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Investigating antioxidant activity of carotenoid compound from *Paracoccus haeundaensis* SAB E11 at the cellular level in *Schizosaccharomyces pombe* ARC039 yeast model

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ABSTRACT

This study aimed to investigate the presence, characterization, and antioxidant properties of carotenoid compounds at the cellular level in the bacteria *Paracoccus haeundaensis* SAB E11. Furthermore, carotenoid synthesis was determined based on the presence of the *crt*Y gene. Characterization of the carotenoid isolated was performed through thin-layer chromatography (TLC), UV-Vis spectrum, high-performance liquid chromatography (HPLC), and Fourier-transform infrared spectroscopy (FTIR). Meanwhile, cellular antioxidant activity was examined using 2,2-diphenyl-1-picryhydrazyl (DPPH) radicals and the organism model yeast *Schizosaccharomyces pombe* ARC039. The results showed that *P. haeundaensis* SAB E11 had a band on the chromatogram at 163 bp, representing 54 amino acid residues of lycopene β-cyclase *crt*Y. TLC, UV-Vis, HPLC, and Fourier transform infrared spectroscopy (FTIR) analyses confirmed carotenoid presence, while the DPPH radicals assay identified antioxidant activity with an IC_{50} value of 203.90 ± 3.12 µg.ml^{-1} At the cellular level, carotenoid concentrations of 28 μg.ml^{-1} and 56 µg.ml^{-1} could enhance the stress tolerance phenotype of *S. pombe* ARC039 against 2 mM H_2O_2. Strong induction of mitochondrial activity was obtained following treatment with the 28 μg.ml^{-1} concentration. After H_2O_2 treatment, there was a 3.18 and 2.55-fold increase in the relative expression of catalase (*ctt1*) and superoxide dismutase (*sod2*) genes of *S. pombe* ARC039, respectively. These data showed that *P. haeundaensis* SAB E11 produced carotenoids with potential antioxidant capabilities manifested at the cellular level by inducing an adaptive oxidative response and mitochondrial activity, as well as increasing the expression of *ctt*1 and *sod*2 genes.

INTRODUCTION

Organismal metabolic processes are inextricably associated with various free radicals such as reactive oxygen species (ROS) and reactive nitrogen species. These highly reactive molecules can cause oxidative damage to cell constituents, including proteins, lipids, and nucleic acids. Oxidants are naturally neutralized by enzymatic (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic (e.g., ubiquinone, amino acids, and albumin) systems. However, an excess of oxidants in cells may generate oxidative stress, which is an imbalance implicated in various degenerative disorders, including cardiovascular, Alzheimer's, and Parkinson's diseases, as well as diabetes [1,2]. An exogenous antioxidant is required to maintain redox homeostasis and can be acquired from the bioactive compounds of plants, fungi, microalgae, and bacteria. This bioactive compound includes carotenoid, a secondary metabolite characterized by the conjugated double-bond structures, which provide attractive colors and reduce free radicals [3]. Carotenoid consists of at least six conjugated

double bonds in a polyene chain that can absorb light and enhance the intensity of the colors, generating shades of yellow, orange, and red [4]. Moreover, the elongated structure of the conjugated double bonds increases the electron-rich condition, which effectively eliminates the free radicals [5].

Carotenoid is a class of isoprenoid pigments with diverse distributions and derivatives, mainly produced by plants and can be found in microalgae, fungi, yeast, and bacteria. In non-photosynthetic bacteria, carotenoid primarily acts as self-defense mechanisms, including photoprotection and radiation protection [6,7]. These self-defense mechanisms are closely related to the ability of bacteria to control oxidants, which can threaten survival. The bacterium *Paracoccus* is widely reported to produce carotenoid with antioxidant properties. Carotenoid extracted from *Paracoccus mercusii* RSPO1 and *P. homiensis* BKA7 have been shown to inhibit 2,2-diphenyl-1-picryhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals [8,9]. Astaxanthin, carotenoid derived from *P. carotinifaciens*, has been comprehensively studied specifically for its health benefits [10–12]. However, investigations on the health-related applications of carotenoid compounds in *P. haeundaesis* remain limited. One study reported the antioxidant activity of cells-free supernatant-mediated gold nanoparticles (AuNPs) of *P. haeundaesis* BC74171[T][13], but the supernatant may not represent the carotenoid compound commonly found in cells.

Paracoccus haeundaesis SAB E11 was used in this study to investigate the carotenoid compound extracted from the bacteria cells. The orange pigment extract of *P. haeundaesis* SAB E11 showed antioxidant activity by effectively scavenging DPPH and ABTS radicals [14]. However, further study is needed to characterize the specific compound from *P. haeundaesis* SAB E11 and its antioxidant effects. To investigate these effects, *Schizosaccharomyces pombe* ARC039 was used as the model organism. This fission yeast has been widely applied in antioxidant assays at the cellular level as a model organism because of the similarity of the mRNA splicing process to metazoans and the identical mitochondrial inheritance mechanism to mammals [15,16]. The antioxidant response of *S. pombe* ARC039 to carotenoid derived from *P. haeundaesis* SAB E11 can be used as a reference for subsequent investigation of carotenoid impact on the biological systems of multicellular organisms.

