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Full Paper

Astaxanthin production and expression of carotenogenic genes in a hyperproducing mutant of *Xanthophyllomyces dendrorhous*

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Abstract: Astaxanthin is produced by *Xanthophyllomyces dendrorhous* as its principle carotenoid but the production in the wild type strain is relatively low. A stable astaxanthin-hyperproducing strain, M34, was obtained in our previous work from N-methyl-N'-nitro-N-nitrosoguanidin mutagenesis. In this study the wild type and M34 strains were compared in terms of growth and carotenoid production. The expression levels and nucleotide sequences of carotenogenic genes *idi*, *crtE, crtYB*, *crtI*, *crtR* and *crtS* of both strains were studied by real-time polymerase chain reaction and polymerase chain reaction respectively. Gene expression results indicated that both *crtE* and *crtS* genes showed significantly higher expressions relative to the wild type throughout the production cycle, the highest being 1.3-fold and 3.8-fold respectively. The six carotenogenic genes exhibited a total of 38 nucleotide changes after mutation, including a missense mutation in *idi* gene (glutamic acid→alanine) and *crtI* gene (histidine→glutamine), and two missense mutations in *crtE* (lysine→arginine; serine→aspartic acid) and *crtR* genes (serine→valine; glutamine→proline).

Keywords: astaxanthin, carotenogenic genes, hyperproducing mutant, nucleotide sequences, *Xanthophyllomyces dendrorhous*

INTRODUCTION

Astaxanthin is a valuable red xanthophyll pigment used in the pharmaceutical and aquaculture industries owing to its potent antioxidant and pigmentation properties [1]. The chemical synthesis of astaxanthin is undesirable due to its dependence on petrochemicals, which are not

environment friendly, and involves a long series of chemical steps. Besides, strict regulations on the safety of synthetic chemicals as food additives and the poor absorption of synthetic astaxanthin may give preference to natural astaxanthin and have already led to the alternative microbial production of astaxanthin [2].

Xanthophyllomyces dendrorhous, a basidiomycetous yeast, has desirable properties and commercial value as it produces astaxanthin as the principle carotenoid, and exhibits rapid metabolism, high cell density and mature fermentation process [3]. However, it has a relatively low content of astaxanthin in the wild type strains. Various approaches have been attempted to improve the astaxanthin yield, including the modification of the biosynthetic pathway through classic random mutagenesis and genetic engineering. Previous studies suggested that an alteration in the mRNA transcription level of genes involved in carotenoid biosynthesis might increase pigment production [4, 5]. However, astaxanthin biosynthesis is a complex process and detailed information on the regulation of the carotenogenic pathway remains unknown. There are only few reports on the genes or enzymes that mediate astaxanthin metabolism, especially the carotenogenic genes.

In our previous study a stable *X. dendrorhous* mutant with a high astaxanthin production capacity had been successfully isolated by N-methyl-N'-nitro-N-nitrosoguanidin (MNNG) chemical mutagenesis [6]. The astaxanthin-hyperproducing mutant, M34, had a 111% increase in total carotenoid content compared to the wild-type strain. In the present study the wild type and M34 strains are compared in terms of growth and carotenoid production. In the novel mutant the expression of six carotenogenic genes involved in the biosynthesis of carotenoids, from the formation of isopentenyl to astaxanthin, is elucidated. Possible mutations in the carotenogenic genes are also characterised to study the effect of mutagenesis on the nucleotide sequences of carotenogenic genes in the mutant.

MATERIALS AND METHODS

Yeast Strain and Culture Conditions

X. dendrorhous DSM5626 from Deutche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) was employed as the wild type strain and the astaxanthin hyperproducing M34 mutant strain was generated from mutagenesis of the parental strain by MNNG treatment [6]. The strains were cultivated in yeast malt medium (1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone) at 20°C and 200 rpm.

Growth and Carotenoid Production Profile

For the cell growth determination of both the wild type and mutant strains, a standard curve (dry cell weight vs optical density (OD)) was constructed. Biomass-to-OD_{600} correlation for *X. dendrorhous* was determined from biomass combined from 50-mL shake flask cultures. Cells were harvested by centrifugation (6000 rpm, 5 min., 4°C), resuspended in water and centrifuged again, and a series of dilutions was prepared in pre-weighed 50-mL polypropylene centrifuge tubes which had been dried at 100°C for one week before pre-weighing. Tubes were dried at 100°C for one week and weighed on an analytical balance with accuracy of 0.1 mg. Tubes were dried and weighed again to confirm stability.

Total carotenoid content was determined spectrophotometrically based on the formula described previously [6]. The measurements of both the OD and total carotenoid content were carried out every 12 hr for the construction of growth curves (dry cell weight vs time) and

carotenoid production curves (total carotenoid vs time). All analyses were performed in triplicate from three independent cultures. The carotenoid production curves were used to determine four-time points, viz. early exponential, late exponential, early stationary and late stationary, for the determination of mRNA levels of the six genes involved in the carotenoid biosynthesis pathway.

RNA Extraction, cDNA Synthesis and Real-Time PCR

The cells from fresh cultures were aliquoted at OD 0.6-1.0 and centrifuged to obtain cell pellets. Total RNA extraction was carried out using a GeneJet RNA purification kit (Thermo Fisher Scientific, USA). The cDNA was synthesised by reverse transcription using a Maxima H-minus cDNA Synthesis kit according to the manufacturer's protocol (Thermo Fisher Scientific, USA). The relative mRNA transcription level analysis of each gene was performed by an Applied Biosystems 7500/7500 Fast Real Time PCR System. The C_T values were normalised using *β-actin* gene as the control. All the oligonucleotides (primers) used in this study are shown in Table 1.

