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ORIGINAL PAPER

Cloning and knockout of phytoene desaturase gene in *Sphingomonas elodea* ATCC 31461 for economic recovery of gellan gum

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Abstract A gene encoding phytoene desaturase (*crtI*) in the carotenoid biosynthetic pathway of *Sphingomonas elodea* ATCC 31461, an industrial gellan gum-producing strain, was cloned and identified. This gene is predicted to encode a 492-amino acid protein with significant homology to the phytoene desaturase of other carotenogenic organisms. Knockout of *crtI* gene blocked yellow carotenoid pigment synthesis and resulted in the accumulation of colorless phytoene, confirming that it encodes phytoene desaturase. Further research indicates that the yield of gellan gum production by *crtI* gene knockout mutants is almost the same as that by the wild-type strain. In addition, a recovery method based on the colorless fermentation broth of the *crtI* gene knockout mutant was investigated. Compared to the volume of alcohol for the parent strain, much less alcohol (30%) is required in this recovery process; thus, the costs of downstream purification of gellan gum can be substantially reduced.

Keywords Carotenoid · Gellan · Phytoene desaturase · *Sphingomonas elodea*

Introduction

Carotenoids are a group of colored terpenoids with antioxidant properties that are widespread in a variety of organisms, from non-phototrophic prokaryotes to higher plants [11]. *Sphingomonas elodea* synthesizes a unique yellow carotenoid product identified as nostoxanthin [17]. To date, however, the carotenoid biosynthetic pathway employed by this organism remains unclear. The initial steps in carotenoid biosynthesis up to phytoene formation are common to all the carotenoids described thus far [27]. Generally, the biosynthesis of carotenoids begins with the condensation of two geranylgeranyl diphosphate molecules to form phytoene. This colorless compound is converted into the red compound lycopene through a series of desaturation reactions catalyzed by phytoene desaturase. Further downstream modification reactions, including cyclization of lycopene, addition of keto groups, and hydroxylation of cyclized carotene, lead to the formation of different carotenoid products [2, 7, 30].

Sphingomonas elodea ATCC 31461 was isolated as a Gram-negative bacterium capable of producing the extracellular polysaccharide gellan gum [19, 20]. This heteropolysaccharide, with an estimated molecular mass of 500 kDa, is based on an acetylated tetrasaccharide repeat unit composed of D-glucose, L-rhamnose, and D-glucuronic acid at a ratio of 2:1:1 [16, 26]. Gellan gum has a number of functional properties that can be readily modified; these include texture versatility, gum stability under heating or pH variations, setting temperature and melting point flexibility, high clarity, dispersibility, and compatibility, as well as low concentration requirements and flavor release [13]. Because of its excellent rheological characteristics and unique structure, gellan gum has a wide range of applications in food, pharmaceutical, and other industries as a texturizing, stabilizing, thickening, emulsifying, and gelling agent [14, 28]. Gellan gum has been used mainly as a stabilizer and suspending agent in the food industry [31]. Furthermore, its potential use as a replacement for gelatin

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and agar makes it the most important commercialized bacterial exopolysaccharide [3], and market demand for it has been increasing.

Although gellan gum exhibits excellent properties, its difficult and costly downstream processing impairs its economic viability. Studies have attempted to improve gellan production [37], as well as to optimize culture conditions [18, 25]. During the gellan fermentation process, the viscosity of the culture medium dramatically increases and reaches a high value at the end of the process. This characteristic not only affects fermentation efficiency but also makes it difficult to separate gellan from the fermentation broth [9]. The general procedure for recovering the biopolymer involves diluting the fermentation broth to reduce viscosity, followed by centrifugation to separate the cells. Isopropanol and cold ethanol are the primary alcohols that have been used for precipitating the biopolymer [4], as well as removing pigments and other impurities. This recovery method necessitates large amounts of alcohol, thereby increasing production costs.

In this study, we cloned the *crtI* gene involved in the carotenoid biosynthetic pathway of *S. elodea* ATCC 31461. Using gene inactivation and HPLC analysis of carotenoids, we determined that this gene encodes phytoene desaturase, which catalyses phytoene to lycopene. We further analyzed gellan gum production using *crtI* gene knockout mutants. A recovery method that is based on the colorless fermentation broth of the *crtI* gene knockout mutant and requires less alcohol precipitation was also investigated.

