Volume 58(2):139-146, 2014
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Expression of a bacterial β-carotene hydroxylase in canthaxanthin producing mutant *Mucor circinelloides* strains

Árpád Csernetics[1]*, Eszter Tóth[1], Anita Farkas[1], Gábor Nagy[1], Ottó Bencsik[1], Palanisamy Manikandan[2,3], Csaba Vágvölgyi[1] and Tamás Papp[1]

[1]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary, [2]Department of Laboratory Medical Sciences, College of Applied Medical Sciences, Majmaah University, Kingdom of Saudi Arabia, [3]Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Coimbatore, Tamil Nadu, India

ABSTRACT Xanthophylls, primarily hydroxyl- and keto-derivatives of β-carotene (such as zeaxanthin, β-cryptoxanthin, canthaxanthin, echinenone and astaxanthin) have powerful antioxidant activity. Due to several positive effects on human and animal health, industrial application of xanthophylls shows an increasing tendency. In our previous study, carotenoid biosynthesis of the β-carotene producing zygomycetes, *Mucor circinelloides* was modified by integrating the β-carotene ketolase encoding gene (*crtW*) of *Paracoccus* sp. N81106 into the genome. The isolated mutants accumulated mainly canthaxanthin and echinenone. Although, the fungus has β-carotene hydroxylase activity the astaxanthin production remained low even under different cultivation conditions, whereby canthaxanthin accumulation was further enhanced. In this study, the β-carotene hydroxylase encoding gene (*crtZ*) of the same bacterium was introduced into these canthaxanthin producing mutants of *M. circinelloides* either on autonomously replicating plasmids or by integrating it into the genome using the REMI method. Increased astaxanthin accumulation was observed in the transformants, which together with zeaxanthin and β-cryptoxanthin content could be further enhanced several fold by the modification of the cultivation conditions. An effective method for the extraction of carotenoids from *Mucor* mycelia grown in liquid culture was also developed. **Acta Biol Szeged 58(2):139-146 (2014)**

KEY WORDS

Paracoccus sp. N81106
β-carotene hydroxylase
heterologous expression
carotenoid extraction
astaxanthin

Mucor circinelloides (Mucoromycotina) is an intensively studied carotenoid producing zygomycetes; well-developed transformation systems are available for the fungus and most of the genes involved in the carotenoid biosynthesis have been isolated and characterized (Velayos et al. 2000a, 2000b, 2003, 2004; Papp et al. 2006, 2010; Csernetics et al. 2011). Carotenoids are tetraterpene molecules and synthesized via the mevalonate – isoprenoid pathway in fungi. Condensation of two molecules of geranylgeranyl pyrophosphate, several dehydrogenation and two cyclisation steps lead to the formation of β-carotene in *M. circinelloides* (Fig. 1.), which is the major carotenoid compound in the fungus, but intermediates (such as lycopene and γ-carotene) are also accumulated (Velayos et al. 2000a, 2000b; Csernetics et al. 2011; Papp et al. 2013). Furthermore, *M. circinelloides* shows a weak β-carotene hydroxylase activity, therefore it is able to synthesize zeaxanthin (3,3′-dihydroxy-β,β-carotene) and β-cryptoxanthin (3-hydroxy-β,β-carotene) (Papp et al. 2006, 2013; Csernetics et al. 2011). At the same time, several microorganisms, such as *Paracoccus* sp. N81106 able to accumulate canthaxanthin (β,β-carotene-4,4'-dione), echinenone (β,β-carotene-4-on) and astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) as well; formation of astaxanthin from β-carotene requires a β-carotene hydroxylase and a ketolase activity (Misawa et al. 1995). These xanthophylls have powerful antioxidant properties and their preventive action against different types of cancer, age-related macular degeneration, heart- or bone disease makes them dietary important (Chew et al. 1999; Nishino et al. 2002; Mares-Perlman et al. 2002; Guerin et al. 2003; Beatty et al. 2004; Yamaguchi 2004; Kumaresan et al. 2008). Besides, they are frequently used in pharmaceutical and cosmetic industry and also in feed additives (Bhosale and Bernstein 2005). Today, most of the commercially available xanthophylls are synthesized chemically. Biosynthesis of astaxanthin is limited to microorganisms and along with other carotenoids an increasing demand can be observed to replace chemical synthesis with natural sources (Bhosale and Bernstein 2005).

In our previous studies, *crtW* and *crtZ* genes of the *Paracoccus* sp. N81106, encoding β-carotene ketolase and hydroxylase, respectively, were expressed in *M. circinelloides*

