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RESEARCH ARTICLE

ASTAXANTHIN SYNTHESIS IN SYNECHOCOCCUS PC7002: OPTIMIZATION AND PRODUCTSECRETIONTO CULTURE MEDIUM

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Abstract

Synechococcus sp. PCC7002 can produce theastaxanthin precursor β-carotene but not astaxanthin.Using a synthetic ketolase-and a synthetic hydroxylase-gene we modifiedSynechococcuscarotene metabolism to produce astaxanthin. The total cellular content of astaxanthin was further increased by over-expressing the intrinsic phytoene synthase. Besides a beneficial step in astaxanthin production our results indicate for some modifications in the suggestedprocess of carotenoid metabolism. In addition, we were expressing an E. coli ABC-transporter to export astaxanthinand decrease intracellular product accumulation. By this, the synthesis was increased further and significant amounts of astaxanthin can be recovered from culture medium without cell destruction.

Introduction:-

In the context of accelerating climate change, decoupling production lines from fossil fuelsis demanded and new production strategies are required.For this purpose, moremicroalgae-based processes are used in industry (Kumar et al. 2020). Many of these photoautotrophic microorganisms require just light, salt, and CO_2 for growth, and their photosynthetic efficiency is up to 10 times higher than that of higher plants(Xu et al. 2019). Photosynthetic fixation of CO_2 is a significant advantage tonon-photosynthetic microorganism-based production chains. Currently,the use of microalgae as production systems gained importance(Gurreri et al. 2023). The focus has primarily been on the prokaryotic microalgae representatives, i. e. cyanobacteria, which are genetically simple compared to the eukaryotic ones. Knowledge from non-photosynthetic microorganisms that are employed in industryat present, can be easily transferred to cyanobacteria(Carroll et al. 2018). Synechococcus sp. PCC7002 is a suitable strain for synthetic biology approaches also due to its natural competence. The completely sequenced 3 Mbp genome permits genetic modification (Chenebault et al. 2023). Synechococcus sp. PCC7002 harbours several neutral integration sites.These have no effect on the metabolism or growth when modified. Transformation vectors can be used to achieve high transformation efficiency and targeted integration of the desired genes through homologous recombination is possible. The strain can grow in both, fresh and brackish water and has a minimum doubling time of 2.6 hours(Ruffing et al. 2016).In contrast to synthetic astaxanthin, the natural has several fold higher biological activities in comparison to the synthetic one. Although, production and extraction from organisms is high-priced natural astaxanthin is precocious due to its exceptional antioxidant properties and health benefits.

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Consequently, it is currently investigatedto employSynechococcusas production platform using synthetic biology. However, often the approaches do not provide optimal yields and do not pay off also because of cost-intensive harvesting and purification processes. Therefore, synthesis as well as harvest should be improved.

Synechococcus sp. PCC7002 is capable to synthesize carotenoidssuch as β-carotene as well as small amounts of zeaxanthin(Hasunuma et al. 2019). The natural carotenoid synthesis pathway could be expanded by a β-carotene ketolase and a β-carotene hydroxylase, enabling the strain to produceastaxanthin. According to Wang et al. (Wang et al. 2017), the origin of the genes for the above-mentioned enzymes can significantly influence yield of astaxanthin. Therefore, it is important to have a suitable combination of β-carotene ketolase and β-carotene hydroxylase genes respectively enzymes. Although the individual ketolases or hydroxylases always catalyse the same reactions, their affinities for different intermediates are not the same. As demonstrated by (Wang et al. 2017), the combination of β-carotene ketolase from Brevundimonas sp. and β-carotene hydroxylase from Alcaligenes sp. PC-1 resulted in the highest astaxanthin yield.

High biomasses and astaxanthin concentration per cell are essential for cost-efficient production of astaxanthin in microalgae. Expenses forharvesting and purification procedures significantly reduce astaxanthin productionprofit. These costs could be avoided if cells would release astaxanthin directly into the culture medium. Membrane transporter permeable for carotenoids have already been demonstrated inE. coli (Doshi et al. 2013). These proteins could potentially also work inSynechococcus sp. PCC7002. Their function would bypass the cost-intensive step of harvestingand at the same time, deplete the cell from possible end-product inhibition in astaxanthin synthesis. As a final step extraction of astaxanthin from culture medium could be achieved from the culture supernatant.

