Isolation of putative geranylgeranyl pyrophosphate synthase type III gene from *Rhodosporidium toruloides* VN1

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Abstract
Carotenoids are valuable pigments with significant applications in industry, thus, the demand for these natural compounds is increasing, especially carotenoids from natural sources. Rhodospordidium toruloides, a red yeast chassis, has the ability to produce carotenoids in high content. But the functions of many genes involved in the carotenoids biosynthesis pathway in this yeast remain poorly understood. In this research, genomic DNA from R. toruloides VN1 was successfully extracted through an SDS-proteinase K method.

The DNA was then used to amplify the putative geranylgeranyl pyrophosphate synthase gene type III (crtE), the product was sequenced using Sanger method and this sequence was cloned into an Escherichia coli strain by a recombinase system for further investigation.

Keywords: *Rhodosporidium toruloides VN1, crtE* (*BTS1*), recombinase assembly.

Introduction
Carotenoids are natural pigments that were widely used in industries, pharmaceuticals as food coloring, antioxidants agents due to their bioactivities and pigmentation properties. Owing to their widespread applications, carotenoid-producing industry is expected to grow stedily in near future. Recently, carotenoids are for 80 - 90 % of global market, which raises environmental and health concerns[2,23].

Consequently, there is a growing demand of carotenoids from natural sources. In nature, carotenoids could be found in plants, microalgae, fungi and bacteria[9,10] while plant-based extraction is unfavorable due to limiting feedstock availability. Microbial carotenoids production presents a more promising alternative. Although microalgae cells accumulate highest quantities of carotenoids (up to 10 % dry cell weight)[15], yeast offers advantages in growth rates, cell biomass titer and abilities to utilize agro-waste as subtrates[2,3].

Red yeast *Rhodosporidium toruloides* (also known as *Rhodotorula toruloides*) or *R. toruloides*, is a promising host for carotenoid production, with an accumulation capacity of up to 3 mg/g dry cell weight[11]. This yeast has the potential to utilize agricultural by-products as carbon sources and the ability to tolerate compounds commonly found in lignocellulosic hydrolysates, such as p-coumaric acid and ferulic acid[8,22]. In our previous research, we isolated *R. toruloides* VN1 which exhibits high yield of carotenoids and shows a unique ability to synthesize astaxanthin, the most valuable pigment of carotenoids[1,18] among *R. toruloides* species. *R. toruloides* VN1 genome was sequenced by whole genome shotgun, however, the biosynthesis pathway to astaxanthin in this red yeast remains unknown[18].

Carotenoids biosynthesis pathway in *R. toruloides* includes three main steps: first isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are synthesized via MVA pathway from acetyl-CoA precursor. Next, IPP and DMAPP are converted into geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) and then, phytoene through GPP synthase, FPP synthase, GGPP synthase and phytoene synthase respectively. Finally, carotenoids such as γ-carotene, β-carotene, torulene, torularhodin etc. are generated with the participation of phytoene dehydrogenase (CAR1) and lycopene cyclase (CAR2), in succession[2,21].

GGPP synthase, encoded by *crtE* (*BTS1*), is one of the key enzymes in directing carbon flux toward carotenoid biosynthesis[20]. Thus, more efforts should be made to investigate the capacity of GGPP synthase and its vital role, particularly in *R. toruloides* cells. In this research, we isolated putative *crtE* gene encoding GGPP synthase type-III from *R. toruloides* VN1 genome, then, this sequence was cloned into a plasmid to result a recombinant *E. coli* DH5α cell line using a recombinase system.

Material and Methods
Microorganisms and cultures: *R. toruloides* VN1 were grown at room temperature at 200 rpm in Hansen medium (50 g/L glucose, 10 g/L peptone, 3 g/L KH_2PO_4, 3 g/L $MgSO_4$). *E. coli* DH5α was used for plasmid construction and cultivated at 37 ºC at 200 rpm in Luria-Bertani broth (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl), supplemented with 50 µg/mL kanamycin, 15 g/L agar if necessary. Super optimal broth with catabolite repression (SOC) for recovery of *E. coli* cells after heat-shock transformation includes: 20 g/L peptone, 5 g/L yeast extract, 3.6 g/L glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

pCEV-G2-Km ymNeongreen was a gift from Bas Teusink (Addgene plasmid # 193959; http://n2t.net/addgene:193959

; RRID:Addgene_193959). Reagents were purchased from the following suppliers: XhoI and rCutSmart™ Buffer 10X from New England Biolabs (UK), HotStarTaq Master Mix Kit from Qiagen (USA), proteinase K from ABT (Viet Nam). *e*Clone kit was a product of Molecular Biotech Lab., University of Science, Vietnam National University of Ho Chi Minh City.