Accepted Dec 11, 2014
*Corresponding author. E-mail: csernetics.arpad@gmail.com

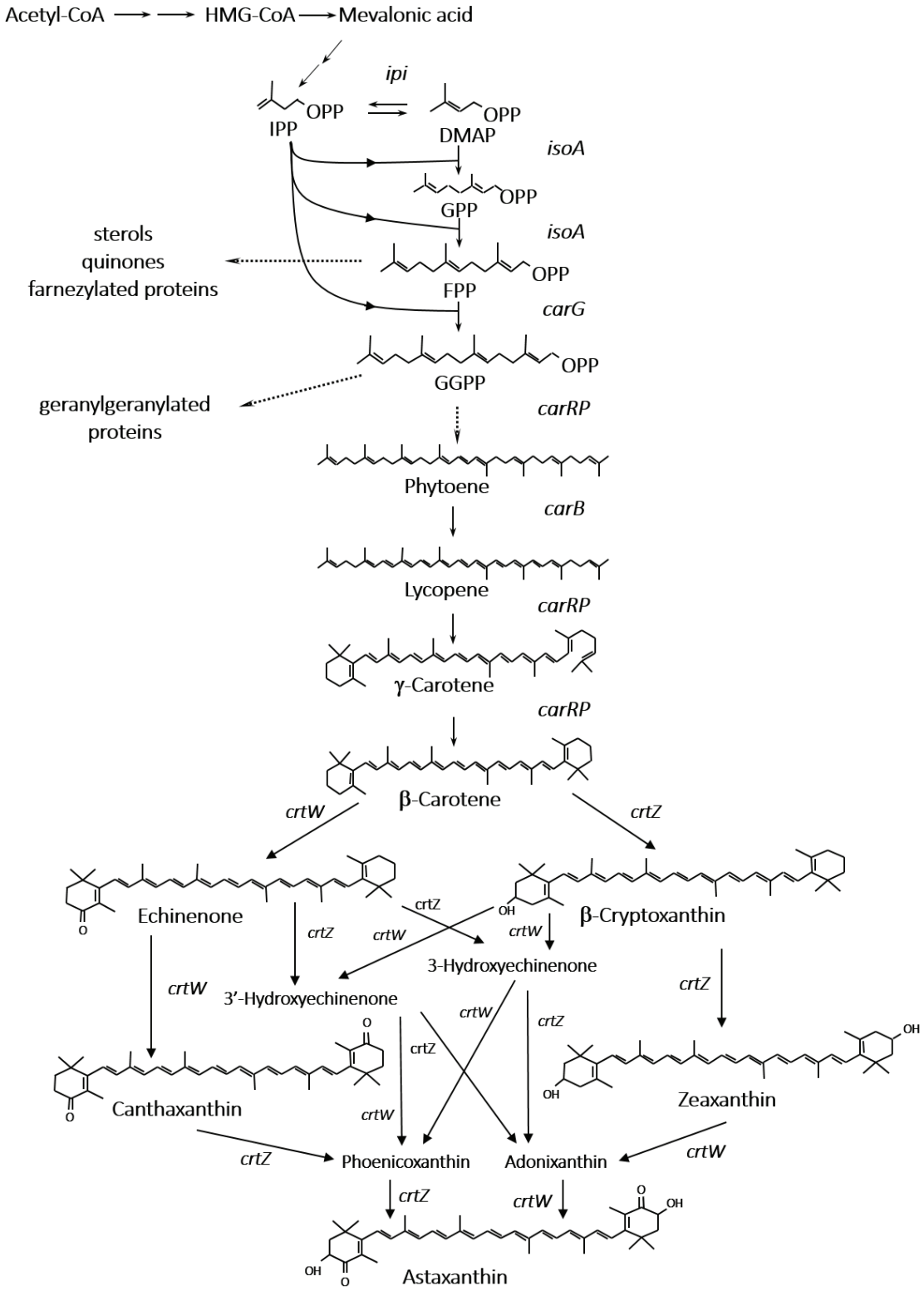

Figure 1. Carotenoid biosynthesis pathway in *M. circinelloides* and possible steps of the β-carotene – astaxanthin conversion in *M. circinelloides* transformants harbouring the *Paracoccus* sp. N81106 *crtW* and *crtZ* genes (based on Misawa et al. 1995). HMG-CoA – 3-hydroxy-3-methylglutaryl-CoA; IPP – isopentenyl pyrophosphate; DMAP – dimethylallyl pyrophosphate; GPP – geranyl pyrophosphate; FPP – farnesyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate. *M. circinelloides* genes: *ipi* – IPP isomerase; *isoA* – FPP synthase; *carG* – GGPP synthase; *carRP* – phytoene synthase/lycopene cyclase; *carB* – phytoene desaturase. *Paracoccus* sp. N81106 genes: *crtZ* – β-carotene hydroxylase and *crtW* – β-carotene ketolase.

MS12 strain on autonomously replicating plasmids (Papp et al. 2006). Introduction of the *crtZ* enhanced the zeaxanthin and β-cryptoxanthin accumulation, while introduction of the *crtW* lead to the presence of canthaxanthin, echinenone and astaxanthin in low amount. Co-expression of the *crtZ* and *crtW* genes increased the canthaxanthin and β-cryptoxanthin production compared to the introduction of only the single *crtW* (Papp et al. 2006). Integration of the *Paracoccus crtW* gene into the *Mucor* genome by double homologous recombination and restriction enzyme-mediated integration (REMI) led to transformants accumulating 123-148 μg g^{-1} canthaxanthin and 117-135 μg g^{-1} echinenone [dry mass] (Papp et al. 2013). At the same time, production of astaxanthin remained low in these transformants. It is supposed that the endogenous β-carotene hydroxylase has poor activity and able to convert canthaxanthin to astaxanthin with low efficiency (Papp et al. 2013). Another explanation could be that *Mucor* β-carotene hydroxylase does not able to catalyse this conversion but the low amounts of zeaxanthin and β-cryptoxanthin accumulated by the fungus may serve as substrates to the *Paracoccus* β-carotene ketolase to form low amount of astaxanthin.