Table 1. List of primers used in this study

Primer name	Sequence (5' → 3')	Target gene
Real-time PCR		
Act_F-RT	CCGCCCTCGTGATTGATAAC	*β-actin*
Act_R-RT	TCACCAACGTAGGAGTCCTT	
Idi_F-RT	TCCGAACCGAAGGACTCAGTTT	*idi*
Idi_R-RT	GGACATCAAGTGGCAGGTCT	
crtE_F-RT	TGTTGGCATGCTACATACCGC	*crtE*
crtE_R-RT	GTTGGGCGAAGCTTGAAGAT	
crtYB_F-RT	TCGCATATTACCAGATCCATCTGA	*crtYB*
crtYB_R-RT	GGATATGTCCATGCGCCATT	
crtI_F-RT	CATCGTGGGATGTGGTATCG	*crtI*
crtI_R-RT	GGCCCCTGATCGAATCGATAA	
crtS_F-RT	ATGGCTCTTGCAGGGTTTGA	*crtS*
crtS_R-RT	TGCTCCATAAGCTCGATCCCAA	
crtR_F-RT	CTGGGAAACAAGACCTACGA	*crtR*
crtR_R-RT	CTGGGAAACAAGACCTACGA	
Conventional PCR		
PCR_idi_F1	TTACCTGCTTCTCTTTCCTC	Upstream *idi* gene
PCR_idi_F2	GACGAGAAGATTACGTTCC	*idi* gene
PCR_idi_R	CTACATCTTCCAGACTTTGT	Downstream *idi* gene
PCR_crtE_F1	GGTTCGGAAGATACGATGAA	Upstream *crtE* gene
PCR_crtE_F2	AACTACGTCTACTTTCTGGC	*crtE* gene
PCR_crtE_F3	CTTAATCGTACTGGGCACAT	*crtE* gene
PCR_crtE_R	TCACAGAGGGATATCGG	Downstream *crtE* gene
PCR_crtYB_F1	TCATCCCCAACAGATAGAGT	Upstream *crtYB* gene
PCR_crtYB_F2	ACCCATTGAGGAAGCTATGT	*crtYB* gene
PCR_crtYB_F3	CTTTACTTCCTCCTTCGCAC	*crtYB* gene
PCR_crtYB_R	TTACTGCCCTTCCCATCC	Downstream *crtYB*

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Table 1. (Continued)

Primer name	Sequence (5' → 3')	Target gene
		gene
PCR_crtI_F1	TCAAACTTCTCGTTCTTCGT	Upstream *crtI* gene
PCR_crtI_F2	TTCAAGCAGACATTCGAAGA	*crtI* gene
PCR_crtI_F3	GATGCCAGAAACAAGATTGG	*crtI* gene
PCR_crtI_R	TCAGAAAGCAAGAACACCAAC	Downstream *crtI* gene
PCR_crtS_F1	GATCCGATTCTTGCCACCTA	Upstream *crtS* gene
PCR_crtS_F2	GTCGATGTAAAGGATTGGGT	*crtS* gene
PCR_crtS_F3	AGACGAACTTAATGCGTTGC	*crtS* gene
PCR_crtS_R	TCATTCGACCGGCTTGAC	Downstream *crtS* gene
PCR_crtR_F1	ATCTCCTCACTCAGGTCAGTC	Upstream *crtR* gene

Note : F = forward primer, R = reverse primer

PCR Amplification of DNA and Nucleotide Sequence Analysis

The PCRs were performed in a SureCycler 8800 using an i-Taq Plus DNA polymerase kit (iNtRON Biotechnology, Korea), in a final volume of 20 µL containing 2 U of i-Taq Plus DNA polymerase, 2 µL of 10x i-Taq Plus PCR buffer, 2 µL of 2.5 mM dNTPs, 10 pmoles of each forward and reverse primers and 50 ng/µL of genomic DNA template. The primers were designed according to the published sequences of carotenogenic genes from a genomic library of the *X. dendrorhous* wild type strain (DSM 5626) in NCBI GenBank database, with accession numbers: *idi* [KR779659], *crtE* [KR779661], *crtYB* [KR779663], *crtI* [KR779665], *crtS* [KR779667], *crtR* [KR779669]. The isolated carotenogenic genes were sequenced and sequence comparison between wild type strain and mutant strain was carried out using BioEdit software and tools on the NCBI website.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad, USA). The statistical significance was analysed by one-way ANOVA. Experiments were performed in triplicate and repeated two times independently. The data were presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Growth and Carotenoid Production Profile

While growing in the medium with glucose as the carbon source, M34 mutant shows better growth than the wild type at all phases of growth even though their growth patterns are similar (Figure 1). Both strains show the highest cell mass at 84 hr when the wild type strain has a cell mass of 7.18±0.02 mg/mL and M34 mutant achieves 7.63±0.09 mg/mL. There is a significant increase in the carotenoid production at the end of the exponential phase and a maximum is observed at the late stationary phase at 84-90 hr with a carotenoid content of 725.61±0.12 µg/g in the M34 mutant, 2.4-fold higher when compared to 296.21±0.11 µg/g in the wild type strain.

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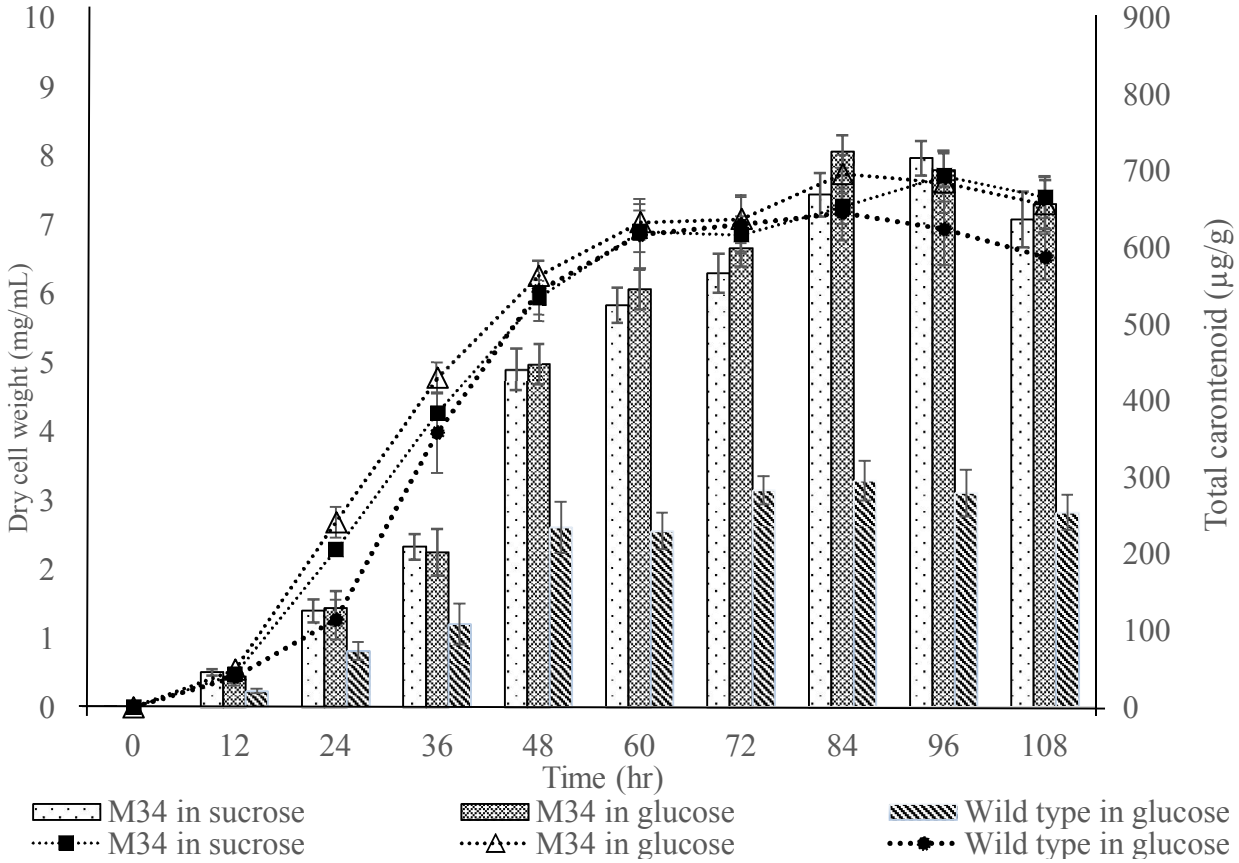