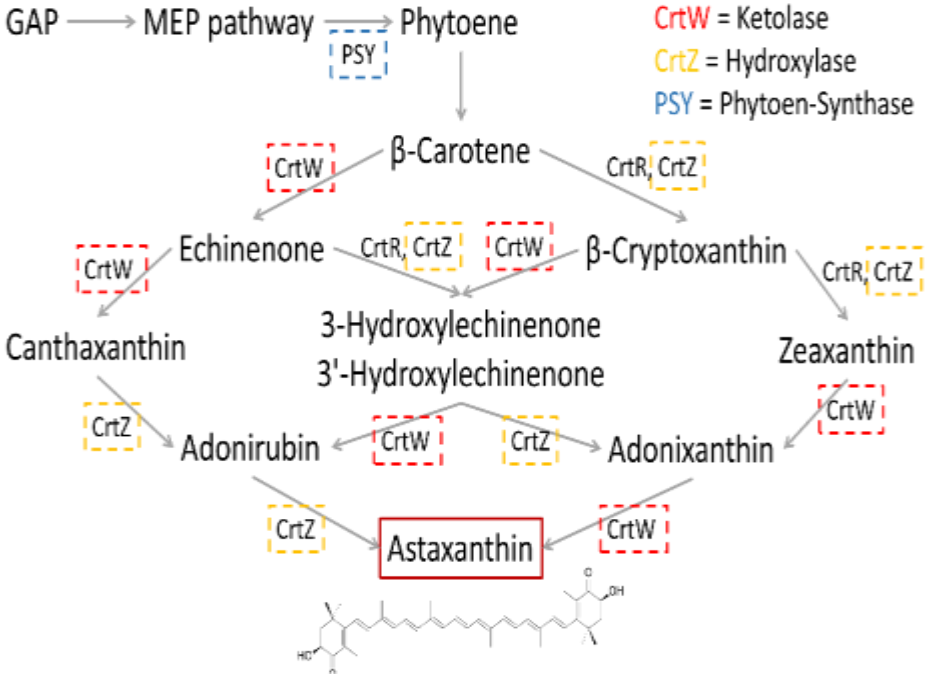

Figure 1:- Carotenoid pathway of Synechococcus sp. PCC7002. Carotenoids and synthesis-enzymes are given. All enzymes framed by dashed line were overexpressed respectivelyintroduced enzymes.

Results and Discussion:-

Synechococcus sp. PCC7002 requires additional enzymes forastaxanthinsynthesis. As indicated by the known metabolism, a β-carotene hydroxylase (CrtZ) and a β-carotene ketolase (CrtW) should be sufficient. Initially, the genes from Brevundimonas sp. were used as described by (Hasunuma et al. 2019). For this purpose, the respective genes were integrated in neutral site 2 and the resulting carotenoid composition was analysed by HPLC. We obtained unexpected results. Expression of β-carotene ketolase (CrtWBrev.) resulted in effective conversion of β-carotene to canthaxanthin. Canthaxanthin yield exceeded that of the β-carotene in comparison to controls (Fig. 2, red vs grey). No activity could be detected for the respective β-carotene hydroxylase (CrtZBrev.). Also, in an E. coli β-carotene-producing system, that we used for enzyme activity inspection,CrtZBrev activity was low. Insteadof

CrtZBrev., β-carotene hydroxylase based onAlcaligenes sp. PC-1 (CrtZA-PC-1) was introduced. This enzyme effectivelyconverted β-carotene to zeaxanthin as indicated by HPLC analysis (Fig. 2).