Materials and methods

Strains, culture media, and culture conditions

Sphingomonas elodea ATCC 31461, purchased from the American Type Culture Collection, and ΔcrtI (*crtI* gene knockout mutant) derived from ATCC 31461 were used in this study. As hosts in genetic manipulation, *Escherichia coli* DH5α and *Escherichia coli* S17-1 were cultured aerobically in LB on a shaker at 200 rpm and 37°C [24]. Unless otherwise specified, the *Sphingomonas* strains were cultured in YPG medium (2% glucose, 0.3% yeast extract, and 0.5% g peptone) at 30°C for 72 h and stored at 4°C. The preculture medium contained: 0.5% NaCl, 0.1% yeast extract, 0.5% peptone, 0.3% beef extract, and 0.5% sucrose. The solid medium contained agar at 15 g l^{-1}. The grown cells were collected by centrifugation. Gellan fermentation was carried out in a medium contained: 0.02% yeast extract, 3% sucrose, 0.2% soy protein, 0.15% K_2HPO_4, 0.1% KH_2PO_4, and 0.06% $MgSO_4{\cdot}7H_2O$. The inoculum was prepared in 250-ml Erlenmeyer flasks containing 50 ml of the preculture medium at 30°C and 200 rpm for 24 h. The fermentations were carried out by inoculating 10% inoculum in 500-ml Erlenmeyer flasks and incubating in a rotary shaker at 30°C and 200 rpm for 48 h. The pH of all the media was adjusted to 7.2 before sterilization. When required, antibiotics were used in the following concentrations (μg ml^{-1}): streptomycin (25), kanamycin (50), ampicillin (15 for *Sphingomonas*, 100 for *E. coli*), and tetracycline (5 for *Sphingomonas*, 25 for *E. coli*).

Batch fermentations were studied in a 6.7-l stirred bioreactor (BiosCADA lab R' ALF plus, Bioengineering AG, Switzerland) with a 4.5-l working volume. The process set points were pH, 7.0 ± 0.1; temperature, 30°C; agitation rate, 400 rpm; and aeration rate, 1 vvm. Both agitation and aeration were kept constant throughout the process. The pH was controlled by the automatic addition of 3 M NaOH or 3 M HCl. Cell growth was measured by optical density at 640 nm of the culture broth with appropriate dilution.

Cloning of the *crtI* gene

To clone the *crtI* gene, the amino acid sequences of several bacterial phytoene desaturases were compared, and two highly conserved regions were found. The corresponding DNA sequence between these two regions was amplified from the genomic DNA of *S. elodea* with degenerate primers DP1 (5′ACSTTYGAYGCNGGBCCSACS3′) and DP2 (5′VCCNGCVCCSGGRTGSGTVCCNGCVCC NAC3′). Sequence analysis and alignment of the degenerate PCR product (about 1,180-bp long) showed that its translated sequence was homologous to bacterial phytoene desaturases from other species. Thus, the amplified product was assumed to be part of the sequence of the *crtI* gene. SiteFinding-PCR was employed to retrieve the full length of the ORF sequence, as previously described [33]. Specific primers were designed according to the 3′-end and 5′-end sequence of the degenerate PCR product. The primer sequences are as follows: 5′GSP1, 5′ATTAGGACAGG CGGTAGAAGGG3′; 5′GSP2, 5′TCCAGCGTCACATC TTCCGAG3′; 3′GSP1, 5′TCGTTCGACGTTCTACGTG3′; and 3′GSP2, 5′ACTATGCGCCCACCGACTTCC3′. From SiteFinding-PCR, a 2.8-kb fragment containing the entire *crtI* gene (1479 bp) was obtained by assembly of the PCR products.