In this study, our aim was to express the *crtZ* gene of *Paracoccus* sp. N81106 in canthaxanthin producing mutants of *M. circinelloides* to enhance the astaxanthin accumulation. The *crtZ* was introduced on autonomously replicating plasmids and, in parallel, it was integrated into the *Mucor* genome using the REMI method. Carotenoid content of the resulting transformants and effect of different culturing conditions on their carotenoid composition were also investigated.

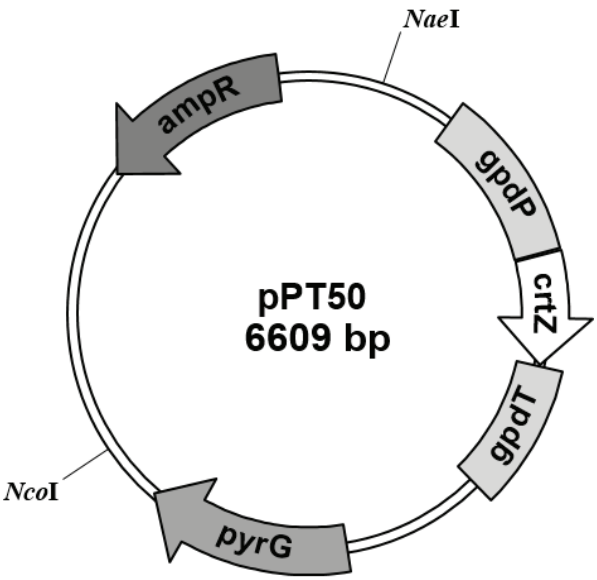

Figure 2. Plasmid construction used to transform the canthaxanthin producing mutants MS12+pCA8lf/1 and MS12+pPT51′R3/2. The plasmid harbours the *Paracoccus* sp. N81106 *crtZ* gene encoding a β-carotene hydroxylase between the *Mucor gpd1* promoter and terminator regions. The *Mucor pyrG* gene is responsible for the complementation of the uracil auxotrophy and *ampR* (gene for ampicillin resistance) for the bacterial selection. The fragment obtained by digesting with the restriction enzymes *Nae*I and *Nco*I corresponds to pPT50′R used for REMI transformation.

Materials and Methods

Strains, media and growth conditions

The *M. circinelloides* strains, MS12+pCA8lf/1 and MS12+pPT51'R3/2, which have *leuA*$^+$, *pyrG*$^-$, *crtW*$^+$ genotype (Papp et al. 2013) and are derived from the *leuA*$^-$, *pyrG*$^-$ mutant MS12 (Benito et al. 1992), were used in the transformation experiments. These strains are auxotrophic for uracil and harbours the *crtW* gene encoding the β-carotene ketolase of *Paracoccus* sp. N81106 (formerly *Agrobacterium aurantiacum*) integrated into their genome by homologous double recombination (MS12+pCA8lf/1) or REMI (MS12+pPT51'R3/2) methods. Both strains accumulate mainly canthaxanthin and echinenone and small amount of astaxanthin (Papp et al. 2013).

Escherichia coli TOP10F$^-$ was used in plasmid amplifications. *E. coli* was cultivated on Luria-Bertani medium (LB, 10 g tryptone; 5 g yeast extract; 10 g NaCl and 20 g agar for solid medium per litre) containing 100 μg mL^{-1} ampicillin at 37 °C.

For growth tests, nucleic acid and carotenoid extraction, *M. circinelloides* strains were cultivated on solid minimal medium (YNB, 10 g glucose; 0.5 g yeast nitrogen base without amino acids; 1.5 g $(NH_4)_2SO_4$, 1.5 g sodium glutamate and 20 g agar per litre) supplemented with uracil and leucine (0.5 mg mL^{-1}) if required. Fungal cultures were grown for 4 days at 25 °C under continuous light. Effect of cultivation temperature on carotenoid accumulation of the fungal strains was tested on YNB plates at 20, 25, 30 and 37 °C. To examine the effect of carbon sources on carotenoid production, glucose was replaced with fructose, trehalose, mannose, galactose, glycerol-L-monoacetate, dihydroxyacetone and L-aspartic acid in a final concentration of 1% in YNB. Combinations of different carbon sources were also tested. The carotenoid content was also determined from liquid cultures when strains were grown in YNB medium without agar for 6 days on 25 °C under continuous light and shaking (110 rpm). Mycelia were separated from the media by filtration using 0.45 μm pore size filters (Millipore).

For growth test, 10^5 spores were inoculated onto the centre of YNB plates. To test the mitotic stability of the transformants solid malt extract medium (MEA, 10 g glucose; 10 g malt extract; 5 g yeast extract and 20 g agar per litre) was used as a complete medium.

Molecular techniques

Purification of plasmid DNA was performed with the Viogene Mini Plus and Midi Plus Plasmid DNA Extraction Systems (Viogene). DNA fragments were purified from agarose gel

Table 1. Different methods and solvents tested to improve the carotenoid extraction efficiency from liquid cultures.