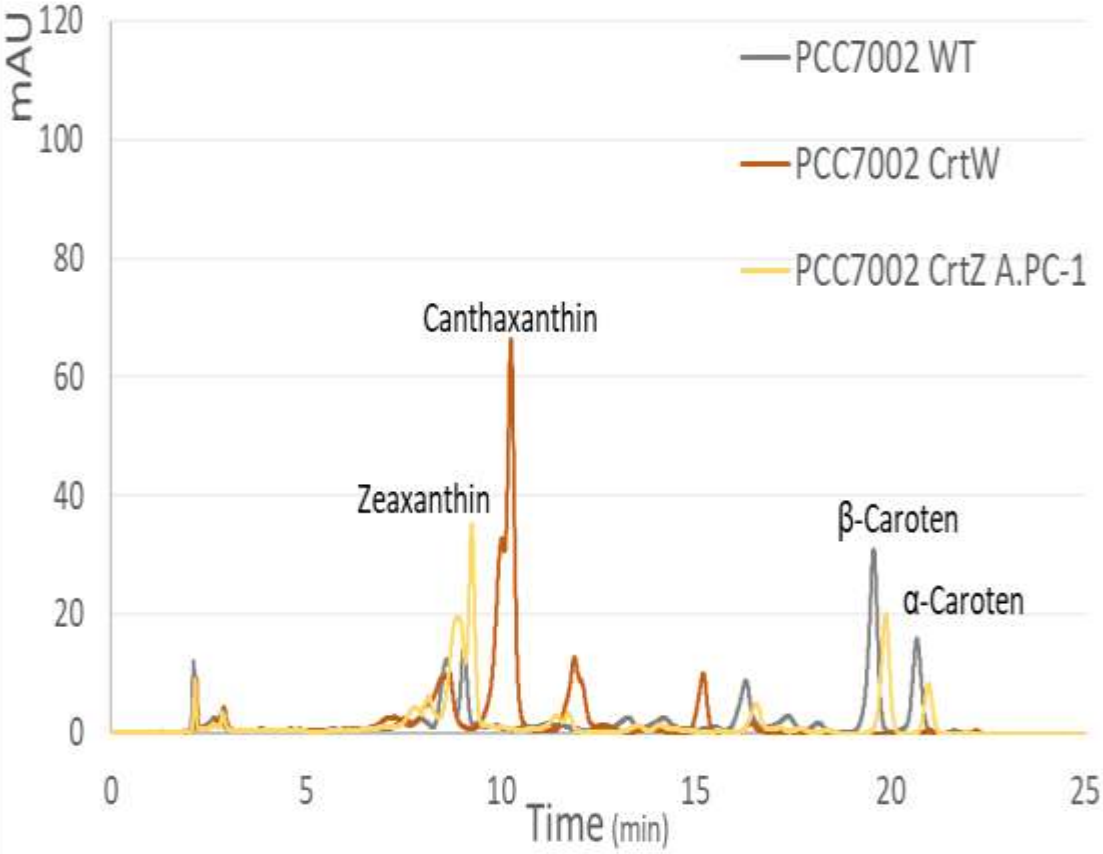

Figure 2:- HPLC-analysis of carotenoid extracts from Synechococcus sp. PCC7002. Control (grey), a modified strain with a synthetic β-carotene ketolase based onBrevundimonas sp. gene (red) and a modified strain with a synthetic β-carotene hydroxylase based onAlcaligenes sp. PC-1gene(yellow). Carotenoid species were indicated at HPLC-Peaks (n=6).

In Saccharomyces cerevisiae,Alcaligenes sp. PC-1 hydroxylase in combination with Brevundimonas sp. β-carotene ketolase produced highest astaxanthin yields (Wang et al. 2017). When hydroxylase and ketolase based onBrevundimonas sp.sequences were present, only the conversion of β-carotene to canthaxanthin could be detected. This was observed irrespective of genomic integration site, promotoras well aswhether the two genes were expressed in combination or separately. Using ketolase derived from Brevundimonas sp.sequences and hydroxylase from Alcaligenes sp. PC-1 (NSIICrtWCrtZ)significant conversion to adonirubin and adonixanthin,the two direct precursors of astaxanthin,was achieved besides small amounts of astaxanthin(Fig.3A). We also noted amounts of β-carotene and α-carotene. In strains with ketolase-genesonly, β-carotene was entirely converted to canthaxanthin.

It is assumed that three genome copies are present in aSynechococcus sp. PCC7002 cell, while the copy number of the endogenous plasmid pAQI is approx. 50. Thus, integration in pAQI should result in increased expression in comparison to genome integration (Pappert et al. 2023). To further increase astaxanthin production the genes were integrated in the plasmid either as a combined genes construct or separately,each gene with its own promotor (pAQICrtWCrtZ).HPLC analysis revealed highest astaxanthin content for the latter. The yield was 1.2±0.13 mg/g dry cell weight (Fig. 3B, n=6).