Figure 1. Dry cell weight (points) and total carotenoid production (bars) of M34 mutant of *X. dendrorhous* for 108 hr in media with glucose or sucrose as carbon source in comparison to wild type strain in glucose medium

The induction of carotenoid biosynthesis upon entering the growth stationary phase indicates that the age and lower growth of the culture are associated with the carotenoid production [7, 8]. Carotenoid production in *X. dendrorhous* is usually stimulated by reactive oxygen species which are present naturally in the cells as by-products of oxidative metabolism processes and these by-products accumulate with time as a function of culture age. There was also a suggestion that enzymatic complexes involved in the carotenoid biosynthesis could be assembled during the log phase (before 24 hr), and cellular resources including energy, cofactors and intermediates such as acetyl coenzyme A and isopentenyl pyrophosphate (IPP) could be mostly consumed in the metabolic pathway towards increment in biomass. After the growth rate diminishes, the metabolic intermediates could then be redirected to focus on carotenoid biosynthesis through the previously assembled carotenogenic complexes [7]. It was further explained that during the late exponential phase, the NADH/NAD^+ ratio increases in the cells due to catabolism situation and together with a low energy demand leads to excess energy that inhibits the tricarboxylic acid cycle activity and subsequently contributes to an oversupply of carbon skeletons for isoprenoid biosynthesis via the mevalonate pathway, leading to increment of pigment production in *X. dendrorhous* [9]. Another possible factor is the presence of MADS-box Transcription Factor Mig1, which is a transcription factor that plays a major role in glucose repression in yeast by blocking the transcription of glucose-repressed genes through binding to upstream sequences in the presence of glucose [10]. The biosynthesis genes *crtYB*, *crtI* and *crtS* are glucose-repressed genes that can be found in *X.*

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dendrorhous [11]. Thus, the low expression of carotenogenic genes leads to low carotenogenesis in the presence of glucose and they are only triggered once the sole carbon source, glucose, has been consumed.

The results of this study also show that the growth profiles of the M34 mutant are similar when it is cultivated with sucrose and glucose as carbon sources. Similar to the findings by Fang and Cheng [12], the growth of the M34 mutant with glucose as carbon source is slightly higher than in sucrose medium (Figure 1). The highest cell mass obtained by culturing in glucose is 7.63±0.09. mg/mL at 84 hr while culturing in sucrose achieves the highest cell mass of 7.61±0.02 mg/mL slightly later at 96 hr. The slight delay might be due to the need for sucrose to be broken down into glucose and fructose before it could be used for metabolism processes [13]. The observation suggests that both carbon sources are suitable for the growth of mutant M34. Similarly, M34 culture with glucose as carbon source gives a slightly higher carotenoid production with the highest value of 725.61±0.12 µg/g at 84 hr while for culture in sucrose, the highest carotenoid production of 717.12±0.08 µg/g is achieved slightly later at 96 hr. The small difference in carotenoid yield with different carbon sources could be due to the deregulation of carotenoid biosynthesis processes and hence the insensitivity to changes in environmental factors such as culture medium [14]. However, overall, carotenoid production in the M34 culture with either glucose or sucrose as carbon source still outpaces that by the wild type culture.

RNA Extraction, cDNA Synthesis and Real-Time PCR

Based on the carotenoid production curve, total RNA of both the wild type and mutant M34 were extracted and quantified from cell pellets after 24 (early exponential), 40 (late exponential), 60 (early stationary) and 90 (late stationary) hr of cultivation in defined medium with glucose as carbon source. cDNA pool obtained from reverse transcription was used for determination of the transcript levels of target genes (*idi*, *crtE*, *crtYB*, *crtI*, *crtS*, *crtR*) in astaxanthin biosynthetic pathway in *X. dendrorhous* (Figure 2). Figure 3 shows results obtained from Real-Time PCR on the relative expression of target genes in the M34 mutant and wild type strain.

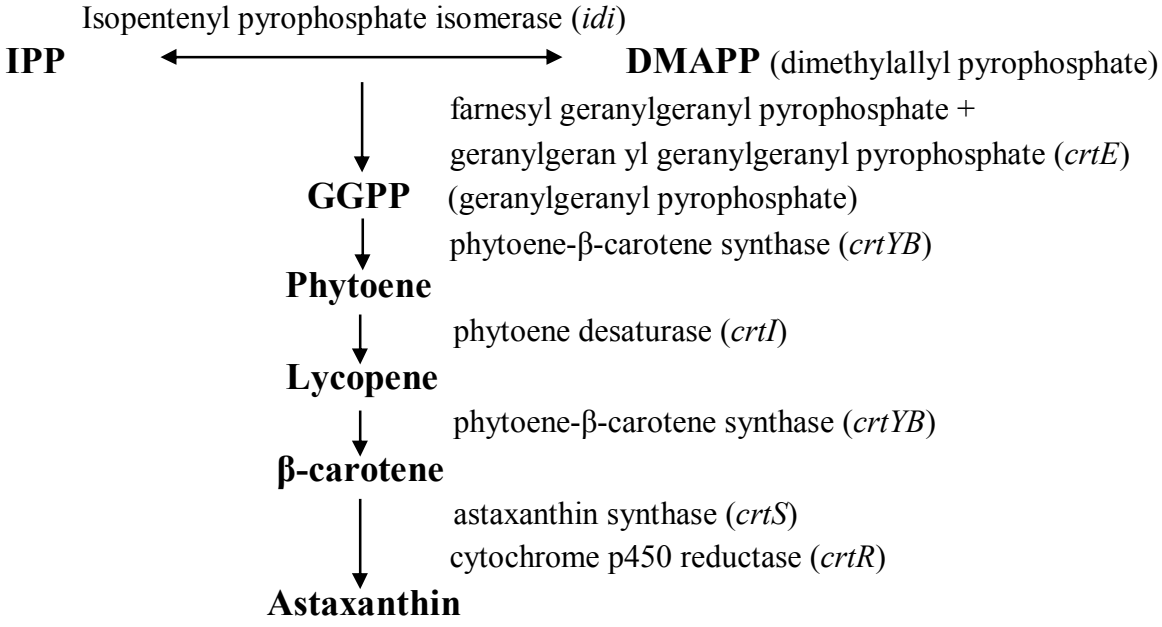

Figure 2. Modified astaxanthin biosynthetic pathway in *X. dendrorhous* [15]