MATERIALS AND METHODS

The microorganisms and media culture

Paracoccus haeundaensis SAB E11 used in this study was the collection of Prof. Aris Tri Wahyudi isolated previously from a marine sponge *Jaspis sp.* (Raja Ampat Island, Southwest Papua-Indonesia) [17]. Additionally, yeast *S. pombe* ARC039 (*h-leu-32 ura4-294*) was provided by Dr. Rika Indri Astuti. *Paracoccus haeundaensis* SAB E11 was cultured in seawater complete (SWC) medium (5 g peptone, 1 g yeast extract, 3 ml glycerol, 750 ml seawater, and 250 ml distilled water). Meanwhile, *S. pombe* ARC039 was routinely cultured in yeast extract supplement (YES) medium (5 g yeast extract, 30 g

glucose, 0.128 g histidine, 0.128 g leucine, 0.128 g adenine, 0.01 g uracil, 0.128 g arginine, and distilled water 1000 ml).

*crt*Y (lycopene β-cyclase) gene analysis

The genomic DNA of *P. haeundaensis* SAB E11 was extracted using the Presto[TM] Mini gDNA Bacteria Kit (Geneaid, Taiwan). The *crt*Y gene of this bacteria was amplified using the specific primer pair *crt*YF (5′-CCA GAA ATT CGT GGG CGT CG-3′) and *crt*YR (5′-ATC GGA ATA GCG CGT GTC CT-3′) [18], while the target DNA fragment was ±163 bp. Polymerase chain reaction was performed under conditions, including pre-denaturation at 95ºC for 5 minutes, followed by 35 cycles of denaturation, annealing, and extension at 95ºC, 58ºC, and 72ºC for 15 seconds, 20 seconds, and 20 seconds, with a final extension at 72ºC for 7 minutes. The confirmed amplicons were sequenced in FirstBase, Malaysia, then the *crt*Y nucleotide sequence was analyzed using BioEdit and identified with the BlastX program on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences of *crt*Y were deduced into amino acids on the Expasy website (https://web.expasy.org/translate/) and correlated using the Clustal Omega website (https://www.ebi.ac.uk/Tools/msa/clustalo/). Additionally, a phenetic tree was constructed in the MEGA.X program using neighbor-joining with 1000x bootstrap. A three-dimensional protein structure model was built on the SWISS-MODEL website (https://swissmodel.expasy.org/interactive), while visualization and analysis of superposition were conducted using UCSF Chimera-X 1.6.1. program.

Carotenoid band detection with thin-layer chromatography (TLC) analysis

TLC analysis was performed following the modified form of the procedure described by Chekanov *et al.* [19]. During this process, a total of 10 µl (5% in methanol) of the carotenoid extract was applied to a silica gel G60F-254 pre-coated aluminum-backed TLC plates (10 × 5 cm) (Merck, Germany) using CAMAG Linomat 5. The plates were eluted with *n*-hexane:acetone (7:3), and then the chromatogram was visualized under UV light at 254 and 366 nm. The carotenoid compound was detected by comparing the band obtained from the pigment extract with a β-carotene standard (C4582, Sigma-Aldrich, Darmstadt, Germany). This comparison was based on the colors and distance of the retardation factor (Rf) presented by the bands.

Characterization of carotenoid compound

Ultraviolet-visible (UV-Vis) spectrum, FTIR, and high-performance liquid chromatography (HPLC) were used to characterize carotenoid compounds. The maximum wavelength was determined based on the procedure by Hagos *et al.* [20] using a UV-Vis spectrophotometer (Metertch SP-8001). For FTIR analysis, carotenoid was pelleted with potassium bromide (KBr) [21] using a Tensor II FTIR Routine Spectrometer (Bruker Optics, USA), which provided an FTIR spectrum in the range of 500–4,000 cm^{-1}. Meanwhile, HPLC analysis was performed using a Hitachi L-2420 system to determine the

retention time of the carotenoid compound peak. The sample preparation was adapted from the method described by Singh *et al*. [22], and each analysis result for carotenoid characterization was compared to a β-carotene standard (C4582, Sigma-Aldrich, Darmstadt, Germany).

Antioxidant activity assay

Antioxidant activity was determined using DPPH radicals according to the method described by Batubara *et al*. [23]. Approximately 100 µl of carotenoid compound at various concentrations was introduced into microplate wells (Costar 96) and 100 µl of DPPH radicals solution (125 µM in methanol) was added, then the mixture was incubated for 30 minutes at 25–27°C in dark conditions. The absorbance of the samples was measured using an ELISA microtiter plate reader (EPOC, USA) at 517 nm. The inhibitory concentration (IC_{50}) was used to express antioxidant activity in the DPPH radical test.

Oxidative stress response assay (spot test)

The oxidative stress response of *S. pombe* ARC039 to carotenoid compound from *P. haeundaensis* SAB E11 was evaluated with spot assay [15]. *S. pombe* ARC039 was pre-cultured in YES broth medium for 24 hours at 27°C, and then transferred into 3 ml sterile YES broth medium at an initial OD_{600} of 0.05 as treatment culture. Carotenoid was added to the treatment culture at several concentrations based on the IC_{50} value of the DPPH radical assay (0.125x, 0.25x, 0.5x, 1x, and 2x). *S. pombe* ARC039 grown in 0.3 % (w/v) YES medium and ascorbic acid (0.1 µg.ml^{-1}) was used as the positive control, while yeast cultured in DMSO was the negative control. A β-carotene standard was applied as the reference sample, and each treatment culture was incubated for 24 hours at 27°C. The spot assay was performed on all treatment cultures at an initial OD_{600} of 1, followed by serial dilutions (10^{-1} to 10^{-4}). A total of 2µl of the $OD_{600}= 1$ and the serial dilutions were spotted in solid YES medium containing 0, 0.5, 1, and 2 mM H_2O_2 as oxidative stress treatment, then cell viability was observed after incubation for 72 hours at 27°C.