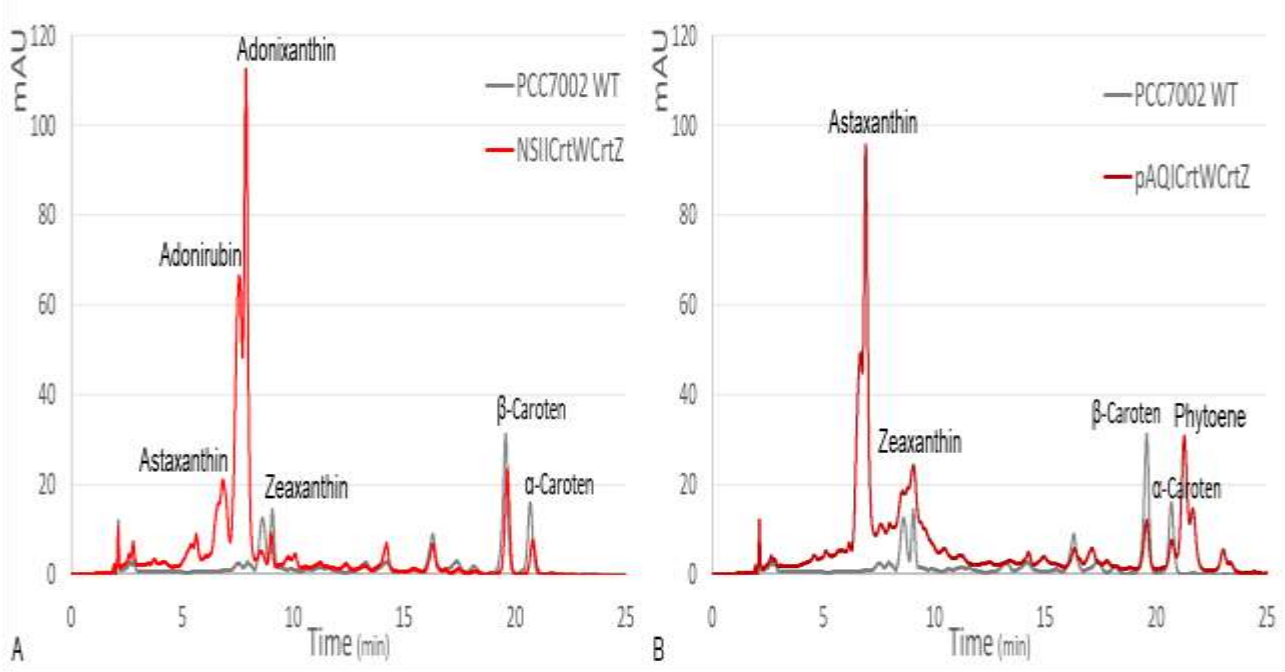

Figure 3:- Results of combined ketolase and hydroxylase in different neutral integration sites. A) controls(grey), modified strain (red) with ketolase and hydroxylase in neutral site 2. B) controls(grey), modified strain (dark red), with both genes in endogen plasmid pAQI.

Our results indicate that hydroxylase activity did not present a pathway bottleneck any more when genes were expressed from plasmid. To further increase the desired product, we added an additional phytoene-synthase (PSY) gene to neutral integration site 2 or 1 under control of the psbAI promoter to enable PSY enzyme overexpression. HPLC analysis revealed an increase in α- and β-carotene concentration irrespective of integration site. Neutral site 1 integration (NSIPSY) resulted in three-fold carotene concentration increase and a five-fold increase was detected for neutral site 2 (NSIIPSY).Consequently, the concentration of astaxanthin per cell was further increased(Fig. 4b).In comparison, 1.71±0.32 mg astaxanthin per g dry cell weight (DCW)was obtained inastaxanthin-forming strains without additional PSY (CrtWCrtZ 4)and 2.38±0.17 mg astaxanthin per g dry cell weight inastaxanthin-producing strains with additional phytoene synthesis (CrtWCrtZPSY 3).Furthermore, an increase in the concentration of canthaxanthin and carotene was observed in these strains.

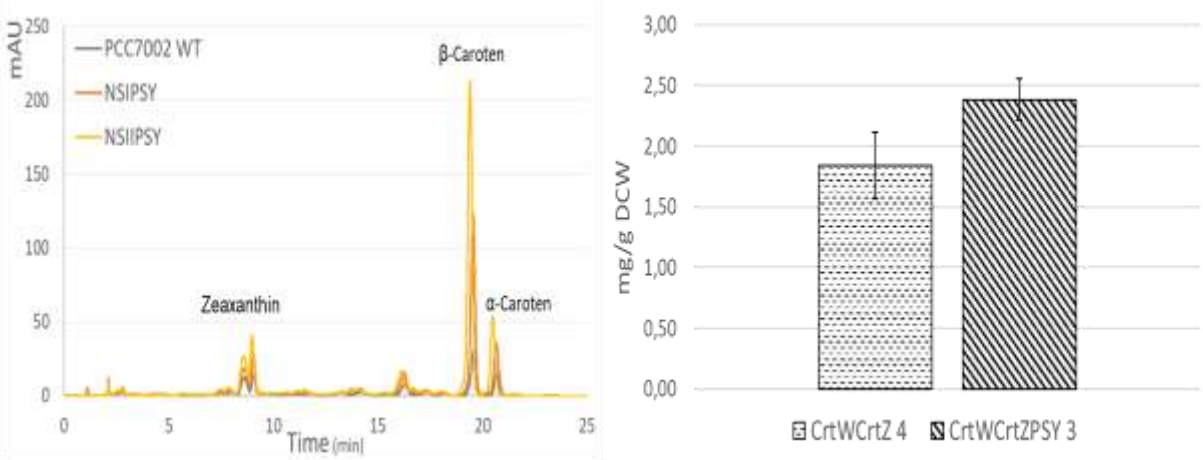

Figure 4:-Overexpression of phytoene synthase in Synechococcus sp. PCC7002. A) controls (grey), modified strain (orange) with phytoene synthase in neutral site 1and modified strain (yellow)with phytoene synthase in neutral site

2.B) chart shows the exploit of astaxanthin in mg per g dry cell weight (DCW).From an astaxanthin forming strain (left dotted column) and from a strain with additional PSY (rightdashed column).

