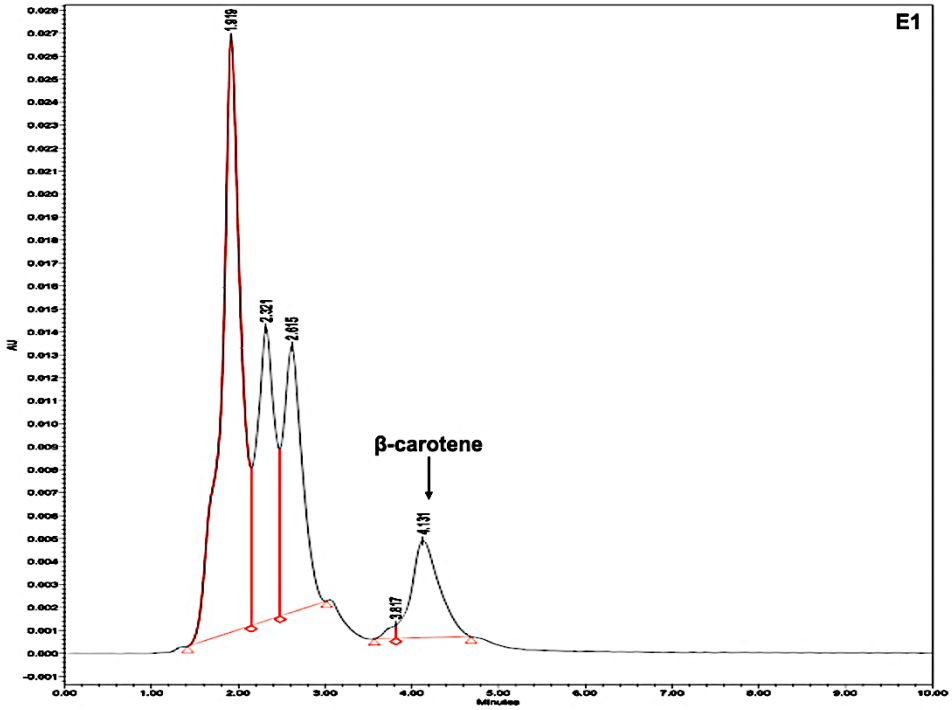

Figure 5.10 : HPLC chromatogram for GM *C. reinhardtii* E1 showing peak arbitrary values and retention time (min)

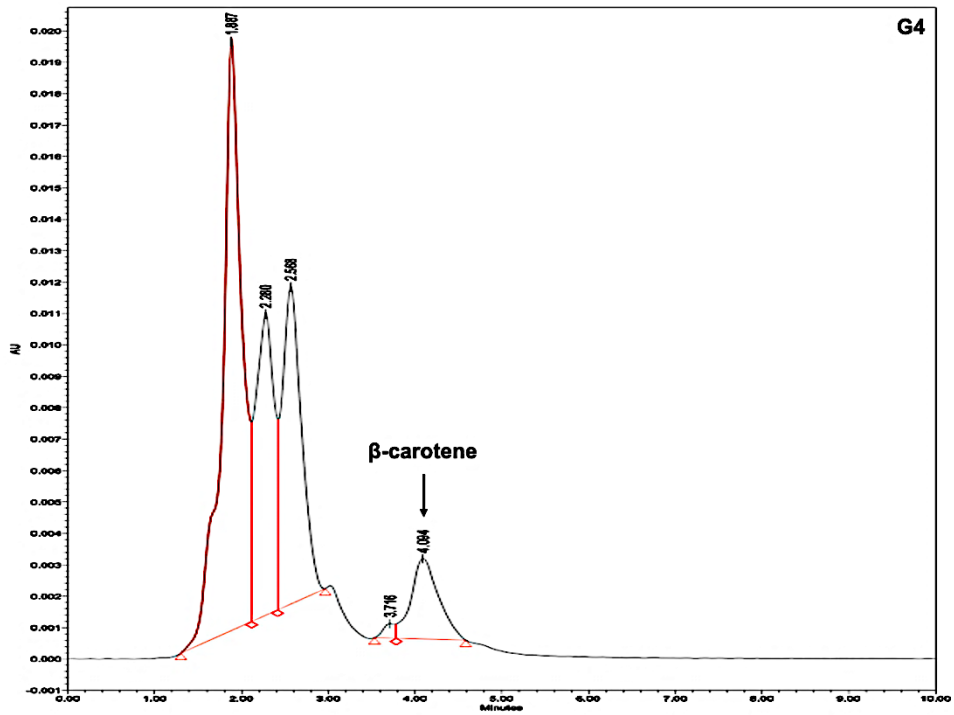

Figure 5.11 : HPLC chromatogram for GM *C. reinhardtii* G4 showing peak arbitrary values and retention time (min)