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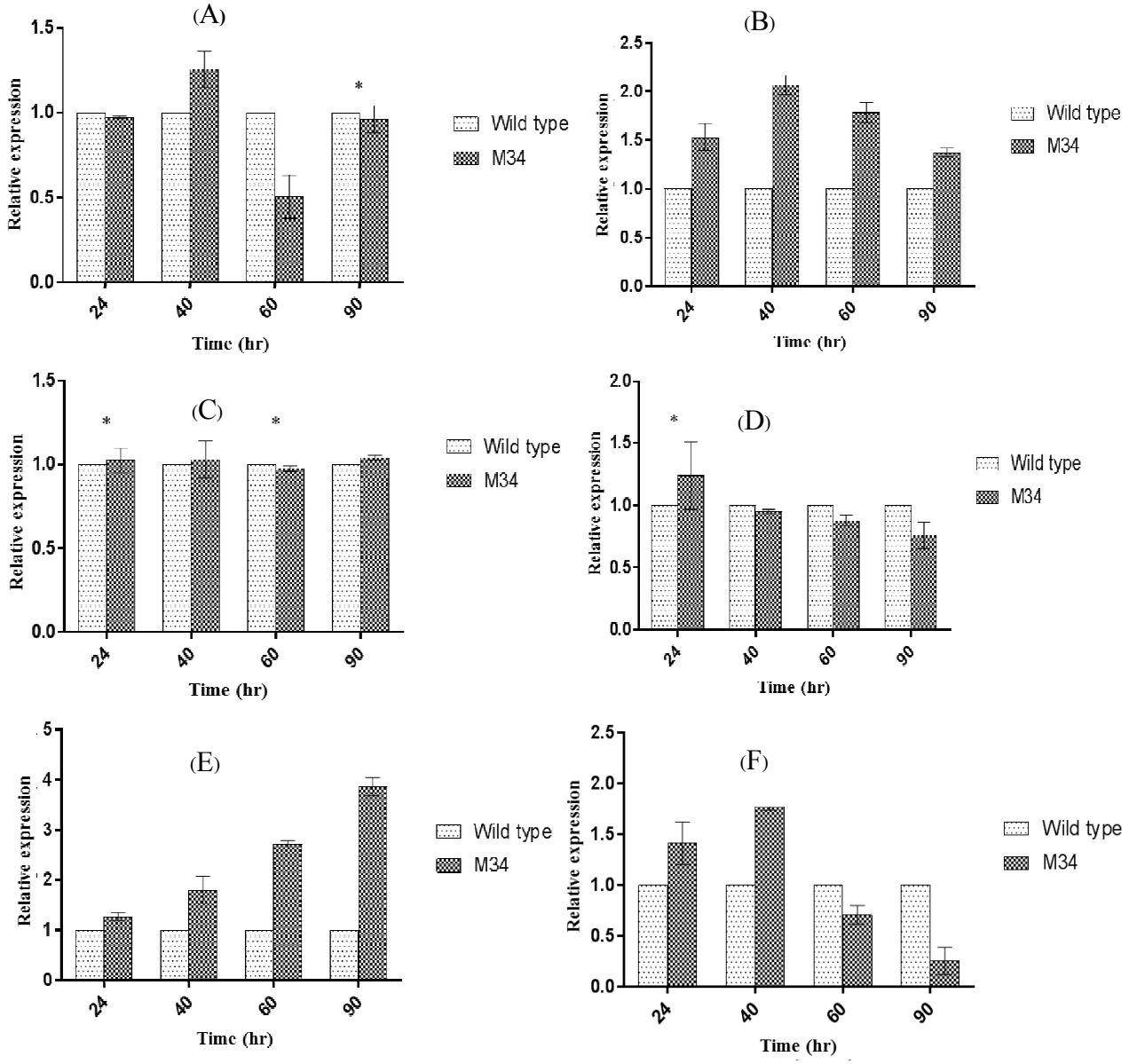

Figure 3. Real-Time PCR analysis of six carotenoid biosynthesis genes: (A) *idi*; (B) *crtE*; (C) *crtYB*; (D) *crtI*; (E) *crtS*; (F) *crtR* in wild type and M34 strains of *X. dendrorhous* grown in medium with glucose as carbon source. Each transcript level is normalised with respect to transcript level of *β-actin* gene and then to wild type strain (=1). Data are shown as mean ± SD from triplicate cultures. Asterisk indicates $p>0.05$.

Figure 3(A) shows that there is lack of distinct pattern in the expression of *idi* gene in both the wild type strain and M34 mutant of *X. dendrorhous* throughout the carotenoid production cycle. The lowest value is shown when the total carotenoid content increases in the early stationary phase, while at the end of the stationary phase there is lack of significant differences between the strains. A decrease in *idi* expression during the stationary phase has also been reported previously in other carotenoid-overproducing *X. dendrorhous* mutants [7, 16]. Visser et al. [17] reported that when the isopentenyl pyrophosphate isomerase encoding gene (*idi*) is overexpressed in *X. dendrorhous*, the astaxanthin content and total carotenoid production decrease, suggesting that the alteration in *idi* gene expression is not associated with the changes in carotenoid production. The *idi* gene is present in the early steps of astaxanthin biosynthetic pathway in *X. dendrorhous*. This may explain the

increase in *idi* mRNA level in the late exponential phase when astaxanthin is actively accumulated. However, this gene is not exclusive to the carotenogenic pathway, but is also involved in other metabolic processes necessary for yeast survival. This may explain the high *idi* expression level in late stationary phase as it might play a role in yeast survival in this phase.

For *crtE* gene expression, the M34 mutant shows an increment from early to late exponential phase and starts to decrease when entering the stationary phase. The expression in the M34 mutant is 1.4- to 2-fold higher compared to the wild type strain, especially during the late exponential phase where a 2-fold expression is observed (Figure 3(B)), suggesting a direct involvement of *crtE* in the higher astaxanthin production in M34 mutant. The product of *crtE* gene catalyses the conversion of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) to geranylgeranyl pyrophosphate (GGPP), which is involved in the initial stage of carotenoid synthesis. This result is in agreement with the study of Breitenbach et al. [18] and Lodato et al. [7], in which the overexpression of *crtE* and the presence of GGPP synthase at a high level cause the diversion of metabolite flow from the sterol pathway towards the carotenogenic pathway, leading to a higher carotenoid yield.