During the transformations, it became apparent that strains modified with the same vector performed differently in terms of both growth rate and carotenoid yield. Therefore, relative integration number (rI number) was determined by PCR-analysis. This indicates for the ratio of unmodified neutral integration sites to sites with inserted target genes. After selection with triple antibiotic boosting, the relative integration number was between 0.7 and 2.5. A relative integration number of 1 would indicate that half of the genome copies were altered, and the other half not. Relative integration numbers coincided with astaxanthin yields. I. e. astaxanthin yield was increased with a higher rI number. Consequently, strains were subjected to stronger selection. Positive strains were plated out by four additional selection series. Resultingindividual colonies were transferred to new liquid cultures. Concentration of antibiotics was doubled in each step, from 100 µg/ml to 800 µg/mL.Final PCR analysis revealed rl values of 1.8 to 5.1 (genome integration) or3.6 to 63.1 (pAQ1) andconfirmed the expected increasein all selected strains (Fig. 5). Comparison of PCR products show that during the extended selectionprocess genomes withNSII sites lacking gene integration were reduced or disappear.Strains with an increased rI number showed increased astaxanthin concentrations (Fig. 5B). For strain pAQICrtWCrtZastaxanthin yield was 1.2±0.13 mg/g dry cell weight and after the fourth round of selection it was 1.84±0.27 mg/g dry cell weight. For strain pAQICrtWCrtZPSY, an astaxanthin yield of 1.33±0.14 mg/g was detected prior to the extended selection and after fourth round of extended selection, the yield was 2.38±1.7 mg/g dry cell weight. Thus, further selection improved astaxanthincontent by 52% respectively, 78 %.

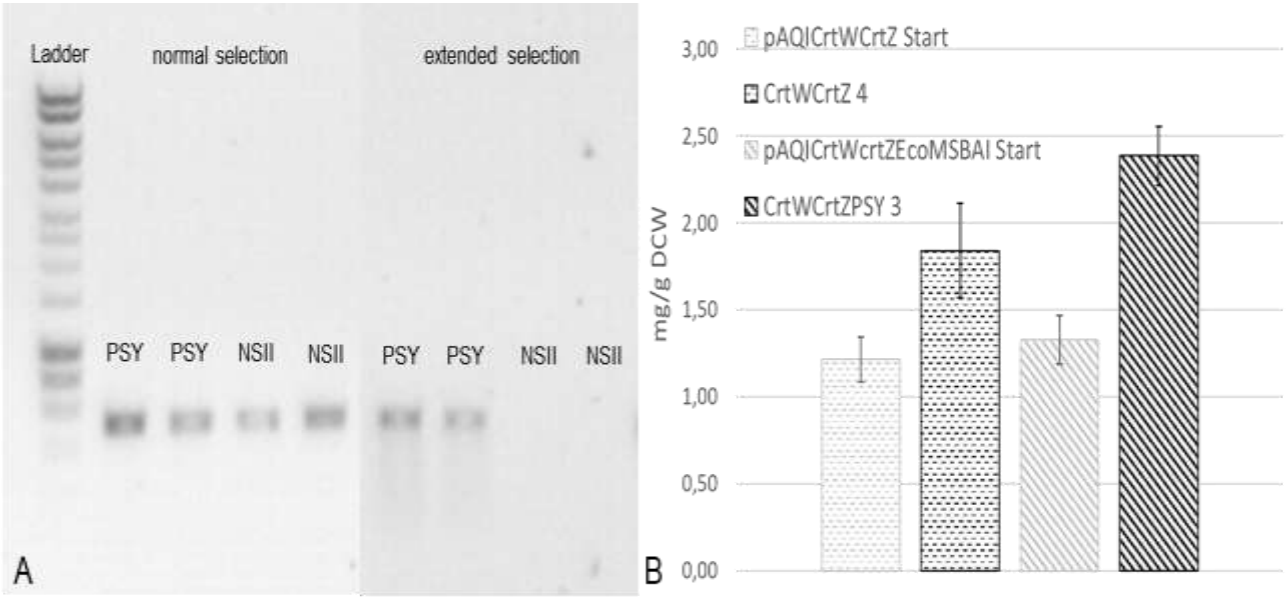

Figure 5:- Relative integration number. A)agarose gel of PCR productsafter PSY transformation and selection or extendedselection. B) astaxanthin yield of a representative astaxanthinproducing strain (CrtWCrtZ 4, left dotted columns) and a strain with additional PSY (CrtWCrtZPSY 3,dashedright columns. Grey columns: selection, black columns: extended selection.