DNA extraction: Briefly, *R. toruloides* VN1 was cultured in Hansen medium for 12-16 hours at room temperature and harvested by centrifugation at 6000 xg for 5 minutes, then, washed in TE buffer 10X (Tris-HCl 100 mM, EDTA 10 mM, pH 8.0) for three times. Cells were incubated at 50 ºC for an hour with 200 µL lysis buffer (Tris-HCl 10 mM, EDTA 1 mM, SDS 0.5 %, NaCl 100 mM) and 1 µL proteinase K, followed by centrifugation at 10000 xg for 5 minutes. Supernatant was inversed with 200 µL PCI (phenol: chloroform: isoamylalcohol = 25:24:1) (pH 8.0) and aqueous layer was collected by centrifugation at 13000 xg and 4 ºC for 10 minutes. This step was repeated twice.

Next, the upper layer was transferred to a tube containing ice-cold ethanol 99 % (to get the final concentration of ethanol of 70 %), the tube was kept at - 20 ºC for at least 30 minutes to precipitate DNA. Subsequently, genomic DNA was pelleted by centrifugation at 13000 xg and 4 ºC for 10 minutes and washed with 500 µL ethanol 70 %, collected by re-centrifugation at 13000 xg and 4 ºC for 5 minutes.

The DNA pellet was dried at 50 ºC for 10 minutes and then resuspended in 50 µL TE buffer 1X for storage at - 20 ºC. The concentration of DNA was analyzed by 1 % agarose gel electrophoresis using GelAnalyzer 19.1.

Polymerase chain reaction composition: The putative GGPP synthase gene type III was amplified from *R. toruloides* VN1 DNA genome using F_4, R_3 primer (Table 1). Polymerase chain reaction (PCR) was performed in 20-µL-volume consisting of 10 µL master mix 2X, 20 ng DNA template and 0.5 µM of each primer. The PCR cycling condition consists of an initial denaturation step at 95 ºC for 15 minutes (follow manufacturer's instructions), then 40 cycles with denaturation at 95 ºC for 30 seconds, anealing at 54 ºC for 30 seconds and extension at 72 ºC for 1 minutes 30 seconds, with final extension at 72 ºC for 10 minutes. The amplicons were evaluated by 1 % agarose gel electrophoresis using GelAnalyzer 19.1.

Preparation of competent *E. coli* DH5α cells: The preparation of competent *E. coli* DH5α cells was carried out prior to transformation and was adapted from the procedure of Green and Sambrook[6].

First the cells were collected at OD_{600nm} of 0.35 by centrifugation at 8000 xg for 5 minutes, followed by washing in $CaCl_2$ (100 mM) twice. The *E. coli* cells were incubated at 4 ºC in $CaCl_2$ 100 mM for 2 hours before performing transformation.

Recombinant plasmid construction: Plasmid pCEV-G2-Km ymNeongreen was digested by XhoI in rCutSmart™ buffer with total volume of 30 µL at 37 ºC for 16 hours, following manufacturer's instructions. Linearized plasmid was then purified by ethanol precipitation and re-suspended in H_2O.

Recombination reaction was set up in a 15 µL volume, containing 1.5 µL *e*Clone buffer 10 X, 0.5 µL *e*Clone enzyme, 100 ng of purified linearized plasmid and a PCR fragment-to-plasmid molar ratio of 3 : 1. The mixture was incubated at 37 ºC for 30 minutes and then stored at - 20ºC.

To perform the transformation, 2.5 µL of cloning reaction was added to 100 µL competent *E. coli* DH5α. The mixture was incubated at 4 ºC for 10 minutes, then transferred to 42 ºC water bath for 1 minute 30 seconds, followed by a 2-minute incubation at 4 ºC. Afer heat shock, *E. coli* cells were recovery by adding 1 mL SOC medium, incubating at 37 ºC with shaking at 200 rpm for an hour.

The cells were then collected by centrifugation at 5000 xg for 5 minutes and plated on LB agar with kanamycin. Colonies that appeared on the plate, were screened using colony PCR. Selected colonies were cultured in LB with kanamycin and DNA plasmid was extracted via alkaline lysis with SDS, as previously described[6] for sequencing[16]. PCR products were analyzed by 1 % agarose electrophoresis using GelAnalyzer 19.1.