Extraction method	Pre-treatment		Extraction solvent
Conventional [a]	-		Acetone Petroleum ether DMSO, partition in petroleum ether DMSO, partition in hexane
Reduction of the moisture content	Lyophilisation for 45 min. Drying at 40 °C overnight		Acetone and petroleum ether
	Desiccation with Na_2SO_4	10% 20% 50% 100%	
Pre-treatment with lactic acid	0.2 M 0.3 M 0.4 M 0.5 M 2 M 3 M 4 M 5 M	heating to 120 °C heating to 50 °C	Acetone

[a] The method, which was used for solid cultures and described in Materials and Methods, is regarded as the conventional method.

using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific). General procedures for plasmid DNA manipulation, transformation of *E. coli* and Southern blotting were performed by following standard methods (Sambrook et al. 1989). For fungal DNA preparation, mycelium was disrupted with a pestle and mortar in liquid nitrogen and DNA was isolated according to Iturriaga et al. (1992). *CrtZ* gene probe for Southern hybridization was constructed by amplifying a DNA fragment from the plasmid pPT50 using the crtZ1 (5' ATG ACC AAT TTC CTG ATC 3') and crtZ2 (5' CGT GCG CTC CTG CGC CTC 3') primers and the digoxigenin-based PCR DIG Probe Synthesis Kit (Roche). The DIG Nucleic Acid Detection Kit (Roche) was used for immunological detection of the nucleic acid blots, following the instructions of the manufacturer.

Plasmid construction and transformation

The plasmid pPT50 harbouring the *crtZ* gene of *Paracoccus* sp. N8106 under the control of the promoter and terminator regions of the *Mucor* glyceraldehyde-3-phosphate dehydrogenase (*gpd1*) gene was used in all transformation experiments (Fig. 2) (Papp et al. 2006). As a selection marker, the plasmid holds the *Mucor pyrG* encoding the orotidine-5 '-phosphate decarboxylase, which complements the uracil auxotrophy of *M. circinelloides* strains MS12+pCA8lf/1 and MS12+pPT51'R3/2.

For PEG/$CaCl_2$-mediated transformation, protoplasts of *M. circinelloides* were prepared following the next steps: spores of MS12+pCA8lf/1 and MS12+pPT51'R3/2 were harvested from four days old cultures and inoculated into small drops onto cellophane sheets placed on YNB media supplemented with uracil and grown at 25 °C for 16 h. Colonies were washed in protoplast-forming solution (10 mM sodium-phosphate buffer, pH 6.4; 0.8 M sorbitol; 1.5% home-made snail enzyme) and incubated at 25 °C for 3 h under continuous gentle shaking. Protoplasts were separated from the mycelia by filtration through 3 sheets of gauze. Protoplasts were collected by centrifugation (3200 rpm, 15 min, 4 °C), washed with SMC buffer (50 mM $CaCl_2$; 10 mM 3-(N-morpholino) propanesulfonic acid and 0.8 M sorbitol) and re-suspended in 0.25 ml SMC. Thereafter, the PEG/$CaCl_2$-mediated transformation of MS12+pCA8lf/1 and MS12+pPT51'R3/2 protoplasts with circular plasmid was performed according to van Heeswijck and Roncero (1984). For REMI, the pPT50 plasmid was digested with *Nae*I - *Nco*I enzymes (to avoid the re-circularization) and the resulting pPT50'R linear fragment, which harboured the *crtZ* gene fused with the *gpd1* promoter and terminator regions and the *pyrG* gene, was introduced into the recipient MS12+pCA8lf/1. The REMI transformation of protoplasts was performed according to the PEG/$CaCl_2$-mediated transformation method, except that 30-30 U restriction enzymes used for preparation of the linearized DNA were added to the protoplasts together with 5-10 µg linear fragment and the solution was incubated for 0.5 h on ice and 1.5 h at 33 °C. Transformants were selected on the basis of auxotrophy complementation on YNB solid medium. Introduction of the pPT50 plasmid into MS12+pCA8lf/1 and MS12+pPT51'R3/2 resulted the MS12+pCA8lf/1+pPT50 and MS12+pPT51'R3/2+pPT50, while introduction of the pPT50'R fragment into MS12+pCA8lf/1 resulted the MS12+pCA8lf/1+pPT50'R transformants.

Carotenoid extraction and analysis

Carotenoids were extracted from 500 mg mycelial powder

with 700 µl acetone and vortexing (Papp et al. 2006). This extraction step was repeated until the pellet was found to be devoid of pigments. Extracts were collected in 50 ml Falcon tube, combined and partitioned in an equal volume of 10% diethyl ether in petroleum ether. To facilitate the separation 2 ml distilled water was added and centrifuged (3200 rpm, 8 min, 4 °C). The petroleum ether fraction was dried under nitrogen. To determine the dry mass, 500 mg mycelial powder was dried at 70 °C overnight.

The carotenoid extraction was performed from liquid cultures, as well. Filtrated *Mucor* mycelia was disrupted with liquid nitrogen and was partitioned to test different extraction methods, such as lyophilisation, drying at 40 °C or desiccation with sodium-sulphate with different concentrations, solvents (such as acetone, petroleum ether and DMSO) and acid pre-treatment combined with heating (0.2-5 M lactic acid at 50-120 °C) (Table 1). Other steps of the extraction were performed as described above.

