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# The potency of yellow pigment extract from the marine bacterium *Pseudomonas oryzihabitans* SAB E-3 as an antioxidant agent

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**Abstract.** *Putri DA, Astuti RI, Wahyudi AT. 2024. The potency of yellow pigment extract from the marine bacterium* Pseudomonas oryzihabitans *SAB E-3 as an antioxidant agent. Biodiversitas 25: 2565-2575.* Metabolic processes in cells can produce byproducts in the form of Reactive Oxygen Species (ROS). Excessive amounts of ROS are one of the causes of oxidative stress. Antioxidants are known to act as free radical-scavenging compounds. Pigment-producing bacteria associated with sponges have been widely reported to have antioxidant compounds. In this study, the marine bacterium *Pseudomonas oryzihabitans* SAB E-3 produced intracellular yellow pigments with potential antioxidant activity. Therefore, this study aimed to assess the *in vitro* antioxidant and mitochondrial activity at the cellular level and identify chemical compounds. The intracellular pigments produced by *P. oryzihabitans* SAB E-3 were extracted using methanol, acetone, chloroform, and ethyl acetate. The methanol pigment extract contained the highest number of phenolic and flavonoid compounds, which may have contributed to its antioxidant activity. Furthermore, the active fraction obtained from the bioautographic TLC and harvested using preparative TLC had stronger antioxidant activity against DPPH radicals (75.71 µg/mL) than the crude pigment extract. However, the ABTS radical-reducing activity was lower (226.59 µg/mL) than the crude pigment extract. The maximum wavelength of the pigment extract was 453 nm, indicating the presence of carotenoids. In addition, the lowest concentration of the crude pigment extract (50 µg/mL) and selected active fractions (18.75 µg/mL) increased yeast *Schizosaccharomyces pombe* ARC039 tolerance to oxidative stress and induced mitochondrial activity. Active fraction one extract analyzed using LC-MS/MS contained piceatannol, resveratrol, isorhapontigenin, isoliquiritigenin, liquiritin, and 2-Omethylisohemigossylic acid lactone, which may contribute to its antioxidant activity. Therefore, the yellow pigment extract and the active fraction produced by *P. oryzihabitans* SAB E-3 are potential candidates for natural antioxidant sources.

**Keywords:** Antioxidant, LC-MS/MS, marine bacteria, *Schizosaccharomyces pombe*, yellow-pigment extract

## INTRODUCTION

Marine ecosystems are home to immense biodiversity and are yet to be fully explored. The unique characteristics of the ocean, such as hydrostatic pressure, temperature differences, and high salinity levels, promote the development of biological adaptations in the production of secondary metabolite compounds (Corinaldesi et al. 2017). Marine organisms, such as sponges, can host a variety of microorganisms, including bacteria, through symbiotic mutualistic relationships (Webster and Thomas 2016). In these relationships, bacteria can produce secondary metabolites that could contribute to the host defense system. In contrast, the host provides nutrients and a place to live for bacteria. Several bacterial symbionts have been reported to produce secondary metabolites (Zhang et al. 2022). Bacterial pigments are one of the secondary metabolites that are currently attracting attention. Bacterial pigments offer several advantages over other organisms, including rapid growth in metabolite production, independence from weather conditions, and easier molecular manipulation (Chandra et al. 2020). In addition, bacterial pigments possess nutritional, therapeutic, non-carcinogenic, and biodegradable properties compared to synthetic pigments (Agarwal et al. 2023). Consequently, bacterial pigments are attractive for the study of secondary metabolites.

Pigments synthesized by bacteria serve as cellular adaptation mechanisms that enable their survival within their habitat (Poddar et al. 2021). Bacterial pigments have demonstrated the ability to protect bacterial cells from damage caused by ozone exposure compared to bacteria that lack pigmentation (de Ondarza 2017). In addition, pigments in bacteria have various applications in industrial and pharmacological fields. Pigments produced by bacteria such as melanin, prodigiosin, pyocyanin, and carotenoids have been widely reported to have biological activities. For instance, carotenoid pigments, a group of yellow to red pigments, have been established to exhibit biological activities, such as antioxidant, antimicrobial, anticancer, and sun-protective properties (Venil et al. 2013; Choksi et al. 2020).