Results and Discussion

Isolation of putative geranylgeranyl pyrophosphate synthase type III gene: In this study, DNA genome was extracted from *R. toruloides* VN1 cells using a simple lysis buffer containing SDS with addition of proteinase K and purified by ethanol precipitation.

Table 1
List of primers

Primers	Sequence	Target
F_4	ATGTCGCTGGACTGGTACGA	Yeast genomic DNA
R_3	GACGGCTACATTCAGACTTTGG	Yeast genomic DNA
F_c	GAACCCTTAATATAAATGTCGCTGGACTGGTACG	Yeast genomic DNA
R_c	GTCGAAAACGAGCTCCATCGCCTCAACCTCAAC	Yeast genomic DNA

The underline indicates the homologous tail for recombinase-cloning.

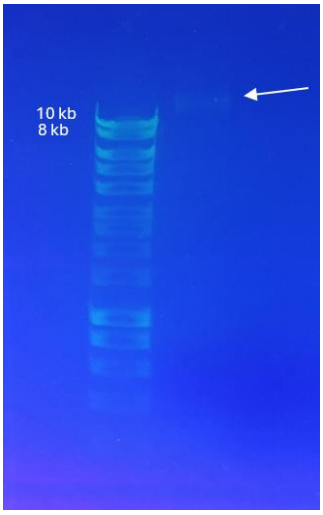

Figure 1: DNA genome from *R. toruloides* VN1
The arrow points to the genomic DNA band.

On the gel, there was a blurred band with large size (> 10000 bp), thus, following this procedure, we have successfully isolated DNA genome from *R. toruloides* VN1 biomass (Fig. 1). It is important that cells should be washed several times in EDTA-contain buffer to suppress the activity of intracellular nucleases[4]. Proteinase K is also crucial in *R. toruloides* VN1 DNA extraction[14]. In this study, we used 1 µL proteinase K for extraction but it remains to be investigated that what is the best concentration. The concentration of DNA is still low (< 50 ng/µL) compared to other methods[6,7,12,17].

Putative *crtE* sequence could be acquired on DDBJ/EMBL/GenBank databank under accession number SJTE00000000, contig SJTE01000075. This sequence was found to be highly homologous to previously reported geranylgeranyl diphosphate synthase genes and was therefore used for primer design. PCR reaction was performed to amplify *crtE* from genomic DNA of *R. toruloides* VN1. Furthermore, the extracted DNA remained stable for at least 3 months (data not shown).

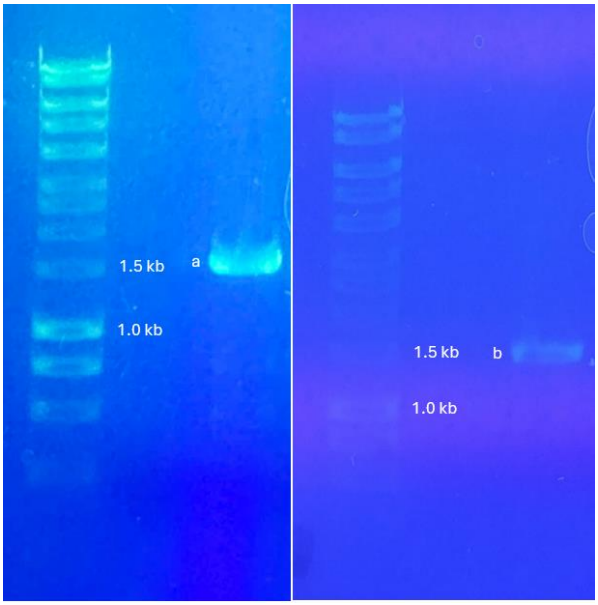

Figure 2: PCR result for the isolation of putative *crtE*
There was a band at 1500-bp-size in both agarose gel (a, b); (a) was the product amplified using F_4, R_3, (b) was the cloning fragment amplified using F_c, R_c.

PCR reaction amplified a product at the size of putative *crtE* (1509 bp) using either primer F_4, R_3 or F_c, R_c pairs. This indicates that the *crtE* gene may have been isolated using these primers. The DNA extraction protocol of this study was simple and easy, as it did not require the use of lyticase, zymolyase or glass beads to break the yeast cells[4-6]. It was shown to be compatible with PCR-applications.