Under medium light intensity (60 µmol/m²/s) transgenic *C. reinhardtii* has demonstrated a 2.74 to 5.39-fold increase in β-carotene content compared with the WT microalgae as shown in Figures 5.12 and 5.13. The β-carotene concentration in the WT, transgenic lines A21, E1, and G4 *C. reinhardtii* was found at 0.78 ± 0.24, 3.08 ± 0.08, 4.26 ± 0.30, and 2.17 ± 0.26 mg/g DCW, respectively. HPLC results have suggested that there was a 289.91% (3.89-fold), 438.97% (5.39-fold, p-value < 0.0001), 174.59% (2.74-fold, p-value = 0.0006) increase in the β-carotene concentration in A21, E1, and G4 transgenic lines as compared to WT *C. reinhardtii*. These results have shown that the β-carotene content was improved for all transgenic strains compared to WT

C. reinhardtii. This study shows that the *C. reinhardtii* transgenic lines could synthesize high-value pigments without requiring external induction.

The transgenic line A21 has shown astaxanthin production which is a remarkable result considering the slow growth of existing commercial astaxanthin producer *H. pluvialis*. The astaxanthin yield in the A21 transgenic line reached 1.47 ± 0.063 mg/g DCW which is greater than the previous study where chloroplast transformation was used for co-expression of *bkt* and *crt* genes in *Dunaliella* species [Lin *et al*., 2019b]. Astaxanthin peak was absent in the HPLC chromatogram of WT *C. reinhardtii* and the other two transgenic lines. Some unidentified pigments at retention times of 1.9 min, 2.3 min, and 2.6 min, were also found elevated in GM *C. reinhardtii* compared to WT, which need further identification (Figure 5.8 - 5.11). Astaxanthin production can be further enhanced by light exposure as shown by Huang *et al*. (2022). It is also crucial to consider that microalgae growth in GM lines is slightly retard than the WT lines which may affect the overall pigment production. However, the higher productivity of these pigments may offset the overall production cost.

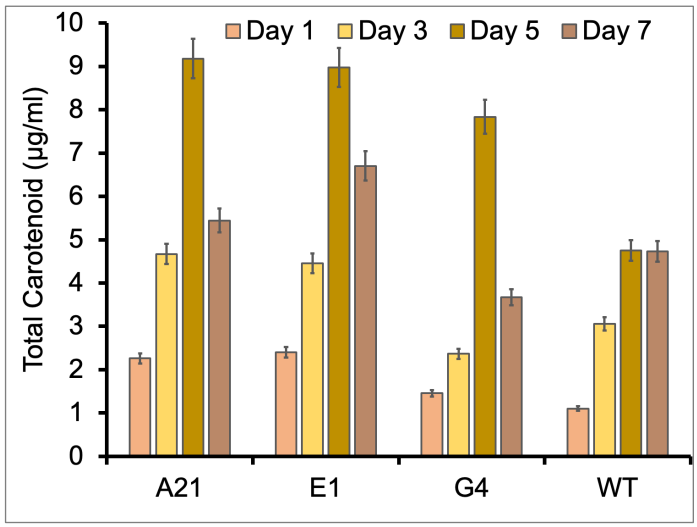

Figure 5.12 : The schematic shows the total carotenoid concentration over a period in a batch cultivation system

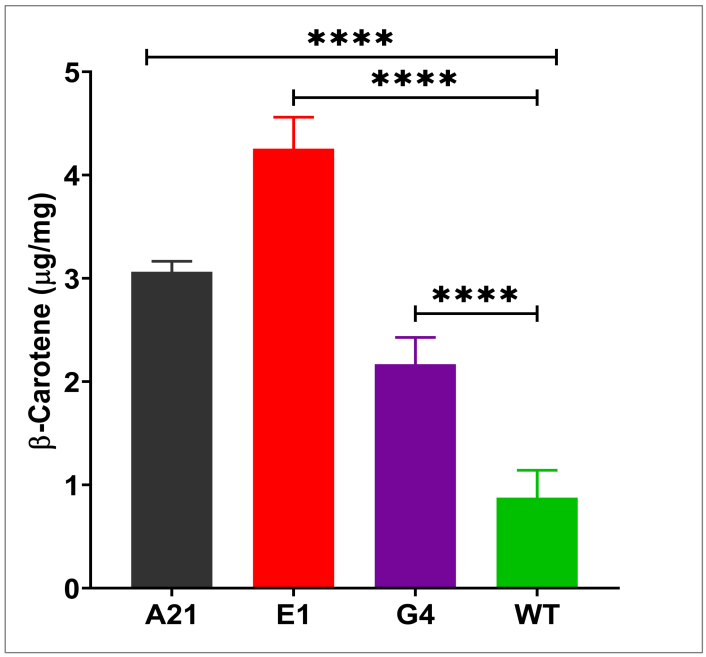

Figure 5.13 : The schematic shows the β-carotene concentration in different GM lines A21, E1, G4, and WT *C. reinhardtii*