Another step to boostastaxanthin production would be to eliminate the end-productfrom reactions. Employing a suitable transporter to withdrawintracellular astaxanthinto the outside could be a feasible way. We chose ABC transporter MsbA1 from E. coli K-12(Doshi et al. 2013)and expressed a codon optimized gene under cpc550 promoter. As a result, we obtained no transformed bacteria and assumed that the transporter proteins in transformed cells were lethal. Driving transporter gene expression by atheophylin inducible promoter provided transformed cells but did not lead to detectable expression of the transporter after promotor induction.By using the psbA1 promoter and integrating the gene for the ABC transporter into the NSII, strains showed slower growth, but also a function of the ABC transporter: Liquid cultures of astaxanthin-producing strains (Strain A) and strains with an additional ABC transporter (Strain AT) were overlaid using dodecane. The cultures were inoculated from pre-cultures to an optical density$_{730nm}$ of 0.5 and cultivated for three days without overcoating. After overcoating with dodecane, samples were

taken after 5 or 11 days. For sampling, dodecane supernatant was removed, carotenoids were extracted,and the yield determinedby HPLC analysis. In addition, the carotenoid yield from cell extracts was measured. Two carotenoids could be detected in the supernatant for the ABC transporterstrain (Fig. 6),astaxanthinin the main HPLC peakand a minoramount of zeaxanthin. After five days overlay,astaxanthin yield was in average 0.22±0.065 µg/ml. After another five days overlay (14 days of cultivation), yield was increased to 0.45±0.11 µg/mL (Fig. 6B). A strain without ABC transporter does not significantly extrude astaxanthin (0.02µg/mL irrespective of overlay time).

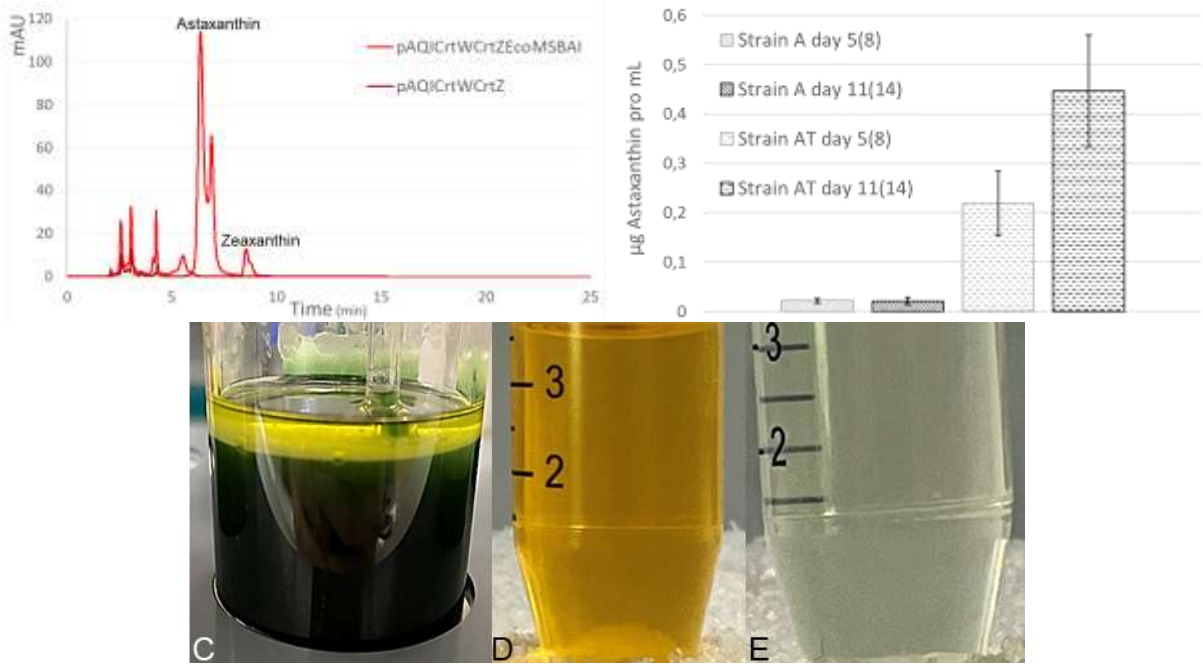

Figure 6:- Astaxanthin in culture overlay. A) HPLC-chromatogram of dodecane overlayer (eight days overlay) strain A in dark red and strain AT in red. B)Quantity of dodecane overlayer astaxanthin exploit.C)Strain AT with dodecane overlayer (eight days overlay).d)Removed dodecane overlayer from strain AT.E) Dodecane overlay of strain A.