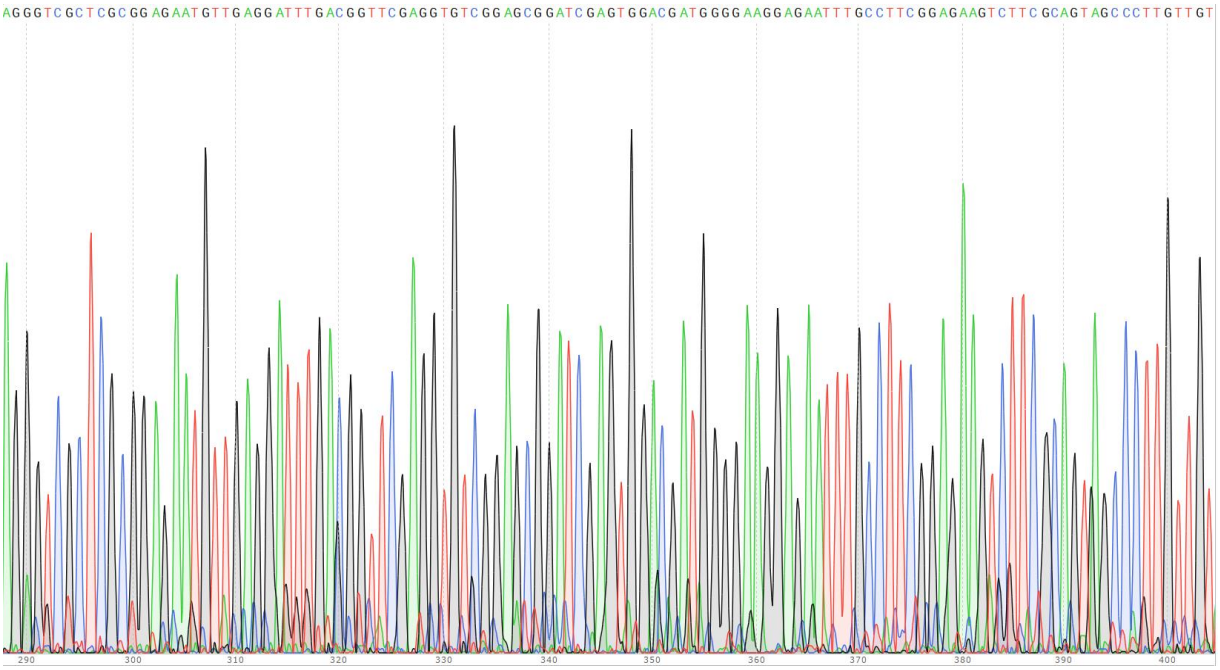

Figure 3: Sanger sequence chromatogram of *crtE* (from base pairs 1110 to 1220)

Recombination of *E. coli*: To transform recombinant vector to *E. coli* cells, the cells were first prepared competent chemically with $CaCl_2$, then, they were treated with heat-shock to receive the DNA. The procedure of this study should result in at least 10^5 transformants per µg plasmid pCEV-G2-Km ymNeongreen.

Sequence of putative geranylgeranyl pyrophosphate synthase type III gene: After PCR amplification, amplicon *crtE* was sequenced using Sanger method. Figure 3 shows the Sanger sequence result of *crtE* from 1110 to 1220 bp. Alignment of this sequence with the sequence published on SJTE01000075 was conducted and they are highly homologous (data not shown). Finally, we can conclude that *crtE* was achieved using our primer pairs (F_4, R_3 and F_c, R_c).

Conclusion

In this study, the genomic DNA of *R. toruloides* VN1 was successfully extracted from yeast culture using a simple lysis buffer containing SDS with proteinase K. Despite low yield of genomic DNA, this method is still compatible with PCR-applications. From the DNA isolated, we have amplified putative *crtE* gene and cloned to pCEV-G2-Km ymNeongreen vector by a recombinase enzyme, resulting in a recombinant *E. coli* DH5α cell line. This will further research the functions of gene in the carotenoid pathway of red yeast *R. toruloides* VN1.

Acknowledgement

We are grateful to Prof. Dr. Dang Thi Phuong Thao, (University of Science, Vietnam National University of Ho Chi Minh City) for kindly providing us the *e*Clone recombinant kit.

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(Received 09th December 2024, accepted 11th January 2025)