Construction of plasmids and ΔcrtI mutant

To construct the *crtI* gene knockout mutant, we used the suicide vector pLO3 provided by Lenz [22]. Primers crtI-FW1 (5′AGTGAGCTCCGAGGACACCTATTACA G3′) (*Sac*I site underlined) and crtI-RV1 (5′TATCTA GAGCGCATCAGCGGCTCCAG3′) (*Xba*I site underlined) were used to amplify 555 bp of the adjacent upstream

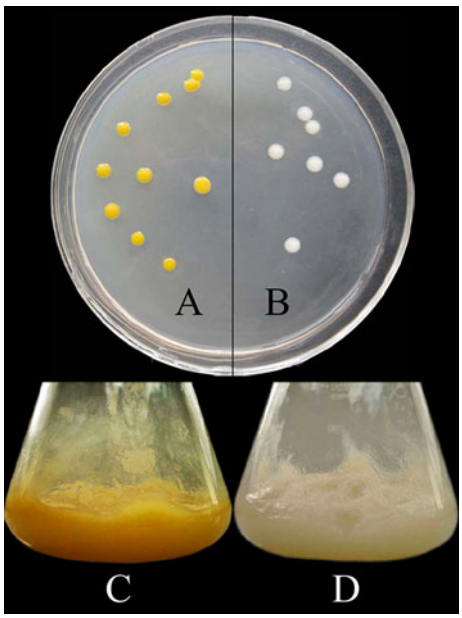

Fig. 1 Pigmentation of ATCC 31461 and ΔcrtI strains. Colony morphology (72 h of cultivation on YM plate at 30°C) of ATCC 31461 (**a**) and ΔcrtI (**b**). Fermentation broth (48 h, at 30°C and 200 rpm) of ATCC31461 (**c**) and ΔcrtI (**d**)

flanking sequence. Primers crtI-FW2 (5′CGTCTAGATT GGCGTGAACATCCAAGCC3′) (*Xba*I site underlined) and crtI-RV2 (5′GACTGCAGAAGCCGACCTTGCCC ATAT3′) (*Pst*I site underlined) were used to amplify 699 bp of the adjacent downstream flanking sequence. The PCR products were digested and cloned into pLO3 to construct the recombinant plasmid pLO3-I. Recombinant DNA techniques were performed following standard methods [29] or as instructed by suppliers. The recombinant plasmid was transformed into *E. coli* S17-1, and the resultant strain was named *E. coli* S17-1/pLO3-I.

Transfer of pLO3-I from *E.coli* S17-1/pLO3-I to *S. elodea* was performed by triparental filter mating using *Escherichia coli* HB101/pRK2013 as the helper strain [8, 10]. The triparental filter mating method was performed as described [15]. The ΔcrtI mutants were isolated by selecting for loss of sucrose (8%) toxicity, encoded by the *sacB* gene of pLO3 [12], followed by PCR screening for those with the correct excision. Diagnostic PCR, using primers I1 (5′GTCTATTGCCTGCCGTTC3′) and I2 (5′GGCTGATAGCGTGTTTTC3′), was used to confirm each constructed strain.

The genetic stability of the mutants was determined by continuous passage experiment. Mutants were continuous inoculated into fresh preculture medium and incubated at 30°C and 200 rpm for 24 h.

Isolation and analysis of carotenoids

The pigments were extracted with acetone/methanol (2:1) under N_2 at 4°C for 4 h and centrifuged to collect the supernatant. The pigments were re-extracted three times to ensure that all pigments were in the liquid phase. Subsequently, the supernatants were evaporated under a vacuum to remove all possible traces of the solvents and then saponified as described [17]. The pigment extracts were stored under N_2 at −80°C after subsequent drying under a vacuum. One part of the carotenoids was resuspended in 1 ml methanol and analyzed by HPLC using an Agilent 1200 high-performance liquid chromatography (Agilent, USA) equipped with a diode-array detector. A Hypersil ODS-C18 column (4.6 mm × 250 mm, 5 μm) was used