For high-performance liquid chromatography (HPLC), samples and standards were analysed by using a modular Shimadzu low-pressure gradient HPLC system. The dried samples were re-dissolved in 100 µl tetrahydrofuran supplemented with butylated hydroxytoluene (100 µg mL^{-1}) directly before the analysis and 2 µl was subjected to HPLC analysis on a Phenomenex Prodigy column (4.6 x 250, ODS 3 µm). The separation was performed with a gradient (where min/solvent A%/solvent B% was 0/99/1; 8/60/40; 13/46/54; 15/0/100; 18/0/100; 21/99/1; 25/99/1) using 4% water-96% methanol as solvent A and 100% methyl-terc-butyl ether as solvent B, at a flow rate of 1 mL min^{-1}. The detection wavelength was 450 nm and the column thermostat temperature was 35 °C. For identification of carotenoids the following standards were used: astaxanthin, lycopene, β-carotene (Sigma), β-cryptoxanthin, canthaxanthin, zeaxanthin (Carl Roth) and echinenone (DHI Water and Environment). For γ-carotene, standard was purified by HPLC from *Mucor azygosporus*.

Results and discussion

Introduction of the *crtZ* gene into MS12+pCA8lf/1 and MS12+pPT51'R3/2 strains

Introduction of the pPT50 circular plasmid into canthaxanthin and echinenone producing MS12+pCA8lf/1 and MS12+pPT51'R3/2 mutants resulted in 20-25 transformants/10^5 protoplasts. The transformation frequency was similar to those in our previous studies, in which autonomously replicating plasmids harbouring the bacterial *crtW* and *crtZ* genes were used to transform the MS12 strain (Papp et al. 2006; Csernetics et al. 2011). The REMI transformation method was applied successfully for zygomycetes firstly by our research group (Papp et al. 2013). Similarly to those experiments, two to three colonies could be isolated per transformation of 10^6 MS12+pCA8lf/1 protoplasts using the pPT50'R linear DNA. Southern hybridization analysis verified the presence of the exogenous DNA in the transformants (results not shown), which proved to be mitotically stable on selective (YNB) and non-selective (MEA) media after ten consecutive cultivation cycles and no differences in their growing ability was observed compared to the recipient or the wild-type strains. Five-five isolates per transformation experiments were selected for further analysis.

Carotenoid content of the transformants

Carotenoid content of five-five transformants was analysed at the 4th and 10th cultivation cycle after the transformation by HPLC. Strains MS12, MS12+pCA8lf/1 and MS12+pPT51'R3/2 were used as controls (Table 2). After the 4th cultivation cycle, low amount of astaxanthin and decreased canthaxanthin and echinenone content were observed in all transformants. After the 10th consecutive cultivation cycle on YNB, MS12+pCA8lf/1+pPT50/1-5 and MS12+pPT51'R3/2+pPT50/1-5 accumulated 737 and 611 µg g^{-1} [dry mass] total carotenoid and 6 and 14 µg g^{-1} [dry mass] astaxanthin, respectively. Astaxanthin content was around 1-2% of the total carotenoids in these transformants. Compared to the recipient strains, significantly increased echinenone, β-carotene and γ-carotene content and decreased canthaxanthin content were also observed, mainly in MS12+pPT51'R3/2+pPT50/1-5 (Table 2). Integration of the *crtZ* gene into the genome of MS12+pCA8lf/1 resulted in 741 µg g^{-1} [dry mass] average total carotenoid content and, similarly to the transformants harbouring the *crtZ* gene on autonomously replicating plasmids, canthaxanthin, echinenone and β-carotene remained the major carotenoid components. These integrative transformants accumulated significantly higher amounts of total carotenoids, as well as canthaxanthin, echinenone and zeaxanthin than those harbouring the *crtW* and *crtZ* genes on autonomously replicating plasmids (Papp et al. 2006). However, they produced similar amounts of astaxanthin. As higher level of astaxanthin accumulation was observed by introducing the *crtZ* gene on autonomously replicating plasmids than by integrating it into the genome (Table 2), MS12+pCA8lf/1+pPT50/1-3 and MS12+pPT51'R3/2+pPT50/1-3 transformant were selected for further investigations.

Effect of the cultivation conditions on the carotenoid accumulation

Carotenoid accumulation of MS12+pCA8lf/1+pPT50/1-3 and MS12+pPT51'R3/2+pPT50/1-3 was analysed at different cultivation temperatures and compositions of media (Table 3). Effect of carbon sources on the carotenoid production of MS12+pCA8lf/1 was analysed in our previous study and the tested carbon sources were selected based on these results (Papp et al. 2013). Cultivation on fructose, trehalose and mannose as sole carbon sources increased

Table 2. Total carotenoid content and composition of the transformants, MS12 and the recipient MS12+pCA8lf/1 and MS12+pPT51′R3/2 strains.