Mitochondria activity assay

The mitochondrial activity assay was conducted based on the best concentration of carotenoid compound that showed a response to oxidative stress, as described by Lesmana *et al*. [24]. Yeast treatment cultures were prepared as previously described for the oxidative stress response assay. Furthermore, the cells were rinsed using 0.1 M phosphate buffer pH 7 and added with 300 nM Rhodamin B. The mitochondrial activity was observed under the Olympus BX51 fluorescence microscope, with *S. pombe* ARC039 grown in 0.3% (w/v) YES medium serving as the positive control, while DMSO was the negative control.

Anti-oxidative genes expression analysis

Antioxidant gene expression analysis was conducted following the method applied by Cahlia *et al*. [25]. The optimal concentration of carotenoid compound in the oxidative stress tolerance assay was selected and prepared as described in the oxidative stress response assay. DMSO was used as the negative control, while the treatment cultures were grown for 24 hours, and 1 mM H_2O_2 was added an hour before harvesting the cell. The total RNA of *S. pombe* ARC039 was extracted with a Direct-zol RNA Microprep Kit (Zymo, USA). Furthermore, cDNA was obtained by reverse transcription using the ReverTraAce™ qPCR RT Master Mix Kit (Toyobo, Japan). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using a QuantStudio 5 instrument and Thunderbird SYBR qPCR master mix (Toyobo, Japan) as a fluorescent signal. The primer sequences of target genes were *ctt*1 (F5'-TCG TGA CGG CCC TAT GAA TG-3') (R5'-AGC AAG TGG TCG GAY TGA GG-3'), *sod*2 (F5'-ATT TGG AGG GAG GTT GCC-3')(R5'-GAT TGA TGT GAC CAC CGC CA-3'), and *act*1 (F5'-CGG TCG TGA CTT GAC TGA CT-3')(R5'-ATT TCA CGT TCG GCG GTA GT-3'). The qRT-PCR was performed in 40 cycles of denaturation at 95°C (15 seconds), annealing at 55°C (30 seconds), and extension at 72°C (30 seconds). Moreover, the relative expression level was determined by normalizing the cycle threshold (Ct) value of *ctt*1 and *sod*2 genes to the Ct of *act*1 as the housekeeping gene.
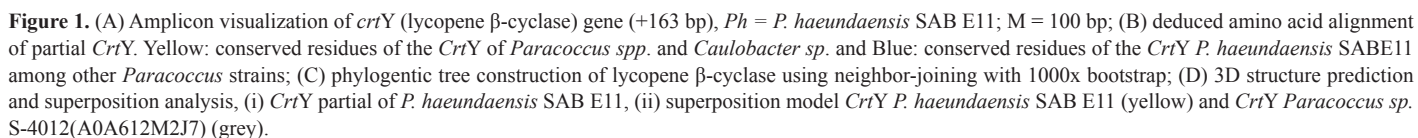
Statistical analysis

The experiment in this study was performed in three independent replicates, and the results were reported as mean ± standard deviation after conducting the statistical analysis with RStudio software version 2023.09.1 ± 494. One-way analysis of variance was used to compare the mean values at 95% confidence levels. Subsequently, Duncan's multiple range test was performed, with a *p*-value ≤ 0.05 being considered significant.

RESULTS AND DISCUSSION

The *crt*Y (lycopene β-cyclase) gene

Paracoccus haeundaensis SAB E11 was detected to comprise the *crt*Y gene (±163 bp), as shown in Figure 1A. The partial *crt*Y gene sequences were deposited in the DNA Data Bank of Japan under accession number LC813284. The sequences were highly similar to those of *P. mercusii* lycopene β-cyclase (similarity: 100%; *E*-value: 3e-17; accession number: WP_282029193.1). According to the result, the bacteria produced the protein lycopene β-cyclase, a critical enzyme in carotenoid biosynthesis forming two β-ionone cyclic rings at the end of lycopene polyene chains to generate β-carotene [26]. Furthermore, β-carotene is a precursor of oxygenated carotenoid (xanthophyll) and pro-vitamin A, which are essential for health. In this study, partial nucleotide sequences were deduced into 54 amino acids, among which the *crt*Y amino acid sequence of *P. haeundaensis* SAB E11 was correlated with the reference sequences of lycopene β-cyclase *crt*Y. The reference sequences were obtained from *Paracoccus* PAMC 22219 (WP_042246542.1), *P. mercusii* (WP_282029193.1), *Paracoccus sp*. 228 (WP_046000944), *Paracoccus sp*. 08 (WP_256481049.1), *Paracoccus sp*. N81106 (P54974.1), *Paracoccus sp*. NBH48 (WP_255522031.1), *P. haeundaensis* BC74171 [27], MULTISPESIES: *Paracoccus sp*. 08 (WP_127898119.1), and *Caulobacter sp*. (MBP7703974.1) as an out-of-group species. Based on Figure 1B, amino acid

Figure 1. (A) Amplicon visualization of *crt*Y (lycopene β-cyclase) gene (+163 bp), *Ph* = *P. haeundaensis* SAB E11; M = 100 bp; (B) deduced amino acid alignment of partial *Crt*Y. Yellow: conserved residues of the *Crt*Y of *Paracoccus spp.* and *Caulobacter sp.* and Blue: conserved residues of the *Crt*Y *P. haeundaensis* SABE11 among other *Paracoccus* strains; (C) phylogentic tree construction of lycopene β-cyclase using neighbor-joining with 1000x bootstrap; (D) 3D structure prediction and superposition analysis, (i) *Crt*Y partial of *P. haeundaensis* SAB E11, (ii) superposition model *Crt*Y *P. haeundaensis* SAB E11 (yellow) and *Crt*Y *Paracoccus sp.* S-4012(A0A612M2J7) (grey).