Antioxidants are essential for preventing and delaying Reactive Oxygen Species (ROS)-induced cell damage. ROS exist in the form of hydroxyl radicals (·OH), superoxide anions ($O_2$·), and hydrogen peroxide ($H_2O_2$), which are generated by biological processes. ROS can act as signaling agents in proper quantities to prompt adaptive responses in cells. However, an accumulation of ROS exceeding the body's natural antioxidant defenses can lead to oxidative stress and cellular damage. High ROS levels can lead to various degenerative diseases, including

premature aging, diabetes, cancer, cardiovascular disease, and Alzheimer's disease (Bardaweel et al. 2018; Shields et al. 2021). To counteract the negative effects of ROS, it is essential to find compounds that can inhibit free-radical reactions by inhibiting the oxidation process and transforming free radicals into by-products that are eliminated from the body (Vladkova et al. 2022). Although several synthetic antioxidants are currently available, they may have adverse effects such as toxicity and carcinogenicity (Yang et al. 2018). Therefore, it is crucial to discover natural antioxidant compounds that are safer than synthetic alternatives.

Natural antioxidants, such as pigment compounds, are one alternative that can be used to overcome the adverse effects of ROS. It is supported by several studies that have reported that pigment-producing bacteria associated with marine sponges have *in vitro* antioxidant activities, including the yellow pigment-producing bacterium *Virgibacillus* sp. 19. PP.Sc.13 associated with the sponge *Sinularia* sp., which has antioxidant and antimicrobial activities (Kusmita et al. 2023). Similarly, *Paracoccus haeundaensis* SAB E-11, a marine bacterium with orange pigmentation, exhibits potent antioxidant properties against DPPH and ABTS radicals (Abubakar et al. 2022). In addition, one type of carotenoid pigment that acts as an antioxidant is myxol and saproxanthin, produced by the *Flavobacteriaceae* group of bacteria (Agarwal et al. 2023).

Previous studies have successfully isolated the sponge-associated bacterial strain SAB E-3, which produces a yellow pigment (Abubakar et al. 2011). The aim of this study was to investigate the *in vitro* antioxidant activity of bacterial pigments and follow it up at the cellular level using *Schizosaccharomyces pombe* ARC039 as a model organism, as well as to identify compounds that may contribute to it. Therefore, the results of this study indicate that the yellow pigment from *Pseudomonas oryzihabitans* SAB E-3 has potential as an antioxidant that can be further developed in the pharmaceutical field.

## MATERIALS AND METHODS

### Materials

The SAB E-3 bacterium, used as a pigment source, are bacteria associated with *Jaspis* sp. sponges collected from Raja Ampat Island, Papua, Indonesia (Abubakar et al. 2011). These bacteria were cultured on sea-water complete (SWC) medium with the composition: peptone 5 g, glycerol 3 mL, yeast extract 1 g, distilled water 250 mL, and sea-water 750 mL. In addition, the fission yeast *Schizosaccharomyces pombe* ARC039 ($h^-$ *leu1-32 ura4-*294), obtained from Astuti et al. (2021), was cultured on YES medium with the composition: glucose 30 g, yeast extract 5 g, uracil 0.01 g, arginine 0.128 g, adenine 0.128 g, histidine 0.128 g, and leucine 0.128 g.

### Characterization and identification of 16S rRNA gene of SAB E-3 isolate

The bacterium SAB E-3 was rejuvenated on SWC agar medium for five days at a temperature of 27°C. The morphology of the colonies was examined. Subsequently, the revitalized isolates were subjected to Gram staining and hemolytic tests. The genomic DNA of isolate SAB E-3 was extracted using the Presto™ Mini gDNA Bacteria Kit following the manufacturer's procedures (Geneaid, Taiwan). The 16S rRNA gene was amplified using the forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). The total PCR reaction volume was 50 µL, consisting of 25 µL MyTaq™ HS Red Mastermix 2x (Bioline, UK), 5 µL of each primer (10 pmol), 5 µL genomic DNA (~100 ng), and 10 µL Nuclease-Free Water (NFW). PCR was conducted in 35 cycles under the following conditions: pre-denaturation (94°C, 5 min), denaturation (94°C, 30 s), annealing (55°C, 45 s), elongation (72°C, 1 min 30 s), and post-elongation (72°C, 10 min). The PCR product was visualized using agarose gel (1%, w/v) and then sequenced using First Base (Selangor, Malaysia). Subsequently, the nucleotide sequences were aligned to the GenBank database using the BLASTn program from the NCBI website (http://blast.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis 11 (MEGA 11) program with the neighbor-joining method (bootstrap 1000x).