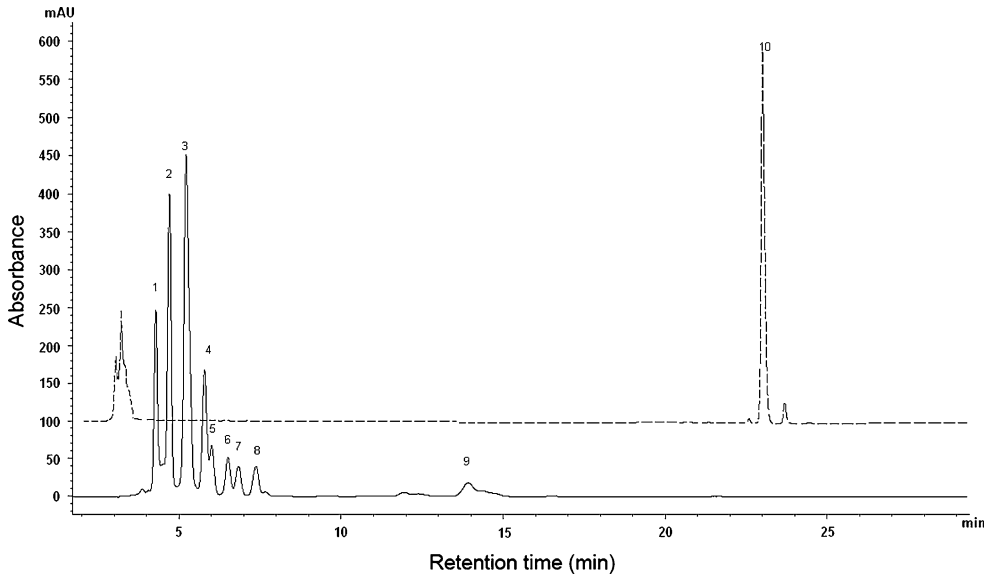

Fig. 2 HPLC elution profiles of the pigments extracted from the wild-type (*solid line*, absorbance at 475 nm) and the *crtI* gene knockout mutant (*dashed line*, absorbance at 284 nm) of ATCC 31461. No peaks were detected in the elution profiles of ΔcrtI by HPLC at 475 nm. *Peaks 1–9*, nostoxanthin and its metabolic intermediate. *Peak 10*, phytoene

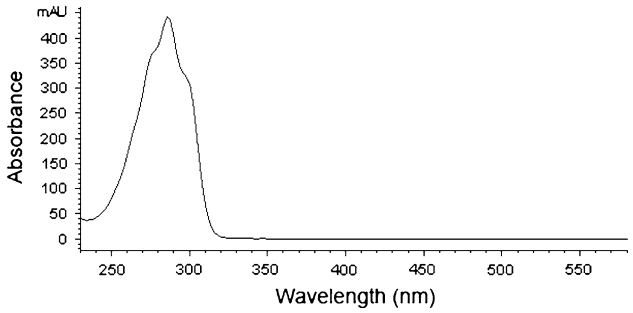

Fig. 3 Absorption spectra of the *peak-10* carotenoid in methanol. The absorption maxima were 276 (shoulder), 284, and 294 nm (shoulder)

and eluted with H_2O/acetonitrile/methanol/2-propanol (85:10:5, by volume) at a flow rate of 1 ml min^{-1}. The LC-mass spectrum of the carotenoids was recorded on an Agilent 1200 series LC/MSD Trap SL mass spectrometer system using atmospheric pressure chemical ionization (APCI). The system was controlled and data were analyzed on a computer equipped with LC/MSD Trap Software 4.2 (Bruker). The MS parameters were set as follows: ion source, APCI; nebulizer, 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 l min^{-1}; APCI temperature, 350°C; HV capillary, 3,500 V; and scan range, 100–2,200 *m/z*. Nitrogen was used as the drying gas.

Polysaccharide and viscosity determinations

The fermentation broth was diluted with distilled water and heated for 15 min in a boiling water bath, then centrifuged at 15,000 × *g* for 45 min at 25°C [36]. The supernatant was then added with three volumes of alcohol followed by vigorous mixing and kept overnight at 4°C. After several washings with alcohol, the polymer was separated by centrifugation at 10,000 × *g* for 45 min. The amount of gellan produced was determined by measuring the dry weight of the polymer recovered from the culture medium [5]. Viscosity of the culture broth was determined at 25°C using a Brookfield viscometer model RVDV-II + P (No. 4 spindle at 60 rpm).

Recovery of gellan gum

Both broths from ATCC 31461 and ΔcrtI were first heated to 95°C for 30 min to kill the cells and deactivate the enzymes. The broths were then treated with enzymes, including lysozyme (20,000 unit l^{-1}) and protease (100,000 unit l^{-1}), at 37°C for 2 h, to degrade the solid cellular debris to soluble compounds, thus improving transmittance. After this, two volumes of 99% alcohol were added to the broth of ATCC 31461 as previously described [21]. Alternatively, the broth of the ΔcrtI after enzyme treatment was mixed with 10 g l^{-1} $CaCl_2$ solution (dissolved in appropriate ddH_2O before use) followed by precipitation with 30% alcohol (v/v) instead of two volumes of 99% alcohol. Finally, the mixture was filtered in a plate and frame filter press to recover the gellan.