alignment showed that most of the partial residues of *crt*Y from *P. haeundaensis* SAB E11 were highly conserved in the *Paracoccus spp.* reference strains. However, certain different amino acid variations were observed with *Caulobacter sp.* The diversity of *crt*Y among species is regulated by the evolutionary process of carotenoid biosynthesis, which leads to mutations in nucleotide sequences. Lycopene β-cyclase is divided into three groups according to the amino acid sequences, namely *crt*Y and *crt*L type, heterodimeric type, and the unique CruA/CruP type. As *P. haeundaensis* SAB E11 belongs to the Proteobacteria phylum, it has *crt*Y type which is also found in *Pantoea agglomerans*, *Rhodobacter sphaeroides*, and *Sphingomonas spp* [27–30]. The *crt*L type is majorly present in Cyanobacteria, but heterodimeric lycopene β-cyclases (*crt*Yc and *crt*Yd) are found in *Brevibacterium linens* and *Mycobacterium aurum*.

Figure 2. Thin layer chromatograph (TLC) of carotenoid compound *P. haeundaensis* SAB E11 compared with β-carotene standard (C4582 Sigma-Aldrich), (A) in visible light, (B) UV light 254 nm, and (C) 566 nm. Ph: *P. haeundaensis*, S: β-carotene standard.

Meanwhile, CruA/CruP type is characteristic of the green sulfur bacterium (*Chlorobium tepidum*) [31].

A phenetic tree of lycopene β-cyclase *crt*Y was constructed based on the deduced amino acid sequences of *P. haeundaensis* SAB E11 and the reference strains. According to Figure 1C, *P. haeundaensis* SAB E11 comprising lycopene

β-cyclase *crt*Y was grouped along with other *Paracoccus* strains. As shown by node numbers 84 and 97, lycopene β-cyclase *crt*Y *P. haeundaensis* SAB E11 and *Paracoccus* PAM (WP042246542.1) were closely related to other *Paracoccus* strains, although clustered separately. The partial lycopene β-cyclase *crt*Y amino acid sequences of *P. haeundaensis* SAB
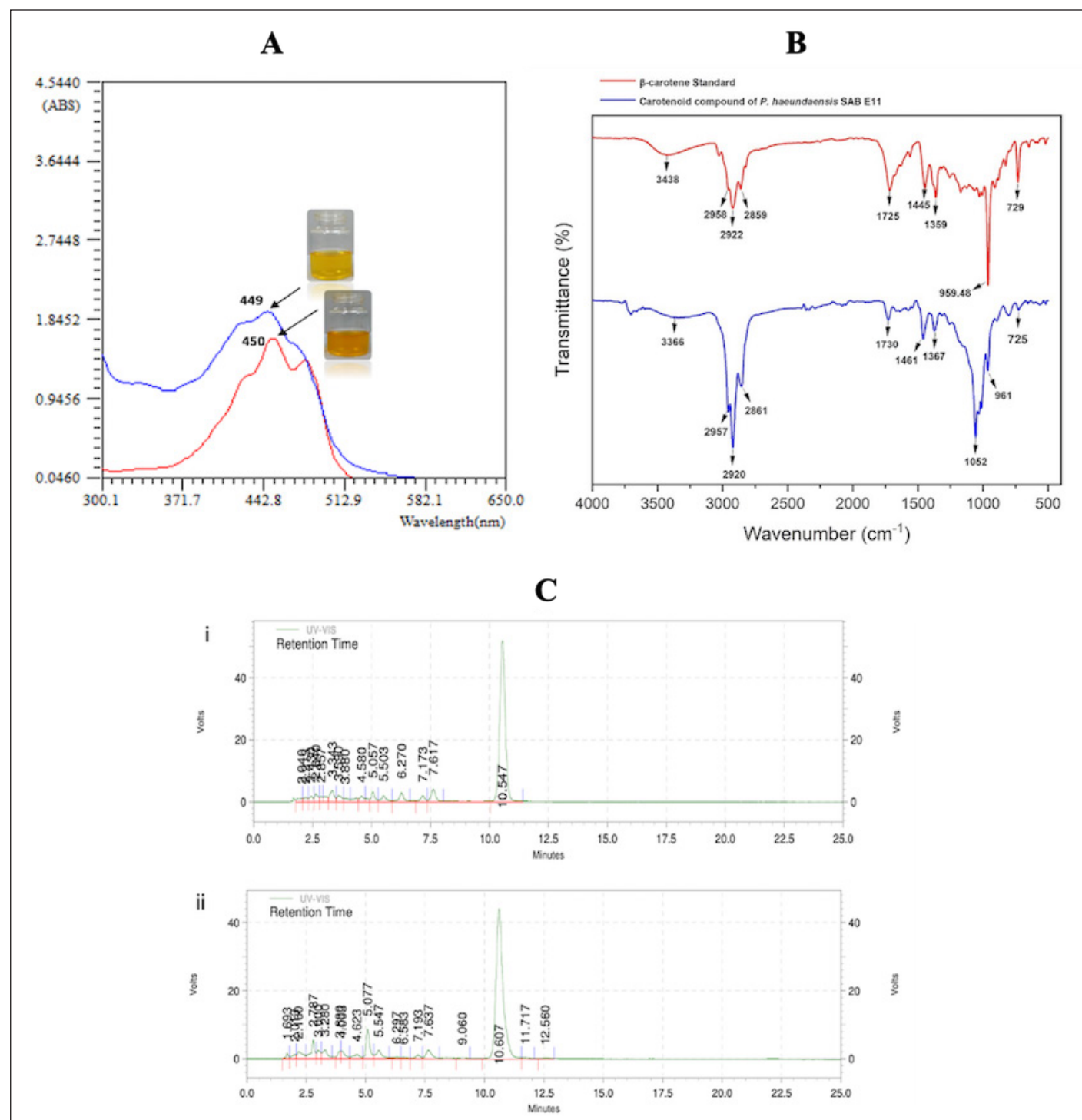
Figure 3. Characterzation of carotenoid compound of *P. haeundaensis* SAB E11, (A) Maximum wavelength measurement of β-carotene standard (C4582, Sigma-Aldrich, Darmstadt, Germany) (red line) and the carotenoid compound (blue line). (B) FTIR pattern of β-carotene standard (red line) and carotenoid compound *P. haeundaensis* SAB E11 (blue line). (C) HPLC chromatogram of (i) β-carotene standard and (ii) carotenoid compound *P. haeundaensis* SAB E11.
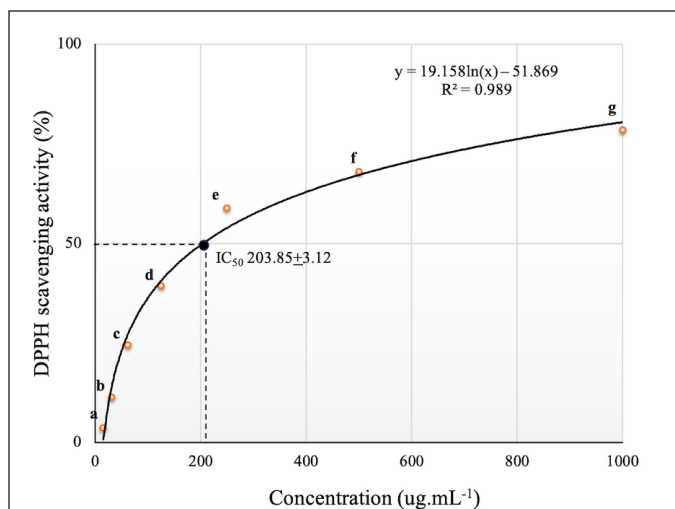
Figure 4. IC$_{50}$ of *P. haeundaensis* SAB E11 carotenoids was presented by the mean $\pm$ SD ($n = 3$). The mean value of each percentage inhibition in different letters indicators a significant distinction ($p < 0.05$) based on the DMR test.