Strain	Total carotenoid	β-carotene	β-cryptoxanthin	Zeaxanthin	Echinenone	Canthaxanthin	Astaxanthin	Lycopene	γ-carotene
MS12	422±25	268±18	25±3	6±2	-	-	-	14±3	23±4
MS12+pPT51′R3/2	511±27	56±11	16±4	10±2	103±4	110±10	2±1	11±2	32±4
MS12+pPT51′R3/2+pPT50/1-5	611±55	201±23	11±1	12±2	137±15	36±11	14±2	19±6	65±10
MS12+pCA8lf/1	534±29	38±10	27±5	4±2	114±11	151±19	2±1	21±5	34±4
MS12+pCA8lf/1+pPT50/1-5	737±75	146±15	24±4	14±4	203±36	103±14	6±4	24±7	87±15
MS12+pCA8lf/1+pPT50′R/1-5	741±112	236±37	14±4	6±3	171±15	145±47	3±1	12±1	36±11

Average values (μg g^{-1} [dry mass]) were calculated from carotenoid content of five transformants in three independent carotenoid extractions and measurements.

the total carotenoid, zeaxanthin and echinenone content of MS12+pPT51'R3/2+pPT50/1-3 in comparison with cultivation on glucose. Astaxanthin accumulation significantly increased on fructose, L-aspartic acid and mannose; on the latter, 28 μg g^{-1} [dry mass] astaxanthin content was measured (Table 3). Glucose, mannose, trehalose and fructose had also positive effect on the carotenoid content of both MS12+pCA8lf/1 and MS12+pPT51'R3/2 (Papp et al. 2013).

Effects of combinations of carbon sources (such as dihydroxyacetone and L-aspartic acid combined with glucose, trehalose and mannose) on the carotenoid content of MS12+pPT51'R3/2+pPT50/1-3, MS12+pCA8lf/1+pPT50/1-3 and the recipient strains were also tested. Most combinations resulted in slight increase in the total carotenoid content. However, glucose combined with dihydroxyacetone led to the highest total carotenoid content in all strains (Table 3), approximately 2-3 times higher amounts were measured than on glucose or dihydroxyacetone, separately. Simultaneously, enhanced β-cryptoxanthin, canthaxanthin and echinenone production was detected in MS12+pCA8lf/1 and MS12+pPT51'R3/2 and increased astaxanthin, zeaxanthin, β-cryptoxanthin, canthaxanthin and echinenone accumulation was observed in MS12+pPT51'R3/2+pPT50/1-3 and MS12+pCA8lf/1+pPT50/1-3 (Table 3). Application of glucose and dihydroxyacetone supposedly resulted the induction and increased activity of the *gpd1* promoter, which enhanced the expression of the *crtW* and *crtZ* genes. Dihydroxyacetone as sole carbon source resulted decreased total carotenoid content, however, the proportion of xanthophylls within it increased in MS12+pPT51'R3/2+pPT50/1-3.

We also tested the effect of the cultivation temperature on the carotenoid content. Temperature is an important environmental factor affecting the biosynthesis of carotenoids (Bhosale 2004). Cultivation at 30 °C had a positive effect on the total carotenoid and astaxanthin content compared to cultivation at 25 °C in the case of MS12+pPT51'R3/2+pPT50/1-3. The transformants were able to survive at 37 °C as well, with lower production of carotenoids. Similarly, transformants harbouring the *Paracoccus crtW* gene integrated into the genome (MS12+pCA8lf/1 and MS12+pPT51'R3/2) were able to survive at 37 °C, in contrast with the wild-type MS12 (Papp et al. 2013). Xanthophylls, including astaxanthin has better antioxidant activity than β-carotene and may protect cells against increased levels of reactive oxygen species and may contribute in higher thermo tolerance through decreasing the fluidity and stabilizing the structure of the membranes (Palozza and Krinsky 1992; Tjahjono et al. 1994; Liang et al. 2009).

Development of carotenoid extraction method for liquid cultures

For fermentation developments, it would be advantageous if carotenoid and xanthophyll production in liquid culturing would be developed and optimized. Therefore, carotenoid production of our transformants was also tested in liquid cultures. It was observed that the extraction method used for mycelia harvested from solid media (Papp et al. 2006; Csernetics et al. 2011) is not effective for those from liquid media. Relatively high amounts of pigments remained after several extraction steps. It was presumed, that high moisture content of mycelia or formation of thicker cell wall in liquid media may cause the low efficiency of the carotenoid extraction. Thus, different methods were tested to improve the extraction efficiency. The conventional method was used as a control (Table 1).

To reduce the moisture content, lyophilization for 45 min. or overnight drying at 40 °C was applied together with acetone and petroleum ether extractions. Sodium-sulphate, which has desiccating effect, was also tested. It was applied in 10, 20, 50 and 100% (m/m) of mycelial mass combined with acetone extraction. Among the tested processes, lyophilisation combined with acetone extraction proved to be the most effective. In case of the astaxanthin producer *Phaffia rhodozyma*, which has thick cell wall, DMSO or acid pretreatment is used for cell lysis before carotenoid extraction (Sedmak et al. 1990; Xiao et al. 2009). Therefore, different acids were tested to enhance cell lysis and avoid degradation of astaxanthin and lactic acid was found to be the most appropriate for this purpose (Ni et al. 2008). Extraction with DMSO and partition of carotenoids in hexane instead of

Table 3. Carotenoid composition of transformants under different cultivation conditions.