Results and discussion

Sequence analysis of the *crtI* gene

The complete nucleotide sequence of the 2.8-kb DNA fragment containing the *crtI* gene was deposited in GenBank under accession number HQ202920. The phytoene desaturase gene (*crtI*) contained 1,479 bp and encoded a 492-amino acid polypeptide. Similar to other *Sphingomonas* genes, *crtI* exhibits high G + C average content (67%) and a high frequency of G or C in the position of codons [35].

An alignment of the deduced amino acid sequence of *crtI* in the GenBank database reveals that this CrtI shares a larger degree of sequence identity with the phytoene desaturase of *Erythrobacter longus* (68%), *Paracoccus haeundaensis* (60%), and many other CrtI, suggesting the similar role of this protein in carotenoid synthesis. The

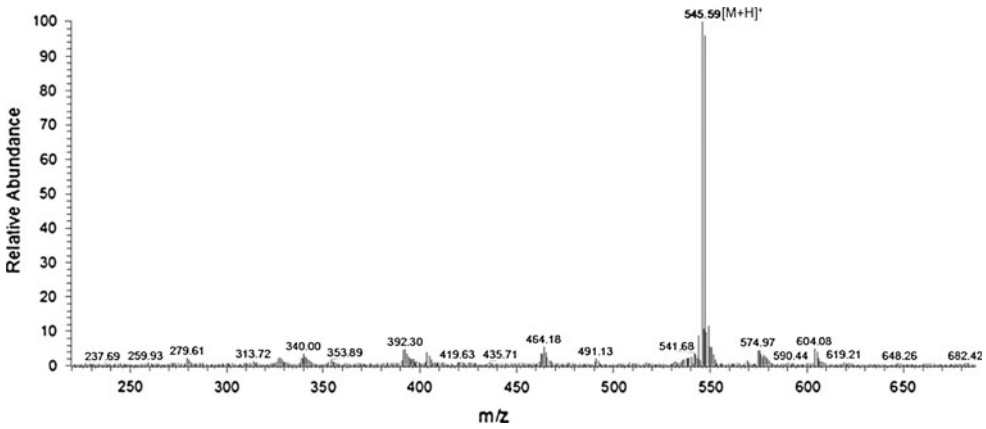

Fig. 4 Mass spectrum with APCI of the peak-10 carotenoid. Ion source, APCI; nebulizer, 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 l min^{-1}; APCI temperature, 350°C; HV capillary, 3,500 V; and scan range, 100–2,200 *m/z*. Nitrogen was used as the drying gas