E11 in a three-dimensional structural protein were visualized using the SWISS-MODEL automated mode. Superposition analysis identified the presence of overlapping structural parts between the model and lycopene β-cyclase *crt*Y proteins of *Paracoccus sp.* S-4012 shown in Figure 1D. The *crt*Y prediction model was assumed to be acceptable and trustworthy, as evidenced by the template sequence identity percentage, global model quality estimate (GMQE) value, and prediction local distance difference test (pLDDT) score. *crt*Y had a high sequence identity (72.55%) with the AlphaFold DB model of lycopene β-cyclase *crt*Y *Paracoccus sp.* S-4012 protein template (A0A6I2M2J7.1.A). Additionally, the GMQE value of *crt*Y was 0.86, where GMQE required a score between 0 and 1, with a higher number signifying higher reliability [32]. In this study, the protein structure of the template modeled by the AlphaFold DB method had a pLDDT score of 94.94%, denoting high confidence in the residue structure [33].

Characteristics of carotenoid in *P. haeundaensis* SAB E11

Carotenoid compound of *P. haeundaensis* SAB E11 was analyzed with TLC, UV-VIS, HPLC, and FTIR. TLC was performed using 5 % crude methanolic extract of intracellular *P. haeundaensis* SAB E11. According to Figure 2, one orange band with an Rf value of 0.88 appeared black under UV light at both 254 nm and 566 nm. The β-carotene standard (C4582, Sigma-Aldrich) showed identical band colors and Rf values. These TLC characteristics were previously observed in *Citrococcus parietis* AUCs and *Rhodotorula toruloides* ATCC 204091 [34,35]. Additionally, the UV-visible spectrum of the carotenoid compound from *P. haeundaensis* SAB E11 showed a maximum peak at 449 nm. For comparison, the β-carotene standard (C4582, Sigma-Aldrich) was observed at 450 nm (Fig. 3A). A study reported that the wavelength range profile of carotenoid compounds was between 400 and 500 nm. The maximum peak wavelength of a carotenoid compound of *P.*

haeundaensis SAB E11 at 449 nm was identified as β-carotene [36,37].

The carotenoid compound of *P. haeundaensis* SAB E11 from the single band (Rf 0.88) of TLC was analyzed using FTIR and HPLC. The FTIR spectra showed a comparative pattern between the β-carotene standard and carotenoid compound at 500–4,000 cm^{-1}. The β-carotene standard pattern was dominated by three major peaks at 2,922 cm^{-1} (C−H), 1,725 cm^{-1} (C=O), and 959 cm^{-1} (C−C). Meanwhile, the carotenoid compound showed four significant peaks, three of which were identical to the β-carotene standard pattern (Fig. 3B). These included three peaks at 2,861, 2,920, and 2,957 cm^{-1}, representing asymmetric and symmetric modes of the C−H groups. The remaining one was a sharp peak at 1,052 cm^{-1} (CH$_3$), which was absent in the β-carotene standard and it might be attributed to the rocking vibration of the (RH) C=C (RH) groups of the synthesized carotenoid [8]. HPLC analysis confirmed the dominant peaks of the carotenoid compound and β-carotene standard at 10.607 and 10.547 minutes, respectively [31]. These peaks had comparable values, including the area and height with high similarity values of 95.26 % and 85.86 %, respectively (Fig. 3C). The characteristic data obtained were insufficient for the definitive identification of carotenoid compounds. Therefore, additional investigation is needed to purify carotenoids and determine their structure.