Strain	Carbon source/ temperature	Total	β-carotene	β-crypto-xanthin	Zeaxan-thin	Echinenone	Canthax-anthin	Astaxan-thin	Lycopene	γ-carotene
MS12+pPT51′R3/2+pPT50	Glucose	611±55	201±23	11±1	12±2	137±15	36±11	14±2	19±6	65±10
MS12+pCA8lf/1+pPT50		737±75	146±15	24±4	14±4	203±36	103±14	6±4	24±7	87±15
MS12+pPT51′R3/2+pPT50	Fructose	694±72	233±21	10±2	16±2	165±22	23±4	17±4	16±5	68±25
MS12+pCA8lf/1+pPT50		705±81	134±19	19±3	11±1	195±31	97±11	6±1	22±7	81±31
MS12+pPT51′R3/2+pPT50	L-aspartic acid	400±49	72±9	7±1	13±3	101±14	19±2	17±3	10±2	44±12
MS12+pCA8lf/1+pPT50		259±25	32±7	9±2	6±2	64±12	47±13	5±2	6±1	26±15
MS12+pPT51′R3/2+pPT50	Trehalose	806±92	309±31	21±4	16±4	159±18	29±7	12±3	17±5	71±23
MS12+pCA8lf/1+pPT50		409±31	57±15	12±3	10±2	108±27	80±9	6±2	10±1	33±11
MS12+pPT51′R3/2+pPT50	Mannose	734±62	175±19	7±1	17±2	202±20	39±4	28±3	21±4	80±21
MS12+pCA8lf/1+pPT50		497±59	75±11	23±7	10±3	143±19	74±9	5±1	19±6	59±15
MS12+pPT51′R3/2+pPT50	Galactose	635±65	261±31	17±2	13±2	123±13	22±2	8±2	12±2	50±18
MS12+pCA8lf/1+pPT50		579±53	124±22	15±3	16±3	150±17	86±7	7±1	17±7	54±11
MS12+pPT51′R3/2+pPT50	Glycerol-L-mono-acetate	465±35	200±16	14±3	9±4	67±9	23±11	6±1	6±2	22±3
MS12+pCA8lf/1+pPT50		588±57	201±21	8±2	12±4	124±23	75±17	5±2	13±5	45±7
MS12+pPT51′R3/2+pPT50	Dihydroxyacetone	395±41	54±7	16±5	29±5	70±16	69±21	11±2	5±1	17±4
MS12+pCA8lf/1+pPT50		207±16	24±6	6±1	8±2	30±7	58±22	5±1	4±2	11±3
MS12+pPT51′R3/2+pPT50	Glucose, on 20 °C	614±44	262±31	20±7	10±2	90±17	20±2	6±1	20±7	51±11
MS12+pCA8lf/1+pPT50		653±71	168±19	13±5	13±4	171±19	68±11	3±1	19±5	71±9
MS12+pPT51′R3/2+pPT50	Glucose, on 30 °C	694±61	206±11	18±4	12±4	105±23	31±17	30±5	21±4	73±27
MS12+pCA8lf/1+pPT50		507±67	94±19	16±4	10±3	98±21	49±15	6±2	32±9	87±24
MS12+pPT51′R3/2+pPT50	Glucose, on 37 °C	447±36	199±14	10±2	4±1	26±6	3±1	4±1	16±6	55±31
MS12+pCA8lf/1+pPT50		500±81	182±19	9±1	3±1	73±11	16±7	1±0.5	23±12	73±37
MS12+pPT51′R3/2	Glucose + Dihydroxyacetone	959±189	36±15	123±25	10±2	161±24	300±55	2±1	133±34	128±29
MS12+pPT51′R3/2+pPT50		989±183	62±24	102±31	30±7	223±35	115±42	35±4	74±19	128±44
MS12+pCA8lf/1		1171±227	44±11	151±31	2±1	225±41	443±71	3±1	161±41	159±33
MS12+pCA8lf/1+pPT50		1032±205	50±23	113±27	27±8	211±45	299±34	29±3	125±21	137±54

Average carotenoid values (μg g^{-1} [dry mass]) were calculated from biological replicates in three independent carotenoid extractions and measurements.

petroleum ether significantly increased the carotenoid yield. In our tests, 0.2-0.5 and 2-5 M lactic acid was added to the mycelial powder and the resulting samples were heated to 120 °C and 50 °C, respectively. After a centrifugation step, the extraction was continued with the repeated addition of acetone and vortexing. In most of the tested cases, mycelia became completely colourless after 6-8 extraction steps. Finally, extraction with DMSO or 4 M lactic acid combined with heating to 50 °C were found to be the most effective for carotenoid extraction, which resulted 120-200% effectiveness in comparison with the conventional method. Additionally, HPLC analysis verified that these conditions did not resulted in significant degradation of pigments. Carotenoid composition of MS12+pPT51'R3/2+pPT50/1-3 was analysed from mycelia cultivated in liquid YNB medium (containing glucose as carbon source) using the above described method. Total carotenoid content was around 398 μg g^{-1} [dry mass] and 10-25% increase in the proportion of canthaxanthin, echinenone, β-cryptoxanthin, zeaxanthin and astaxanthin content was observed, however it was still below of the xanthophyll content of mycelia harvested from solid media.

Acknowledgement

The research of Árpád Csernetics and Csaba Vágvölgyi was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/2-11/1-2012-0001 'National Excellence Program'. The infrastructure and research equipment was supported by OTKA NN 106394.

References

Beatty S, Nolan J, Kavanagh H, O'Donovan O (2004) Macular pigment optical density and its relationship with serum and dietary levels of lutein and zeaxanthin. Arch Biochem Biophys 430(1):70-76.

Benito EP, Díaz-Mínguez JM, Iturriaga EA, Campuzano EA, Eslava AP (1992) Cloning and sequence analysis of the *Mucor circinelloides pyrG* gene encoding orotidine-5'-monophosphate decarboxylase: use of *pyrG* for homologous transformation. Gene 116(1):59-67.