Conclusion and Outlook:-

An optimized synthesis of astaxanthin in the blue algaeSynechococcus sp. PCC7002was achieved by stepwise improvement. The use of synthetic genes encoding the protein sequence bya codon usage suitable for the specific microalgae made efficient enzyme synthesis possible. Non optimized sequences did not lead to significant increase in respective products. It was apparent that the choice of enzyme sequences is crucial for successful astaxanthin synthesis. If enzyme sequences from Brevundimonas sp.were used as template for the synthetic genes, no significant astaxanthin synthesis was observed. This is in contrast toresults of Hasunumaet al. (Hasunuma et al. 2019)who observed significant amounts of astaxanthin.However,the genes were targeted to the endogenous plasmid in contrast to our approachwhere NSII in the genome is target site.Therefore,the gene copy number was comparably lower (5 versus 50), and the enzyme was less abundant, which could explain the observed difference in astaxanthin synthesis.By ourapproach, we were able to evaluate the effect of different enzymes. When using Brevundimonas astaxanthin precursors accumulated. Target substance was present to just minor amounts.It turns out that a combination of gene sequences originating from Brevundimonasor Alcaligenes was most effective. This effect has been described elsewhere for yeast (Wang et al.2017) and we could reproduce it in Synechococcus. The results suggest the existence of a preferred direction of the synthesis pathway (Fig.1). When the astaxanthin precursors were enriched, the intermediates formed by the reaction of β-carotene with ketolase were always also enriched. Canthaxanthin was found in higher concentration than zeaxanthin, as well as adonirubin compared to adonixanthin. Furthermore, we could show thatoverexpression of phytoene synthase removed one of the synthesis bottlenecks. Accordingly, the concentration of β-carotene and astaxanthin increased. Overexpression of phytoene synthase increased astaxanthin content, but significant amounts of phytoene were also detected in the cells. This suggests that the conversion of phytoene into β-carotene by additionalphytoene desaturasemight increaseastaxanthin respectively carotene synthesis even further. Another reason for non-maximal synthesis could also be end-product inhibition

caused by intracellular accumulation of synthesis products. A transporter exporting astaxanthin into themediumcould also reduce end-product concentration. It would havethe additional beneficial effect thatthe cells don't have to be disrupted and astaxanthin could be harvested directly from growth medium. Doshi et al. (Doshi et al.2013)demonstrated the function ofMsbafrom E. coli and homologuesin E.coliforcarotenoid export. We used the ABC-transporter sequence as template for a syntheticSynechococcus gene.The successful expression leads to no reduction in intracellular astaxanthin concentrations. In addition, we observed a significant efflux of astaxanthin into the growth medium. This suggests that astaxanthin synthesis is atoptimum and, as expected, inhibited by end-product accumulation.Highly pure astaxanthin could becollectedwithoutharvesting of biomass either by use of organic solvent or directly. The system would enable a constant permanent production.

Materials and Methods:-

Culture Conditions

Synechococcus sp. PCC7002 was cultivated using A^+ medium, as described by Stevens et al. 1973. Pre-cultures were inoculated from cryostocks or A^+ agar plates. A thawed cryostock was added to 50 mL of A^+ medium or a colony from an A^+ agar plate was dissolved in the liquid medium using an inoculation loop. The pre-cultures were cultivated at 30°C and 100 µmol/s/m^2 white light 6500k. For experiments, 200 mL cultures were inoculated from the pre-cultures (OD_{730} 0.1). The cultures were maintained at 30°C and exposed to white light (300 µmol/s/$m^2$6500k) with a light/dark cycle of 16/8 hours. All cultures were grown in bubble column reactors and continuously supplied with 1% CO_2.

Vector Construction and Strain Transformation

The synthetic genes were cloned downstream with cpc560 or psbA1 promoter. The expression cassette and antibiotic selection marker were flanked by the upstream and downstream homologous regions for the respective neutral integration sites. Selection markers used were either kanamycin or spectinomycin. Figure XY displays the constructed vectors. The list of primers used can be found in the Supplementary table. The vectors were produced from linear PCR fragments using In-Fusion Snap Assembly master mix (TaKaRa). All vectors were verified for accuracy by PCR and the expression cassette was also sequenced (Microsynth AG). Genes for cloningand transformation were synthesised by BioCat and codon optimised using the codon optimiser from IDT (Integrated DNA Technologies) or the codon table from kazusa.or.jp.

Cultures were harvested during the exponential phase and adjusted to an OD730 of 3.5. Next, 1 µg of plasmid was added to 400 µL of cell suspension, and the mixture was incubated for 5 hours at 37°C in the dark. After incubation, the samples were transferred to A^+ agar plates and left for 38 hours at 30°C and 100 µmol/s/m^2 white light. Following the recovery period, the respective antibiotic was added to plates, and incubated at 30°C and 100 µmol/s/m^2 white light for 10 days. The obtained colonies were dissolved in 25 µL water and then transferred to selection plates with a higher concentration of antibiotics.