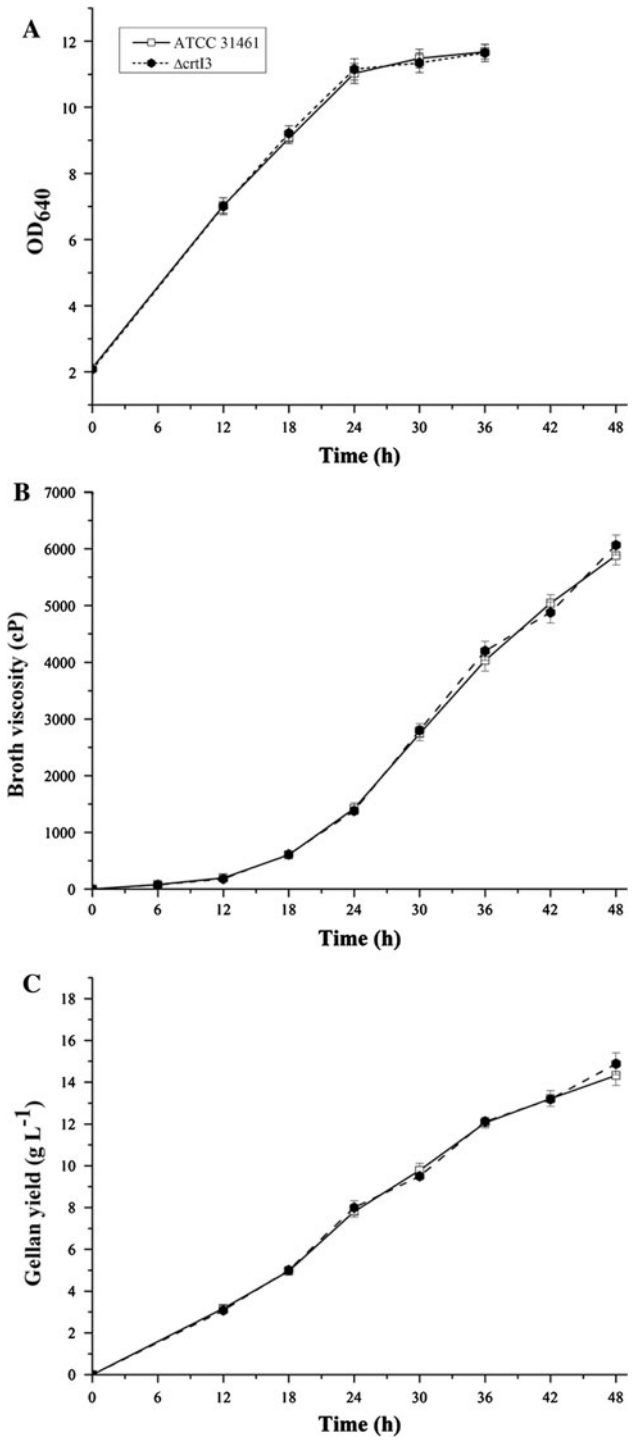

Fig. 5 Fermentation profile of the wild-type and ΔcrtI3 strains in 6.7-l bioreactors for 48 h. Cells were grown under the same fermentation conditions (agitation 400 rpm, aeration rate, 1 vvm). **a** The growth properties; **b** broth viscosity; **c** gellan production. Values are the averages of three parallel samples (*error bars* indicate standard deviations)

motif in the N-terminal region, found in most bacterial CrtI enzymes [27] and perfectly matching the requirements for $\beta\alpha\beta$-binding fold for dinucleotide binding in the desaturation reaction [2, 6], is also present in the sequence of this protein. A second conserved region, located at the C-terminal end of CrtI, is also found in the CrtI of *S. elodea* ATCC 31461. This consensus sequence, which also has a $\beta\alpha\beta$-binding fold, represents conserved structures or functional features required for the interaction between the substrate and the desaturase [1]. These results suggest that phytoene desaturases from different bacteria are conserved and may come from the same ancestor.

Phenotype of ΔcrtI mutants

The predicted phytoene desaturase gene was knocked out, and the mutants were confirmed by PCR and DNA sequencing. Compared to the yellow-pigmented wild-type strain, ΔcrtI mutants were colorless (Fig. 1).

Carotenoid identification

Subsequently, the carotenoid compositions of the wild-type strain and mutants were analyzed by HPLC (Fig. 2). The wild-type strain exhibited several peaks at 475 nm. The major peaks were isolated and identified as nostoxanthin [17] and its metabolic intermediate. No peaks were detected in the elution profiles of ΔcrtI by HPLC at 475 nm, and a main peak (peak-10) was detected at 284 nm. The absorption maxima of peak-10 carotenoid in methanol were 276 (shoulder), 284, and 294 nm (shoulder) (Fig. 3); this absorption spectrum is compatible with that of phytoene [32]. LC-APCI-MS analysis of the peak-10 carotenoid reveals the presence of the phytoene $[M + H]^+$ ion at *m*/*z* 545.59 (Fig. 4); this property was consistent with that of phytoene reported in the literature. Together with the HPLC elution time, absorption spectrum, mass spectrum, and comparison with standard compounds, this peak-10 carotenoid was identified as phytoene [34]. These results show that the mutant inhibited lycopene synthesis and resulted in phytoene accumulation, indicating that an inactive *crtI* gene was responsible for the loss of pigmentation in the ΔcrtI mutants.