Antioxidant activity of carotenoid in *P. haeundaensis* SAB E11

The DPPH radical assay was used to assess the antioxidant activity of the carotenoid compound. The scavenging activity against DPPH radicals increased proportionally with carotenoid concentration. According to Figure 4, the IC$_{50}$ value required to reduce 50 % of DPPH radicals was 203.85 ± 3.12 μg.ml^{-1}, while the IC$_{50}$ of the β-carotene standard was 43.53 ± 0.59 μg.ml^{-1}. These results corresponded with previous studies showing that the carotenoid compound derived from *Kocuria* sp. RAM1, *Virgibacillus* sp., and *Citricoccus parietis* AUC had antioxidant properties [35,38,39]. Carotenoid extract generated from *Paracoccus marcusii* RSPO1 reduced 65% ± 0.06% DPPH and 42% ± 0.7% ABTS radicals at 20 μg.ml^{-1} concentration. Meanwhile, 10 mg/ml β-carotene derived from *P. homiensis* BKA7 reduced DPPH radicals by 72% [8,9]. Carotenoid effectively eliminates free radicals due to the electron-rich conditions, which arise from the elongated structure with conjugated double bonds [5]. The presence of a cyclic structure including two β-ionic rings along with a lipophilic microenvironment provides an opportunity for carotenoids to reduce oxidants through mechanisms such as electron transfer, hydrogen atom transfer, or inhibition of peroxidase on lipids [40,41].
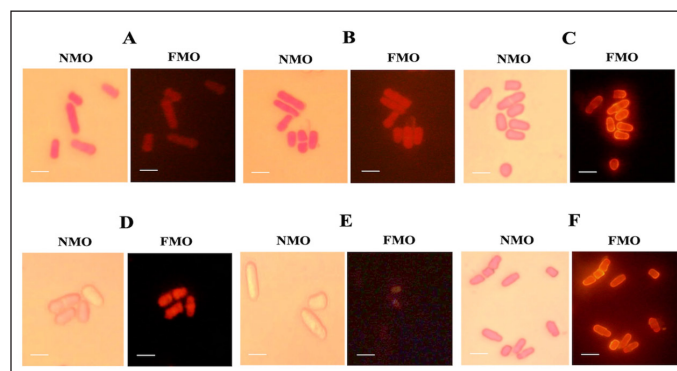
Effect of the carotenoid on *S. pombe* ARC039 cell viability

The antioxidant activity of the carotenoid compound was assessed at the cellular level in *S. pombe* ARC039. Furthermore, yeast viability was determined by colony spot density after 72 hours of incubation in YES medium supplemented with H$_2$O$_2$. The antioxidant assay identified that the carotenoid compound showed lower DPPH radical scavenging activity than the β-carotene standard. However, in
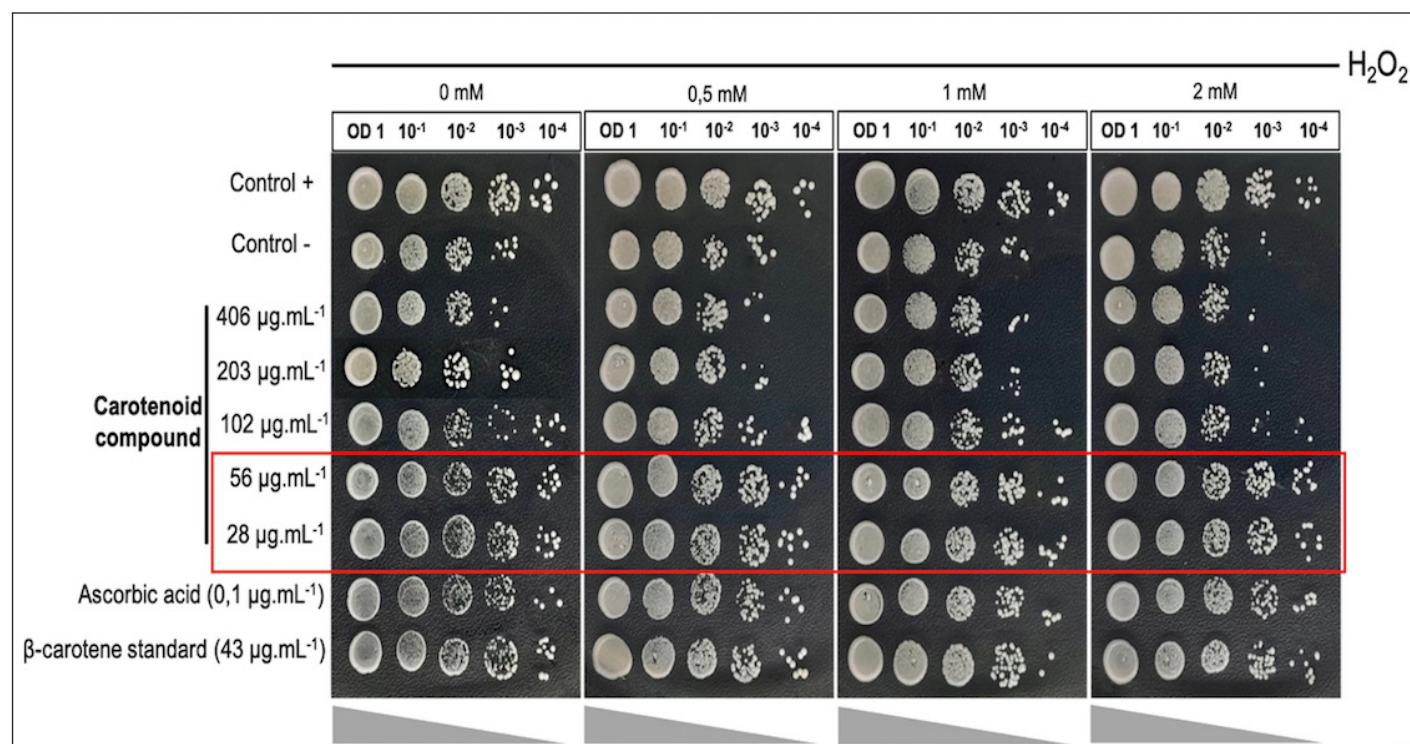
**Figure 5.** The effect of caretenoid compound (406 µg.ml⁻¹, 203 µg ml⁻¹, 102 µg ml⁻¹, 56 µg ml⁻¹ and 28 µg ml⁻¹) on the *S. pombe* ARC093 cell viability with H_2O_2 induced *S. pombe* ARC093 in YES medium (0.3 % glucose) was assigned as positive control, while negative control was prepared in YES medium (3 % glucose Ascorbic acid (0.1µg ml⁻¹) and β-carotene standard (C4582 Sigma-Aldrich) (43µg ml⁻¹) were used as a reference.