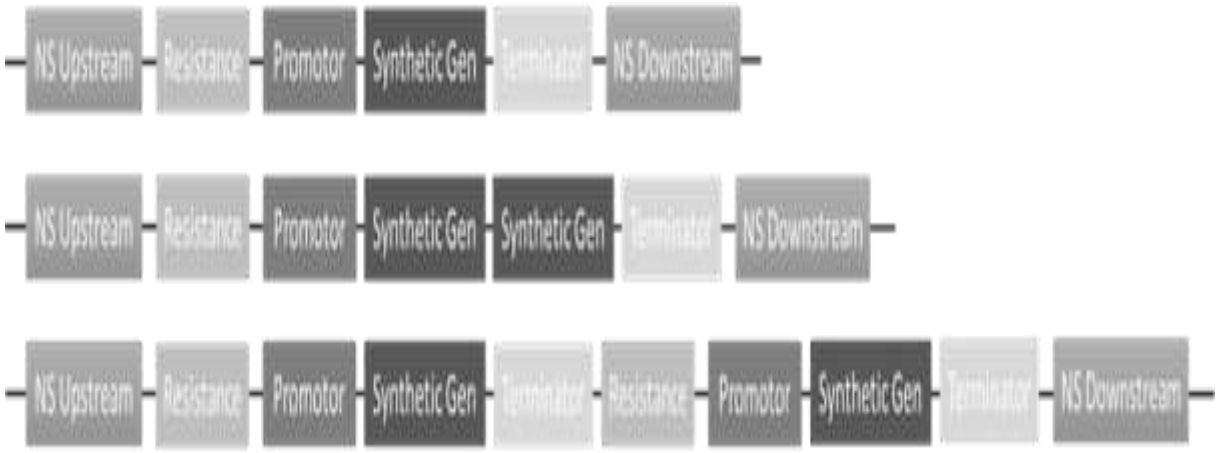

Figure 7:- Schematic representation of the integration cassettes. At the beginning and end the homologous recombination sites (NS Upstream/Downstream) are given. In between the elements that are integrated into the genome or endogenous plasmid pAQIare depicted. Resistance, Spectinomycin or Kanamycin resistance cassette;

Promotor, psbAI or cpc560 promotor; Synthetic Gen, β-carotene-ketolase, β-carotene-hydroxylase or Phytoen-Synthase; Terminator, TerOOP or rbcs terminator sequence

Carotenoid-Analysis

To analyse carotenoids, we conducted HPLC or HPLC-MS analyses. Initially, 50 ml of the culture was centrifuged, and the resulting pellet washed twice with ddH_2O. After lyophilisation, we weighed the same amount of dry weight from the dried cell mass and dissolved it in a mixture of methanol, chloroform, and water (10:3:1). To disrupt the cells, glass beads (0.5 mm) were added. The suspensions were treated twice for 30 seconds at 7 m/s in a BeadBlaster Microtube Homogeniser (Biozym). Carotenoid were chloroform extracted andsaponificated. To achieve this, extracts were dissolved in 300 µL of chloroform and mixed with 300 µL of 2% KOH-methanol. The solution was incubated on ice for 3 hours. 600 µL of 10% NaCl solution was added, and the mixture was centrifuged (2 min, 10k RPM) for phase separation. The chloroform phase was transferred to a new tube after 2 x wash with 10% NaCl solution. The carotenoid extracts were analysed using an Agilent 1100 series HPLC with a YMC carotenoid column. The running agents were obtained from the manufacturer and the gradient was run for 40 minutes.

Organic solvent overcoating

To analyse the functionality of the ABC transporter, 50 mL Synechococcus sp. PCC7002 cultures were inoculated in 200 mL Erlenmeyer flasks OD_{730} 0.5. The cultures were incubated for three days at 30°C and 100 µmol/s/m2 white light under 120 RPM rotation. Subsequently 5 mL dodecane were used as overlay and it was incubated for a further five days. After five days, the dodecane supernatants were removed and replaced with new dodecane, which was removed after another five days. Dodecane samples were evaporated using a SpedVac and the remaining carotenoids were dissolved in 50 µL chloroform.

Determination of relative integration number by PCR

To determine whether the target gene was correctly integrated into the genome or the endogenous plasmid pAQ1 of Synechococcus sp. PCC7002, colonies were analysed using PCR to detect the presence of both the original DNA sequence and the target gene sequence. The Taq DNA polymerase from Biozym was used according to the manufacturer's protocol. The results were then used to determine the relative integration number (rl number). To achieve this, we analysed the agarose gel images using ImageJ and compared the band intensities. We set the intensity of the band of the original DNA sequence as one and categorised the intensity of the band of the target gene relative to it.

Associated content

TheSupportingInformationisavailablefreeofchargeat

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