Gellan gum production and cell growth of the ΔcrtI mutants

Seven independent isolates of the ΔcrtI mutants were chosen to investigate gellan gum production. In general, the yield of gellan gum production by the ΔcrtI mutants was almost the same as that produced by the wild-type strain. Continuous passage experiment indicated that all of the seven mutants were stable after 20 continuous passages. Among the seven mutants, ΔcrtI3 exhibited the highest gellan yield. Thus, the mutant ΔcrtI3 was selected as a preferable gellan-producer for further studies. To further

Table 1 Comparison of the effects of the two recovery methods

Strain	Recovery method	Recovery yield of gellan (%)	Residual carotenoid content (mg g^{-1})
ATCC 31461	The new recovery method	94.5	0.57 (yellow carotenoids)
	The conventional method	93.8	0.06 (yellow carotenoids)
ΔcrtI3	The new recovery method	94.2	0.44 (colorless phytoene)
	The conventional method	93.6	0.04 (colorless phytoene)

evaluate and compare the wild-type and ΔcrtI3 strains, fermentation experiments were carried out in bioreactors. As shown in Fig. 5b and c, broth viscosity and gellan yield of the two strains during the fermentation process was almost identical. Additionally, the gellan gum yielded by ΔcrtI3 was 14.88 ± 0.52 g l^{-1}, almost the same as that produced by the wild-type strain (14.32 ± 0.48 g l^{-1}). Consequently, the ΔcrtI mutant can be used as an industrial gellan gum-producing strain in place of ATCC 31461. The cell growth of ATCC 31461 and the ΔcrtI3 mutant were compared by measuring OD_{640} (Fig. 5a). There was no obvious difference in cell growth between the two strains. These observations suggest that *crtI* gene knockout did not influence gellan gum production and cell growth.

Effects of the new recovery method

The production of gellan gum was a highly viscous microbial fermentation process, which led to difficulties in gellan gum recovery [4]. Moreover, the carotenoids and gellan gum produced by ATCC 31461 were closely bound together, which also makes it difficult to recover gellan gum. Accordingly, large amounts of alcohol were often required in the conventional recovery method to dilute the fermentation broth [23], precipitate the biopolymer [4], and remove the carotenoid pigments. To the best of our knowledge, all gellan gum recovery methods reported include repetitive precipitation, in which a non-aqueous solvent, such as isopropanol, methanol, ethanol, dioxane, acetone, or tetrahydrofuran, is used [4]. These methods require large quantities of organic solvents. Consequently, the significantly high cost of the organic solvents makes this process economically unfavorable.

Based on the colorless fermentation broth of the ΔcrtI mutants (Fig. 1d), we designed and analyzed an economical recovery method, as described above. In this method, a 10 g l^{-1} $CaCl_2$ solution was used to flocculate gellan, followed by precipitation with 30% alcohol (v/v) instead of two volumes of 99% alcohol. Through this new method, the recovery yield of gellan gum (including the unavoidable impurities) obtained from ΔcrtI3 was 94.2%, quite close to that from ATCC 31461 recovered via the conventional method (93.8%). In order to further compare the effects of the two recovery methods, we calculated the residual carotenoid content of dried product. As shown in Table 1, 30% alcohol can not remove most of the yellow carotenoid pigments of ATCC 31461. The residual carotenoid pigments confer a dark yellow color to the product and this will affect the purity and its application performance, so the new recovery method was invalid for the fermentation broth of ATCC 31461 with the presence of yellow pigment in the dried product. The great advantage of the mutants for gellan recovery was that it was non-pigment, and this new recovery process did not include the deacylation procedure; hence, direct recovery from the broth yields the gum in its high acyl form. Yuan et al. pointed out that high acyl gellan gum is calcium stable [38]. Therefore, the residual phytoene content and Ca^{2+} would have a negligible effect on the application performance of gellan gum.

In conclusion, the *crtI* gene encoding phytoene desaturase in the carotenoid biosynthetic pathway of *S. elodea* ATCC 31461 was successfully cloned and identified. Gellan gum produced by the ΔcrtI mutants was almost the same as that produced by the wild-type strain. Consequently, the ΔcrtI mutant can be used as an industrial gellan gum-producing strain in place of ATCC 31461. In addition, the new gellan gum recovery method, based on the colorless fermentation broth of the ΔcrtI mutants, requires only a small amount of alcohol (30%), presenting the possibility of reducing gellan gum downstream purification costs.

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