Figure 6. The effect of the treatments on mitochondrial activity of *S. pombe* ARC039:carotenoid compound of *P. haeundaensis* SAB E11 of (A) 102 µg ml⁻¹; (B) 56 µg ml⁻¹; (D) positive control (0.3 % glucose of YES medium); (E) negative control % glucose of YES medium); (F) reference sample β-carotene standard (43 µg ml⁻¹). Nonfluorescent mitochondrial obderved (NMO), Fluorescent mitochondrial observed (FMO); bars 5 µm.

the cellular level assay, treatment with carotenoid compound maintained yeast viability against H_2O_2-induced oxidative stress until a concentration of 2 mM (Fig. 5). Treatment with carotenoid compound concentrations of 56 µg.ml⁻¹ and 28 µg.ml⁻¹ generated the same colony density as the positive control (CR treatment) and reference sample (ascorbic acid and β-carotene standard). Meanwhile, the negative control prepared with 3% glucose in the YES medium generated a lower colony density compared to the carotenoid compound treatment. Ascorbic acid was applied as the reference sample because previous studies showed the capacity to maintain yeast cell viability under oxidative stress conditions induced by H_2O_2 [24,25]. Additionally, the β-carotene standard was used to support the data obtained for the carotenoid compound. CR treatment was used as a positive control to investigate the oxidative response in yeast and has been widely reported to maintain yeast cell adaptability under low-glucose conditions. This induced a metabolic shift in yeast cells from glucose fermentation to mitochondrial respiration to maximize energy usage in response to limited glucose availability. Consequently, the shift enhanced mitochondrial activity, leading to increased intracellular ROS levels at mild concentrations, which induced an adaptive oxidative response in yeast cells [42]. Under the CR condition, yeast cells inhibited nutrient signaling kinases, such as Sck2 and the Git/PKA1 pathways in *S. pombe* [43,44]. In correspondence with the CR condition, carotenoids tended to trigger the adaptive oxidative response of yeast cells, which showed a colony density reaching 10^{-4} dilutions without low-glucose treatment. Previous studies found that *S. pombe* responded to oxidative stress through key mediators of mitogen-activated protein kinase signaling. The mediators stimulated the core transcriptional response to diverse stress conditions, including the core environmental stress response (CESR) [45,46]. This prediction needs to be supported by another cellular analysis, such as the analysis of mitochondrial activity and the oxidative response of antioxidant genes in *S. pombe* cells ARC039.
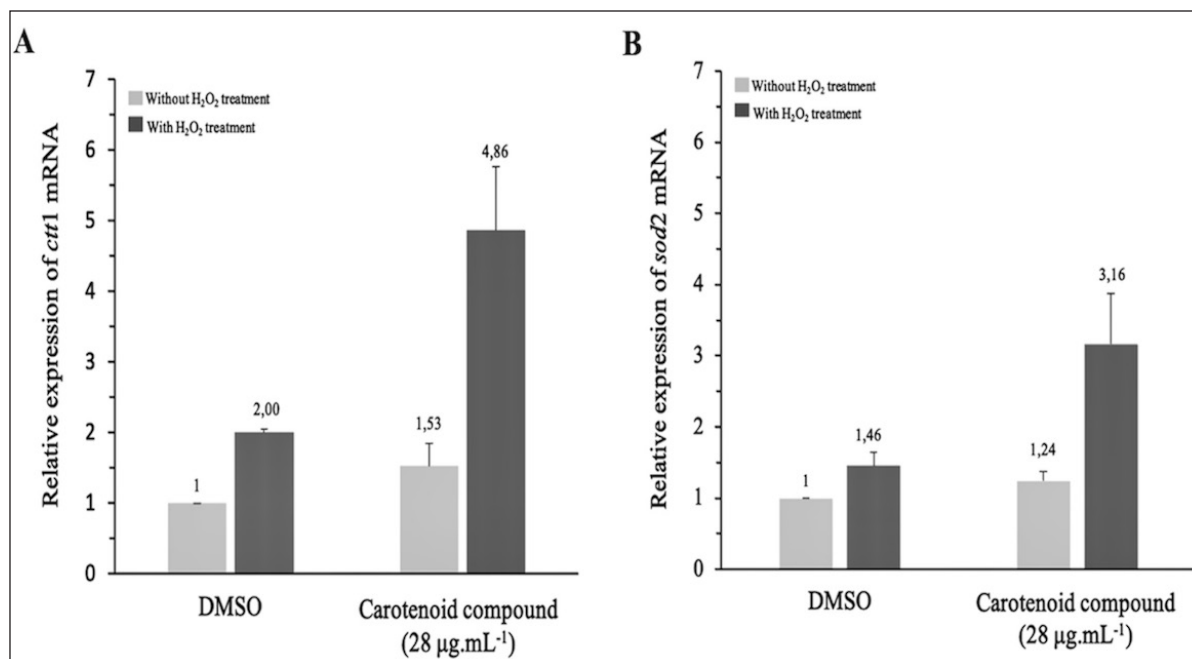
Figure 7. Carotenoid compound *P. haeundaensis* SAB E11 affected the expression of *ctt*1 and *sod*2 genes. (A), The carotenoid compound (28 µg ml⁻¹) induced relative expression of *ctt*1 and (B) *sod*2 genes. DMSO treatment is used as the negative control. The estimation of relative expression value was shown by the mean $\pm$ SD (n = 3).