Bhosale P (2004) Environmental and cultural stimulants in the production of carotenoids from microorganisms. Appl Microbiol Biotechnol 63:351-361.

Bhosale P, Bernstein PS (2005) Microbial xanthophylls. Appl Microbiol Biotechnol 68(4):445-455.

Chew BP, Park JS, Wong M, Wong TS (1999) A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. Anticancer Res 19 (3A):1849-1853.

Csernetics Á, Nagy G, Iturriaga EA, Szekeres A, Eslava AP, Vágvölgyi C, Papp T (2011) Expression of three isoprenoid biosynthesis genes and their effects on the carotenoid production of the zygomycete *Mucor circinelloides*. Fungal Genet Biol 48(7):696-703.

Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. Trends Biotechnol

21(5):210-216.
Iturriaga EA, Díaz-Mínguez JM, Benito EP, Álvarez MI, Eslava AP (1992) Heterologous transformation of *Mucor circinelloides* with the *Phycomyces blakesleeanus leu1* gene. Curr Genet 21:215-223.
Kumaresan N, Sanjay KR, Venkatesh KS, Kadeppagari R-K, Vijayalakshmi G, Umesh-Kumar S (2008) Partially saturated canthaxanthin purified from *Aspergillus carbonarius* induces apoptosis in prostate cancer cell line. Appl Microbiol Biotechnol 80(3):467-473.
Liang J, Tian Y, Yang F, Zhang J, Skibsted LH (2009) Antioxidant synergism between carotenoids in membranes. Astaxanthin as a radical transfer bridge. Food Chem 115(4):1437-1442.
Mares-Perlman JA, Millen AE, Ficek TL, Hankinson SE (2002) The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. Overview. J Nutr 132(3):518S-524S.
Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwara S, Saito T, Ohtani T, Miki W (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J Bacteriol 177:6575-6584.
Ni H, Chen Q-H, He G-Q, Wu G-B, Yang Y-F (2008) Optimization of acidic extraction of astaxanthin from *Phaffia rhodozyma.* J Zhejiang Univ Sci B 9(1):51-59.
Nishino H, Murakosh M, Ii T, Takemura M, Kuchide M, Kanazawa M, Mou XY, Wada S, Masuda M, Ohsaka Y, Yogosawa S, Satomi Y, Jinno K (2002) Carotenoids in cancer chemoprevention. Cancer Metastasis Rev 21(3-4):257-264.
Palozza P, Krinsky NI (1992) Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Arch Biochem Biophys 297:291-295.
Papp T, Csernetics Á, Nagy G, Bencsik O, Iturriaga EA, Eslava AP, Vágvölgyi C (2013) Canthaxanthin production with modified *Mucor circinelloides* strains. Appl Microbiol Biotechnol 97(11):4937-4950.
Papp T, Csernetics Á, Nyilasi I, Ábrók M, Vágvölgyi C (2010) Genetic transformation of zygomycetes fungi. In: Rai M, Kövics G (eds) Progress in mycology, Springer, Scientific Publishers, Jodhpur, pp. 75-94.
Papp T, Velayos A, Bartók T, Eslava AP, Vágvölgyi C, Iturriaga EA (2006) Heterologous expression of astaxanthin biosynthesis genes in *Mucor circinelloides*. Appl Microbiol Biotechnol 69(5):526-531.
Sambrook J, Fitsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor.
Sedmak JJ, Weerasinghe DK, Jolly SO (1990) Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. Biotechnol Tech 4(2):107-112.
Tjahjono AE, Hayama Y, Kakizono T, Terada Y, Nishio N, Nagai S (1994) Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. Biotechnol Lett 16:133-138.
Xiao A, Ni H, Cai H, Li L, Su W, Yang Q (2009) An improved process for cell disruption and astaxanthin extraction from *Phaffia rhodozyma*. World J Microbiol Biotechnol 25:2029-2034.
Yamaguchi M (2004) Osteogenesis promoter containing β-cryptoxanthin as the active ingredient. Japanese patent CT Int Appl 42 pp. WO 2004037236.
van Heeswijck R, Roncero MIG (1984) High frequency transformation of *Mucor* with recombinant plasmid DNA. Carlsberg Res Commun 49:691-702.
Velayos A, Blasco JL, Alvarez MI, Iturriaga EA, Eslava AP (2000a) Blue-light regulation of the phytoene dehydrogenase (*carB*) gene expression in *Mucor circinelloides*. Planta 210(6):938-946.
Velayos A, Eslava AP, Iturriaga EA (2000b) A bifunctional enzyme with lycopene cyclase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides*. Eur J Biochem 267(17):1-12.
Velayos A, Fuentes-Vicente M, Aguilar-Elena R, Eslava AP, Iturriaga EA (2004) A novel fungal prenyl diphosphate synthase in the dimorphic zygomycete *Mucor circinelloides*. Curr Genet 45(6):371-377.
Velayos A, Papp T, Aguilar-Elena R, Fuentes-Vicente M, Eslava AP, Iturriga EA, Álvarez MI (2003) Expression of the *carG* gene, encoding geranylgeranyl pyrophosphate synthase, is up-regulated by blue light in *Mucor circinelloides*. Curr Genet 43(2):112-120.