The *crtYB* gene encodes for two enzymes in the carotenogenic pathway, namely phytoene synthase which condenses two GGPP molecules to phytoene, and lycopene cyclase which converts lycopene to β-carotene. The expression pattern of *crtYB* gene in M34 mutant is almost the same as in the wild type strain, suggesting that mutagenesis does not affect its efficiency and function (Figure 3(C)). Similarly, Niklitschek et al. [19] reported that *crtYB* expression is not associated with the carotenoid production. In contrast, Gassel et al. [8] and Verdoes et al. [20] found that the addition of *crtYB* gene into a mutant of *X. dendrorhous* results in a higher yield of carotenoids, which largely comprise of β-carotene and some intermediates of ketocarotenoid. They found that the overexpression of *crtYB* gene might lead to an increase in the overall carotenoid production, mainly due to higher amounts of β-carotene and echinenone even though the astaxanthin content remains unaffected or even lower.

The *crtI* gene of M34 strain only shows 1.2-fold higher expression in the early exponential phase, followed by a decrease throughout the growth cycle with an expression level lower than wild type strain when the main carotenoid production occurs (Figure 3(D)). The ratio of mature to alternative mRNA for *crtI* is inversely proportional to the age of the culture, decreasing with the aging of the culture. The mature mRNA of *crtI* gene is translated into the functional protein, phytoene desaturase, while the alternative spliced variants have numerous stop codons in their sequence and thus cannot be translated [21]. This may explain the decrease in the *crtI* expression along the growth cycle. It was reported that the overexpression of *crtI* actually increases the amount of monocyclic carotenoids such as torulene and 3-hydroxy-3′,4′-didehydro-β,-ψ-carotene-4-one, while it decreases the amount of bicyclic carotenoids such as echinenone, β-carotene and astaxanthin [22]. Verdoes et al. [20] suggested that the contest between phytoene desaturase and phytoene synthase for lycopene could be the sticking point in the carotenogenic pathway, where phytoene desaturase diverts the flux from astaxanthin towards 3-hydroxy-3′,4′-didehydro-β,-ψ-carotene-4-one synthesis. The high level of *crtI* gene product could be correlated with a high amount of monocyclic carotenoids. Thus, the decreasing *crtI* expression does correlate with the accumulation of astaxanthin in M34 mutant.

The M34 mutant shows consistent increment in *crtS* gene expression and the mRNA level is significantly higher than for the wild type strain, especially in the late stationary phase, when the expression is 3.8-fold higher (Figure 3(E)). The results indicate that the production of astaxanthin

increases with increasing *crtS* expression. The *crtS* gene encodes for astaxanthin synthase, which is necessary for the catalysation of astaxanthin synthesis from β-carotene in four enzymatic steps through ketolation and hydroxylation [23]. Astaxanthin synthase is a cytochrome P450 hydroxylase-type enzyme and requires a cytochrome P450 reductase (CPR) as redox partner for electron transfer. This astaxanthin-producing cytochrome P450 system is distinctive due to the high specificity of astaxanthin synthase in *X. dendrorhous* towards its redox partner, which is the CPR ecoded by *crtR* gene [24].

The expression of *crtR* in M34 mutant shows up to 2-fold increment in the late exponential phase before decreasing drastically to levels lower than the wild type strain in early and late stationary phases (Figure 3(F)). In *X. dendrorhous*, astaxanthin is synthesised when *crtR* gene, encoding the yeast CPR, coexpresses with *crtS* gene [4, 25]. Although astaxanthin synthase has a high specificity for its own CPR, there is no direct correlation between the mRNA expression patterns of *crtS* and *crtR* genes. Similar findings were reported in which the expression levels of *crtR* gene remain at the same level throughout the growth cycle, despite the increment of *crtS* gene expression level, suggesting the *crtR* gene expression has a different regulatory mechanism due to its participation in other metabolic pathways in *X. dendrorhous* [25-27]. The *crtR* gene expression may be regulated by a different regulatory mechanism as CPR encoded by *crtR* gene acts as an electron donor not only for astaxanthin synthase but also for other cytochrome P450s that have been described in *X. dendrorhous*, such as CYP51 (lanosterol 14-demethylase) and CYP61 (C-22 sterol desaturase) [28, 29].

PCR Amplification of DNA and Nucleotide Sequence Analysis

The results suggest that the transcriptional regulation is only partially involved in the carotenoid production. Hence the sequence analysis of the six carotenoid biosynthesis genes was also carried out to further elucidate the presence of complementary mechanisms for the regulation of carotenoid biosynthesis. The mutagenic process in the *X. dendrorhous* has led to alteration in structural genes involved in the carotenogenic process, which could probably affect the phenotype and astaxanthin yield. Table 2 shows the nucleotide changes found in the carotenoid biosynthesis gene sequences of M34 mutant when compared to the wild type strain.

Table 2. Mutations of carotenoid biosynthesis genes in M34 mutant

Gene	No. of nucleotide changes	Location	Amino acid change
idi	2	1048, 2144	2144 : Glutamic acid → Alanine
crtE	13	892, 1655, 1722,1723, 1748, 1751, 1880, 2262, 2268, 2425, 2428, 2470, 2482	892 : Lysine → Arginine 1722 : Serine → Aspartic acid
crtYB	3	2259, 2541, 3072	-
crtI	1	3178	3178 : Histidine → Glutamine
crtS	1	1859	-
crtR	18	1023, 1565, 1622, 1905, 1986, 2163, 2166, 2248, 2337, 2385, 2445, 2508, 2866, 2867, 3055, 3174, 3298, 3478	2866 : Serine → Valine 3298 : Glutamine → Proline

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The mutations caused by MNNG were reported to be usually point mutations with single-base substitutions and without the presence of deletions, frame shifts or insertions [30]. In this study the same is observed for the MNNG mutagenesis and the base-pair mutations cause either silent mutation or missense mutation. The base-pair mutations consist of 6 missense mutations (5 conservative and 1 non-conservative) that lead to amino-acid substitutions and 32 silent mutations that cause no amino-acid substitutions, and there is no nonsense mutation leading to a change in a stop codon. All genes involved in the astaxanthin biosynthesis show at least one nucleotide change, and only the *crtYB* and *crtS* genes do not show any missense mutation. These changes could provide partial explanation for the different patterns of carotenoid production in the wild type strain and M34 mutant.