Mitochondrial activity of *S. pombe* ARC039

The adaptive oxidative response of *S. pombe* ARC039 induced by carotenoid compound was confirmed through mitochondrial activity. Carotenoid from *P. haeundaensis* SAB E11 increased mitochondrial activity, as shown by bright fluorescence (Fig. 6). This compound produced the highest fluorescence intensity in cells at a concentration of 28 µg.ml⁻¹. The CR treatment and β-carotene standard presented the same fluorescence intensity as the value obtained from carotenoid. However, yeast cells treated with 3 % glucose in YES medium and DMSO showed no fluorescence. In this study, the observed fluorescence originated from the reactions between Rhodamine B and cations produced during respiration in the mitochondria [47]. As previously predicted, *S. pombe* ARC039 cells withstand oxidative stress at 3 % glucose in the medium due to the influence of carotenoid. The mitochondrial activity of yeast is probably enhanced to produce ROS, thereby allowing *S. pombe* ARC039 to tolerate ROS level. Exposure to ROS in mitochondria generated an adaptive response to oxidants, as evidenced by the viability of yeast cells that remained alive after 72 hours of incubation. Induction of mitochondrial activity can develop mitochondrial adaptive responses to ROS and induce the CESR system through the transcription factors (TF) Sty1-Atf, Pap1, and Prr1 [16,46]. Sty1-Atf1 modulates the transcriptional response to severe oxidative stress conditions, while Pap1 and Prr1 respond to weak oxidative stress [43,48].

Effect of the carotenoid on anti-oxidative genes expression

Carotenoid compound was used to assess the potential to induce CESR genes in *S. pombe* ARC039. The *ctt*1 and *sod*2 genes present in the CESR system encode the enzymes catalase (Ctt1) and superoxide dismutase (Sod2). These genes are critical components of the detoxification system, functioning as ROS detoxifiers to enhance the oxidative response and promote reduction-oxidation homeostasis in *S. pombe* [46]. At a carotenoid concentration of 28 µg.ml⁻¹, a significant increase was observed in the mRNA level of the *ctt*1 and *sod*2 genes of *S. pombe* ARC039. One hour after exposure to 1 mM H_2O_2, the relative expression of *ctt*1 and *sod*2 increased by 3.18 and 2.55 folds, respectively (Fig. 7). These data suggested that carotenoid compound induced the CESR system in *S. pombe* ARC039 by targeting *ctt*1 and *sod*2. The *ctt*1 gene in *S. pombe* is downstream of the TF Sty1-Atf1 and Pap1, which regulate gene expression differently depending on the H_2O_2 level [49]. Meanwhile, *sod*2 encodes mitochondrial Sod2, which Pap1 modulates in mild oxidative stress [16]. These genes may be regulated simultaneously by Pap1 TF to protect yeast cells from H_2O_2 and the ROS produced. The regulation is probably caused by carotenoid compound-induced increases in mitochondrial activity, leading to an adaptive response to ROS signaling. This result showed a mechanism similar to the type observed in the previous study of antioxidant compounds oxyphyllacinol, valine, and sugiol extracted from the water fraction of the Asteraceae plant. The three compounds induced an adaptive response to oxidative stress and promoted longevity in *S. pombe* ARC039 by modulating Pap1-*ctt*1 and Pap1-*sod*2 [16].

CONCLUSION

In conclusion, this study showed the capabilities of *P. haeundaensis* SAB E11 to produce carotenoid compound, as evidenced by the presence of the *crt*Y gene encoding lycopene β-cyclase. TLC, UV-Vis, HPLC, and FTIR confirmed carotenoid in *P. haeundaensis* SAB E11 requiring more purification through structural analysis, while DPPH radicals assay detected

antioxidant activity. The compound effectively induced the adaptive response of *S. pombe* ARC039 by increasing mitochondrial activity and the relative expression of *ctt*1 and *sod*2 genes. Due to the results, carotenoids can be developed as functional dietary additives or nutraceuticals. However, this preliminary study requires further investigations, particularly regarding the impact on oxidative responses in multicellular organisms.

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AUTHORS' CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work..

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analized are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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