### Pigment production and extraction

Bacterial strain SAB E-3 was cultured in SWC broth for seven days at 27°C in a shaker incubator at 120 rpm. The culture was centrifuged for 15 min at 6000 rpm, and the cell biomass was harvested. Single organic solvents, such as methanol, acetone, chloroform, ethyl acetate, and n-hexane, were used to extract intracellular pigments. The cell biomass was mixed with the most effective solvent and then vigorously vortexed, while the cells were colorless. The mixture was then centrifuged again at 6000 rpm for 15 min. The solvent was evaporated at 40°C using a rotary evaporator (Dawoud et al. 2020).

### Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The total phenolic content in the extract samples was measured using a method described by Rafi et al. (2018). Each crude pigment extract (10 µL) from different solvents was mixed with distilled water 160 µL, 10% (v/v) Folin-Ciocalteu reagent 10 µL, and 20 µL 7.5% (w/v) $Na_2CO_3$. All mixtures were added to a microwell plate (Nunc 96) and then incubated for 30 min in the dark. Absorbance was measured at 750 nm using an ELISA microplate reader (EPOC, USA). Total phenolic content was determined using the gallic acid (GAE) standard curve with the following formula: $y=0.0025x+0.0576$, with $R^2=0.9824$, and the results were represented as mg GAE/g extract.

The total flavonoid content of the sample extract was measured using the method described by Meda et al. (2005). Crude pigment extract (100 µL) was combined with an equal volume of 2% (w/v) $AlCl_3$ and then incubated for 30 min. The total flavonoid content was calculated using the quercetin (QE) standard curve with the following formula:

y=0.0106x+0.1578, with $R^2$=0.9539, and the results were represented as mg QE/g extract.

***In vitro* antioxidant activity of SAB E-3 pigment extract**

The crude pigment extract and active fraction were evaluated for *in vitro* antioxidant activity using two different radicals, 2,2-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich), as described by Prastya et al. (2020a). L-ascorbic acid served as the positive control.

**DPPH assay**

The assay was performed by diluting the pigment extract with methanol at various concentrations (6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL). 100 µL of each pigment extract and DPPH (125 µM in methanol) were added to a microwell plate (Nunc 96). The mixtures were incubated in the dark for 30 min. The reduction in DPPH radicals was quantified at 517 nm using an ELISA microtitre plate reader spectrophotometer (EPOC, US). The percentage inhibition was determined using the following formula:

$$Inhibition\ (\%) = \left[1 - \left(\frac{A_{sample} - A_{control}}{A_{blank} - A_{control}}\right)\right] \text{x}100\% \quad (1)$$

The inhibition values were regressed on a linear regression equation to obtain the value of inhibitory concentration ($IC_{50}$). Where, ($A_{sample}$) is the absorbance of the mixture of radicals and extracts, ($A_{control}$) is the absorbance of the mixture of radicals and ascorbic acid, and ($A_{blank}$) is the absorbance of the radicals and methanol. Each concentration of the extract was tested in triplicate.

**ABTS assay**

The assay was performed by mixing 140 mM potassium persulfate ($K_2S_2O_8$) solution with 7 mM ABTS solution and incubating for 16 h. The mixture was diluted until the absorbance of the radicals became 0.68 to 0.7 at 734 nm. Pigment extracts were diluted in methanol to concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL. Each pigment extract concentration (20 µL) was combined with 180 µL ABTS radicals in microplate wells (Nunc 96) and incubated in the dark for 6 min. The reduction in ABTS radicals was quantified at 734 nm using an ELISA microtitre plate reader spectrophotometer (EPOC, USA). The percentage scavenging effect was determined using the formula (eq 1).