Two nucleotide changes occur at locations of 1048 bp (exon 1) and 2144 bp (exon 5) on the DNA sequence of the *idi* gene. The amino acid at position 16 of the *idi* gene undergoes point mutation. This mutation is a silent mutation where there is no change in either the structure or function of the protein. Meanwhile, the amino acid at position 204 in the *idi* gene is converted from glutamic acid to alanine. This mutation occurs at the end of the active site in the *idi* gene. The amino acid is changed from a polar acidic amino acid which is negatively charged to a non-polar amino acid, causing a non-conservative missense mutation. Glutamic acid belongs to the polar group of amino acids which are negatively charged. It tends to be substituted with another amino acid, especially aspartate, which is also negatively charged. Glutamine, which differs only in that it contains an amino group in place of one of the oxygens found in glutamate, is also a good choice for substitution apart from the lack of a negative charge. Due to its charged and polar characteristics, glutamate usually gains its position on the surface of proteins exposed to the aqueous environment. But when the glutamates are buried inside the proteins, stabilising hydrogen bonds are formed between them with positively charged amino acids establishing salt bridges for protein stability [31]. The change of polar to neutral amino acids usually causes the disruption of protein stability and activity. As we can observe from the mRNA expression results in Figure 3(A), the expression of *idi* gene is reduced at 60 hr. This reduction could have resulted from the change of glutamic acid to alanine. The alanine side chain is usually important for recognition of a specific substrate during interactions with other non-reactive atoms like carbon, but is hardly taking part in protein function since it is very non-reactive [31]. Furthermore, there are two conserved domains in the *idi* gene. The first is located at positions 102 to 165 of the *idi* gene, and the second is from positions 64 to 111, where both of them have shown a very high level of conservation, suggesting an important role for enzyme function [32]. Nucleotide changes at locations 1048 bp and 2144 bp on the DNA sequence of the *idi* gene do not occur in these domains; thus, there should not be any effect taking place in the functions of the *idi* gene.

The *crtE* gene undergoes 13 nucleotide changes but only changes at 892 bp (exon 1) and 1722 bp (exon 4) cause missense mutations, converting amino acids from lysine to arginine and serine to aspartic acid. The conversion located at position 31 (lysine to arginine) does not affect any of the known binding sites on *crtE* gene product (GGPP synthase). Since there is no active site in both exon 1 and exon 4, this suggests that the change of lysine to arginine might instead cause some alterations in the protein structure. Both lysine and arginine, positively charged basic amino acids, are usually located at the surface of the protein and exposed to the environment. This allows them to be involved in the maintenance of protein stability through the formation of electrostatic interactions and hydrogen bonds [33, 34]. Compared to lysine, arginine has more advantages from the aspect of stability as a result of its ability to form electrostatic interactions in three directions

through the presence of a guanidinium group that consists of three asymmetrical nitrogen atoms (N^{ε}, $N^{\eta 1}$, $N^{\eta 2}$), while lysine only interacts in one direction. At the same time, a higher pKa due to the presence of basic residues in arginine contributes to the formation of more stable ionic interactions [35]. Sokalingam et al. [36] reported similar findings that the replacement of surface lysine with arginine in a native green fluorescent protein leads to enhancement of its stability against urea, alkaline pH and detergents without affecting the thermal stability and final structure. For the conversion of serine to aspartic acid at position 148 in the *crtE* gene, the conversion occurs at the substrate binding pocket (66 to 269) of the gene. This suggests that the conversion could affect the activity of GGPP protein. Aspartic acid is chemically similar to the phosphorylated serine, so replacement of serine by aspartic acid could lead to phosphomimetics, which causes the protein to be always in its phosphorylated form, which may mutate the protein into a constitutively active state. From observations, the expression of *crtE* gene in mutant M34 is higher compared to that in the wild type strain, so this suggests that the conversion leads to a positive alteration, contributing to a higher yield of astaxanthin.

The expression pattern of *crtYB* gene is almost similar in both the wild type strain and M34 mutant and this observation indicates no consequence of mutation on the gene, which undergoes 3 nucleotide changes at 2259 bp, 2541 bp and 3072 bp (amino acid at position 335, 429 and 606 respectively) on the gene sequence, all located at exon 5, although all of them are silent mutations that do not cause any amino acid changes. Thus, it is in agreement with the mRNA expression results where the expression of both strains is similar.

The only nucleotide change in *crtI* gene happens at location 3178 bp of the gene sequence, causing a transition of histidine to glutamine at exon 12 (amino acid at position 427) in the *crtI* gene product (phytoene desaturase). Histidine is distinctive in its characteristics compared to other polar amino acids, and it cannot really be well replaced by other amino acids [29]. Thus, this might be the main reason why the conversion of histidine to glutamine causes a slight decrease in the expression of *crtI* along the growth cycle. There are two domains found in the *crtI* gene: (NAD(H)/NADP(H) or FAD(H))$^{-}$binding domain located at amino acid positions 13-52 and carotenoid-binding domain located at amino acid positions 486-513 [37]. The mutation does not occur at the main binding domains of the *crtI* gene, thus suggesting that the conversion of histidine to glutamine does not affect the function of the *crtI* gene.

From the sequence analysis, *crtS* only shows the change from codon GAG to codon GAA at location 1859 bp (exon 5) of the gene sequence, but there is no amino acid change since it still encodes glutamic acid expression. In similar findings by Alvarez et al. [38], the astaxanthin overproduction in VKPM Y2410 overproducing mutant could not be explained by the changes in *crtS* gene sequences because none of the changes caused any amino acid substitution.

The *crtR* gene has the highest number of mutations among the carotenoid biosynthesis genes. Among the 18 nucleotide changes, only two nucleotide changes at locations 2866 and 3298 bp (amino acids in positions 540 and 684) on exon 3 cause amino acid changes from serine to valine and glutamine to proline. The first mutation happens between the FAD binding domain and NAD(P)H binding domain and both domains are not affected by the conversion of the amino acid. However, valine and serine are fairly different in their properties. Serine is characterised as an amino acid with slight polarity and is considerably neutral to undergo mutation. As with other polar amino acids, serine tends to be replaced by other polar amino acids or amino acids with low molecular mass. Serine can be found within the interior or on the surface of a protein. On the other hand, valine is an aliphatic hydrophobic amino acid, which is a non-polar type of amino acid.

Generally, conversion of amino acids from polar to non-polar could affect the function and stability of the protein. In addition, β-branched residues such as valine are invariably poor helix formers [39]. The second point mutation, where glutamine changes to proline, occurs at an NAD(P)H binding domain between amino acids 582 and 709. The non-reactive characteristic of the proline side chain, in addition to its hindrance to adopting many protein main-chain configurations, results in rare involvement of proline in the active or binding sites of most proteins [31]. The conversion of glutamine to proline could affect the helix formation of the protein since glutamine is polar while proline is non-polar, which may lead to lower efficiency. These mutations may partly contribute to the lower *crtR* gene expression in M34 mutant in this study.