Table 5.2 : Comparative Performance of GM Microalgae for Enhanced Carotenoid Content by Manipulating *bkt* and *crt* Genes

S. No.	Algae strain name	Target gene/enzyme	Gene origin	Transformation	Promoter	Algae growth	Pigment concentration	Reference
1	*C. reinhardtii* CC-849 cell-wall deficient	*bkt* and *bch* (*crt*)	*H. pluvialis*	Nuclear transformation (alloy beads)	*Hsp70A/RbcS2* promoter	Not reported	Increase in total carotenoids (29%)	[Zheng *et al*., 2014]
2	*D. virdis*	*bkt* and *crt* in a polycistron	*H. pluvialis*	Chloroplast transformation (microparticle bombardment)	*AtpA*	WT: 45.00 ± 4.27×10^5 per ml, GM: 33.33 ± 5.35×10^5 per ml	β-carotene: 3.0783 ± 0.0231 mg/g DCW, Free astaxanthin: 0.0326 ± 0.006 mg/g DCW	[Lin *et al*., 2019b]
3	*C. reinhardtii* modified strain UVM4	Synthetically redesigned *Crbkt*	*C. reinhardtii*	Nuclear transformation (glass bead agitation)	*Hsp70A/RbcS2*	Not perturbed	2.63.1 mg astaxanthin/l/d and 3.7–4.3 mg ketocarotenoid/l/d	[Perozeni *et al*., 2020]
4	*C. reinhardtii* strain CC-124	*crtYB* (*phytoene-β-carotene synthase–PBS*)	Red yeast *Xanthophyllomyces dendrorhous*	Nuclear transformation (biolistic method)	*Hsp70A/RbcS2*	Equal or high growth in GM than WT microalgae	Increase in β-carotene (38%) and lutein (60%)	[Rathod *et al*., 2020]
5	*C. reinhardtii* CC-4102	truncated *Crbkt*	*C. reinhardtii*	Nuclear transformation (electroporation)	*Hsp70A/RbcS2*	Not reported	-	[Tran and Kaldenhoff, 2020]

6	*C. reinhardtii* CC-125	*bkt* and *chyB* (*crt*)	*H. pluvialis*	Nuclear transformation (electroporation)	35Sde for *bkt* and *Hsp*70A/*Rbc*S2 for *crt*	GM microalgae have slightly (23.39%) lower cell growth than WT microalgae	β-carotene (4.26 ± 0.30 mg/g DCW) and Astaxanthin production 1.47 ± 0.063 mg/g DCW, WT: no astaxanthin	This study

Overall, the study has shown that the overexpression of *H. pluvialis* carotenoid pathway enzymes in *C. reinhardtii* resulted in the overall increase in the production of the carotenoid pigment, making it a very promising approach for microalgae-based biorefinery for therapeutics production. The present study has demonstrated a notable increase in β-carotene and astaxanthin production. At the same time, there is an opportunity to enhance these pigment yields further by implementing a two-stage cultivation strategy, which is expected to be a more effective approach for increasing productivity [Kim *et al*., 2020; Chen *et al*., 2022].

5.3 CONCLUSIONS

The present study establishes *C. reinhardtii* as an exceedingly promising platform, driven by photosynthesis, for efficiently producing high-value carotenoids, explicitly focusing on β-carotene and astaxanthin. This is particularly important compared to the slow-growing *H. pluvialis*, which is commonly used in commercial applications. This study has successfully co-expressed the β-carotene ketolase and β-carotene hydroxylase genes originating from *H. pluvialis* in the nuclear genome of *C. reinhardtii*. This integration has resulted in enhanced carotenoid production in GM *C. reinhardtii*. These results were particularly striking as the GM *C. reinhardtii* exhibited a remarkable 5.39-fold increase in β-carotene yields under constitutive expression. Furthermore, this work demonstrated that ketocarotenoid astaxanthin production reached 1.47 ± 0.063 mg/g DCW. Applying light induction and salt-induced stress conditions can improve carotenoid productivity without compromising the biomass yield. These findings offer valuable insights into the potential of *C. reinhardtii* as a superior and more rapid platform to produce carotenoids, especially astaxanthin, with significant implications for the biotechnological industry and the advancement of sustainable high-value compound production. Moreover, the biocontainment of GM microalgae should always be considered for minimizing the risk associated with transgene escape.