**UV-Vis absorption spectrometry**

The crude yellow pigment extract with the best antioxidant activity was dissolved in methanol and measured in the wavelength range of 330-650 nm using a visible spectrophotometer (UV-Vis Hitachi U-2800) to detect the maximum absorbance (Ashenafi et al. 2023).

**Thin-layer chromatography (TLC) analysis**

The crude yellow pigment was analyzed using a TLC plate coated with silica gel $G_{60}F_{254}$ (10×2 cm, Merck, GER). The crude pigment extract at a concentration of 5% (w/v) was dissolved in methanol, and 10 µL of the solution was applied using a CAMAG® Linomat 5 applicator (Muttenz, CH). The TLC plate was then eluted in a chromatography chamber containing 10 mL of saturated eluent. Nine eluents were selected to properly separate the crude pigment extract components, including methanol, ethanol, acetone, 1-butanol, n-propanol, dichloromethane, chloroform, ethyl acetate, and n-hexane. The eluted plates were visualized under visible light and a UV lamp at 254 and 366 nm to determine the number of spots formed. The two best eluents were combined at ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (v/v). Bioautographic TLC analysis was performed by spraying a 5 mM DPPH solution onto the TLC plate and incubating it for 30 min under dark conditions. The active fraction was characterized by a yellow band, which was suspected to have antioxidant activity. The yellow band was isolated by preparative TLC. Then, the yellow band was scraped, dissolved in methanol, and evaporated to obtain the active fraction (Prastya et al. 2020b). Each fraction was reassayed for antioxidant activity using a previously described method.

**Oxidative stress tolerance assay using spot test**

This assay was used to determine the effects of pigment extracts and active fractions on *S. pombe* ARC039 in response to oxidative stress (Prastya et al. 2020b; Astuti et al. 2021). As a starter culture, *S. pombe* ARC039 was cultured in YES medium for 18 h. The culture was transferred to YES liquid medium (3%, w/v) at an initial $OD_{600}$ of 0.05. Crude pigment extracts and fractions were added to the cultures at various concentrations based on the $IC_{50}$ values of the DPPH assay (0.25x, 0.5x, 1x, and 2x $IC_{50}$). The positive control used was yeast cultured on reduced glucose medium or calorie restriction conditions (0.3%, w/v) and ascorbic acid (5 µg/mL). In contrast, yeast cultured on YES medium (3%, w/v) supplemented with dimethyl sulfoxide (DMSO) without extracts were used as negative controls. Each treatment culture had a final volume of 3 mL. The cultures were then incubated at room temperature for 24 h in a shaker at 120 rpm. The spot test was initiated by measuring the initial $OD_{600}$ value of 1 in each treatment culture and then performing dilutions ($10^{-1}$ to $10^{-4}$). Each dilution was spotted (2 µL) on YES medium containing $H_2O_2$ at various concentrations (0.5, 1, and 2 mM) and incubated for three days at room temperature. Cell viability was observed after three days.

**Mitochondrial activity assay**

The mitochondrial activity of the yeast model organism *S. pombe* ARC039 was evaluated using Rhodamine B as a fluorescent probe (Sigma-Aldrich, US) (Astuti et al. 2021). *S. pombe* ARC039 was cultured on YES medium for 24 h at an initial $OD_{600}$ value of 0.05. The initial starter culture and the extract with the best concentration from the previous step were added to the new YES culture medium. The cultures were incubated for 18 h at room temperature. The yeast cells were then harvested and suspended in 0.1 M phosphate-buffered (pH 7.4). Rhodamine B (final concentration, 200 nM) was added to yeast cells. After 30 min, mitochondrial activity in yeast cells was observed using a fluorescence microscope (Olympus BX51).