CONCLUSIONS

In *X. dendrorhous*, the biosynthesis of carotenoids is intricate due to multilevel regulations in controlling the expression of carotenoid biosynthesis genes, the concentration of related proteins and their enzymatic activities and the directing of the flow of metabolic intermediates between varying pathways. By controlling the expression and altering the sequence of specific carotenoid biosynthetic genes, the carotenoid content in *X. dendrorhous* can be modified significantly to produce a specific carotenoid in higher amounts. This indicates that a change in the ratios of carotenogenic enzymes in *X. dendrorhous* by either induced mutations or metabolic engineering may affect the amounts and composition of carotenoids. An integrated study of cell growth, carotenoid biosynthesis, transcriptional regulation on carotenogenic genes and alteration in gene sequences between a wild-type and a mutant *X. dendrorhous* strain help to elucidate the M34 astaxanthin-overproducing phenotype. This study provides a wider basis of knowledge on astaxanthin biosynthetic genes at molecular level although further work is required to contribute towards the development of *X. dendrorhous* as a cell factory for carotenoid production which may be competitive economically with chemical synthesis.

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REFERENCES

1. R. G. Fassett and J. S. Coombes, “Astaxanthin, oxidative stress, inflammation and cardiovascular disease”, *Future Cardiol.*, **2009**, *5*, 333-342.
2. A. L. Demain and E. Martens, “Production of valuable compounds by molds and yeasts”, *J. Antibiot.*, **2017**, *70*, 347-360.
3. G. I. Frengova and D. M. Beshkova, “Carotenoids from *Rhodotorula* and *Phaffia*: Yeasts of biotechnological importance”, *J. Ind. Microbiol. Biotechnol.*, **2009**, *36*, 163-180.
4. K. Ukibe, T. Katsuragi, Y. Tani and H. Takagi, “Efficient screening for astaxanthin-overproducing mutants of the yeast *Xanthophyllomyces dendrorhous* by flow cytometry”, *FEMS Microbiol. Lett.*, **2008**, *286*, 241-248.
5. A. Marcoleta, M. Niklitschek, A. Wozniak, C. Lozano, J. Alcaíno, M. Baeza and V. Cifuentes, “Glucose and ethanol-dependent transcriptional regulation of the astaxanthin biosynthesis pathway in *Xanthophyllomyces dendrorhous*”, *BMC Microbiol.*, **2011**, *11*, Art.no.190.

6. F. S. Ang, S. Y. Khaw, L. L. Few, W. C. See Too and A. L. Chew, "Isolation of a stable astaxanthin-hyperproducing mutant of *Xanthophyllomyces dendrorhous* through random mutagenesis", *Appl. Biochem. Microbiol.*, **2019**, *55*, 255-263.
7. P. Lodato, J. Alcaino, S. Barahona, M. Niklitschek, M. Carmona, A. Wozniak, M. Baeza, A. Jimenez and V. Cifuentes, "Expression of the carotenoid biosynthesis genes in *Xanthophyllomyces dendrorhous*", *Biol. Res.*, **2007**, *40*, 73-84.
8. S. Gassel, H. Schewe, I. Schmidt, J. Schrader and G. Sandmann, "Multiple improvement of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* by a combination of conventional mutagenesis and metabolic pathway engineering", *Biotechnol. Lett.*, **2013**, *35*, 565-569.
9. C. Chavez-Cabrera, Z. R. Flores-Bustamante, R. Marsch, C., M. del C. Montes, S. Sanchez, J. C. Cancino-Diaz and L. B. Flores-Cotera, "ATP-citrate lyase activity and carotenoid production in batch cultures of *Phaffia rhodozyma* under nitrogen-limited and nonlimited conditions", *Appl. Microbiol. Biotechnol.*, **2010**, *85*, 1953-1960.
10. F. Rolland, J. Winderickx and J. M. Thevelein, "Glucose-sensing and -signaling mechanisms in yeast", *FEMS Yeast Res.*, **2002**, *2*, 183-201.
11. A. Wozniak, C. Lozano, S. Barahona, M. Niklitschek, A. Marcoleta, J. Alcaino, D. Sepulveda, M. Baeza and V. Cifuentes, "Differential carotenoid production and gene expression in *Xanthophyllomyces dendrorhous* grown in a nonfermentable carbon source", *FEMS Yeast Res.*, **2011**, *11*, 252-262.
12. T. J. Fang and Y. S. Cheng, "Improvement of astaxanthin production by *Phaffia rhodozyma* through mutation and optimization of culture conditions", *J. Ferment. Bioeng.*, **1993**, *75*, 466-469.
13. J. Jasman, I. D. Prijambada, C. Hidayat and D. Widianto, "Selection of yeast strains for ethanol fermentation of glucose-fructose-sucrose mixture", *Indones. J. Biotechnol.*, **2012**, *17*, 114-120.
14. P. Lodato, J. Alcaino, S. Barahona, P. Retamales, A. Jimenez and V. Cifuentes, "Study of the expression of carotenoid biosynthesis genes in wild-type and deregulated strains of *Xanthophyllomyces dendrorhous* (Ex.: *Phaffia rhodozyma*)", *Biol. Res.*, **2004**, *37*, 83-93.
15. J. Alcaino, N. Bravo, P. Cordova, A. E. Marcoleta, G. Contreras, S. Barahona, D. Sepulveda, M. Fernandez-Lobato, M. Baeza, and V. Cifuentes, "The involvement of Mig1 from *Xanthophyllomyces dendrorhous* in catabolic repression: an active mechanism contributing to the regulation of carotenoid production", *PLoS ONE.*, **2016**, *11*, Art.no.e0162838.
16. L. M. Castelblanco-Matiz, A. Barbachano-Torres, T. Ponce-Noyola, A. C. Ramos-Valdivia, C. M. Cerda Garcia-Rojas, C. M. Flores-Ortiz, S. K. Barahona-Crisostomo, M. E. Baeza-Cancino, J. Alcaino-Gorman and V. H. Cifuentes-Guzman, "Carotenoid production and gene expression in an astaxanthin-overproducing *Xanthophyllomyces dendrorhous* mutant strain", *Arch. Microbiol.*, **2015**, *197*, 1129-1139.
17. H. Visser, G. Sandmann and J. C. Verdoes, "Xanthophylls in fungi: Metabolic engineering of the astaxanthin biosynthetic pathway in *Xanthophyllomyces dendrorhous*", in "Methods in biotechnology Microbial processes and products" (Ed. J. Barredo), Humana Press, Totowa, NJ USA, **2005**, pp. 257–272.
18. J. Breitenbach, H. Visser, J. C. Verdoes, A. J. J. van Ooyen and G. Sandmann, "Engineering of geranylgeranyl pyrophosphate synthase levels and physiological conditions for enhanced

carotenoid and astaxanthin synthesis in *Xanthophyllomyces dendrorhous*", *Biotechnol. Lett.*, **2011**, *33*, 755-761.

19. M. Niklitschek, J. Alcaino, S. Barahona, D. Sepulveda, C. Lozano, M. Carmona, A. Marcoleta, C. Martinez, P. Lodato, M. Baeza and V. Cifuentes, "Genomic organization of the structural genes controlling the astaxanthin biosynthesis pathway of *Xanthophyllomyces dendrorhous*", *Biol. Res.*, **2008**, *41*, 93-108.
20. J. C. Verdoes, G. Sandmann, H. Visser, M. Diaz, M. van Mossel and A. J. J. van Ooyen, "Metabolic engineering of the carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*)", *Appl. Environ. Microbiol.*, **2003**, *69*, 3728-3738.
21. P. Lodato, J. Alcaino, S. Barahona, P. Retamales and V. Cifuentes, "Alternative splicing of transcripts from *crtI* and *crtYB* genes of *Xanthophyllomyces dendrorhous*", *Appl. Environ. Microbiol.*, **2003**, *69*, 4676-4682.
22. H. Visser, A. J. J. van Ooyen and J. C. Verdoes, "Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*", *FEMS Yeast Res.*, **2003**, *4*, 221-231.
23. K. Ojima, J. Breitenbach, H. Visser, Y. Setoguchi, K. Tabata, T. Hoshino, J. van den Berg and G. Sandmann, "Cloning of the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) and its assignment as a β-carotene 3-hydroxylase/4-ketolase", *Mol. Genet. Genomics*, **2006**, *275*, 148-158.
24. J. Alcaino, M. Fuentealba, R, Cabrera, M. Baeza and V. Cifuentes, "Modeling the interfacial interactions between CrtS and CrtR from *Xanthophyllomyces dendrorhous*, a P450 system involved in astaxanthin production", *J. Agric. Food Chem.*, **2012**, *60*, 8640-8647.
25. J. Alcaino, S. Barahona, M. Carmona, C. Lozano, A. Marcoleta, M. Niklitschek, D. Sepulveda, M. Baeza and V. Cifuentes, "Cloning of the cytochrome p450 reductase (crtR) gene and its involvement in the astaxanthin biosynthesis of *Xanthophyllomyces dendrorhous*", *BMC Microbiol.*, **2008**, *8*, Art.no.169.
26. J. Alcaino, M. Baeza and V. Cifuentes, "Astaxanthin and related xanthophylls", in "Biosynthesis and molecular genetics of fungal secondary metabolites*"* (Ed. J. Martín, C. García-Estrada and S. Zeilinger), Springer Verlag, New York, **2014**, pp. 187-208.
27. M. S. Gutierrez, M. C. Rojas, D. Sepulveda, M. Baeza, V. Cifuentes and J. Alcaino, "Molecular characterization and functional analysis of cytochrome b5 reductase (CBR) encoding genes from the carotenogenic yeast *Xanthophyllomyces dendrorhous*", *PLoS ONE*, **2015**, *10*, Art.no.e0140424.
28. K. Leiva, N. Werner, D. Sepulveda, S. Barahona, M. Baeza, V. Cifuentes and J. Alcaino, "Identification and functional characterization of the CYP51 gene from the yeast *Xanthophyllomyces dendrorhous* that is involved in ergosterol biosynthesis", *BMC Microbiol.*, **2015**, *15*, Art.no.89.
29. I. Loto, M. S. Gutierrez, S. Barahona, D. Sepulveda, P. Martinez-Moya, M. Baeza, V. Cifuentes and J. Alcaino, "Enhancement of carotenoid production by disrupting the C22-sterol desaturase gene (CYP61) in *Xanthophyllomyces dendrorhous*", *BMC Microbiol.*, **2012**, *12*, Art.no.235.
30. J. Ohnishi, H. Mizoguchi, S. Takeno and M. Ikeda, "Characterization of mutations induced by N-methyl-N'-nitro-N-nitrosoguanidine in an industrial *Corynebacterium glutamicum* strain", *Mutat. Res./Genet. Toxicol. Environ. Mutagen.,* **2008**, *649*, 239-244.

31. M. J. Betts and R. B. Russell, "Amino acid properties and consequences of substitutions", in "Bioinformatics for geneticists: A bioinformatics primer for the analysis of genetic data" (Ed. M. R. Barnes), Wiley, Hoboken, NJ, **2007**, pp. 311-342.
32. J. C. Verdoes and A. J. J. van Ooyen, "Isolation of the isopentenyl diphosphate isomerase encoding gene of *Phaffia rhodozyma*; improved carotenoid production in *Escherichia coli*", *Acta Bot. Gallica*, **1999**, *146*, 43-53.
33. K. Yokota, K. Satou and S. Ohki, "Comparative analysis of protein thermostability: Differences in amino acid content and substitution at the surfaces and in the core regions of thermophilic and mesophilic proteins", *Sci. Technol. Adv. Mater.*, **2006**, *7*, 255-262.
34. S. S. Strickler, A. V. Gribenko, A. V. Gribenko, T. R. Keiffer, J. Tomlinson, T. Reihle, V. V. Loladze and G. I. Makhatadze, "Protein stability and surface electrostatics: a charged relationship", *Biochemistry*, **2006**, *45*, 2761-2766.
35. J. E. Donald, D. W. Kulp and W. F. DeGrado, "Salt bridges: Geometrically specific, designable interactions", *Proteins*, **2011**, *79*, 898-915.
36. S. Sokalingam, G. Raghunathan, N. Soundrarajan and S. G. Lee, "A study on the effect of surface lysine to arginine mutagenesis on protein stability and structure using green fluorescent protein", *PLOS One*, **2012**, *7*, Art.no.e40410.
37. J. C. Verdoes, N. Misawa and A. J. J. van Ooyen, "Cloning and characterisation of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthophyllomyces dendrorhous*", *Biotechnol. Bioeng.*, **1999**, *63*, 750-755.
38. V. Alvarez, M. Rodriguez-Saiz, J. L. de la Fuente, E. J. Gudina, R. P. Godio, J. F. Martin and J. L. Barredo, "The crtS gene of *Xanthophyllomyces dendrorhous* encodes a novel cytochrome-P450 hydroxylase involved in the conversion of beta-carotene into astaxanthin and other xanthophylls", *Fungal Genet. Biol.*, **2006**, *43*, 261-272.
39. L. M. Gregoret and R. T. Sauer, "Tolerance of a protein helix to multiple alanine and valine substitutions", *Fold. Des.*, **1998**, *3*, 119-126.