### LC-MS/MS analysis

The chemical compound composition of selected active fractions with antioxidant activity was analyzed using a High-Performance Liquid Chromatography (HPLC) type ACQUITY UPLC®H-Class System with high-strength silica (HSS) LC column type ACQUITY UPLC® HSS C18 (1.8 µm 2.1 x 100 mm) (Waters, USA) and coupled with Xevo G2-S QTof (Waters, USA) in positive ion mode. The mobile phases consisted of distilled water + 5 mM formic acid (A) and acetonitrile + 0.05% formic acid (B). The flow rate used for chromatography was 0.2 mL/min for 23 min. Five microlitres of the selected active fraction extract was injected into the LC column at a separation temperature of 50°C. The spectra were analysed using Masslynx 4.1.

### Statistical analysis

Data analysis was performed using ANOVA followed by Duncan's test. The analysis was carried out using RStudio software.

## RESULTS AND DISCUSSION

### Bacterial characterization

The colonies of the SAB E-3 strain were yellow, and round, and had rough and wrinkled surfaces with entire edges and convex elevations on SWC agar after five days of incubation. It is a Gram-negative bacterium with rod-shaped cells (*coccobacillus*). It has no hemolytic activity (ɣ-hemolysis), when compared to *Staphylococcus aureus* (Sa), which has a yellowish zone around the isolate (β-hemolysis) (Figure 1). Based on 16S rRNA gene identification, SAB E-3 was closely related (99.37%) to *Pseudomonas oryzihabitans* IAM 1568 (E-value 0.00; Query Cover: 100%; Accession Number: NR 115005.1). Phylogenetic tree showing homologous relationships between SAB E-3 and other *Pseudomonas* species based on 16S rRNA gene sequences (Figure 2). The *Pseudomonas oryzihabitans* SAB E-3 sequence has been submitted to NCBI GenBank with accession number OR600238.

### Determination of best extraction solvent

Pigment extraction begins with the separation of the supernatant and cell biomass. Among the five solvents, four were effective for extracting the yellow pigment, as shown in Figure 3. The highest yield was obtained from chloroform pigment extract at 6.41%, followed by methanol (5.83%), acetone (4.84%), and ethyl acetate at 1.02% (w/w) (Table 1).

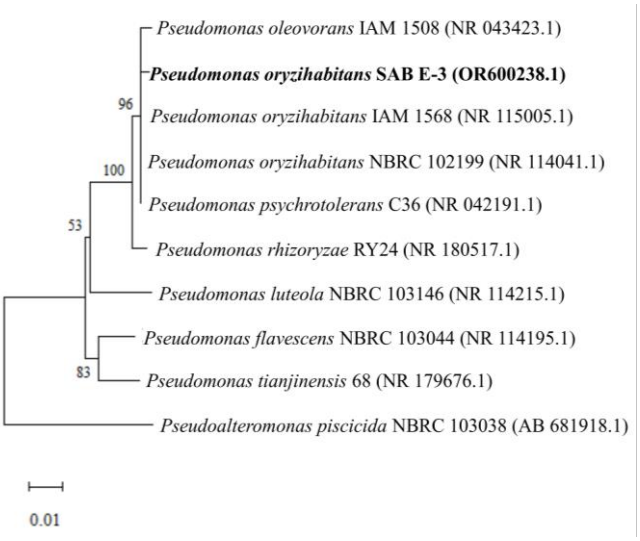


**Figure 2.** A phylogenetic tree of *P. oryzihabitans* SAB E-3 and its related species based on the 16S rRNA gene was constructed using neighbor-joining methods with 1000x bootstraps

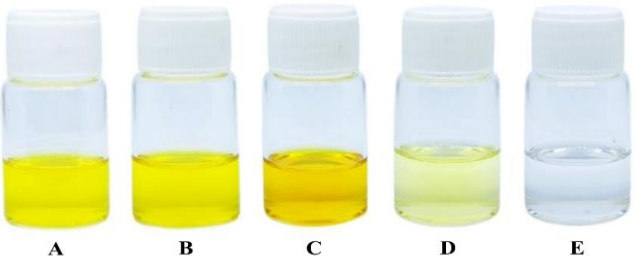


**Figure 3.** The yellow pigment of *P. oryzihabitans* SAB E-3 was extracted using five solvents: (A) methanol; (B) acetone; (C) chloroform; (D) ethyl acetate; and (E) n-hexane

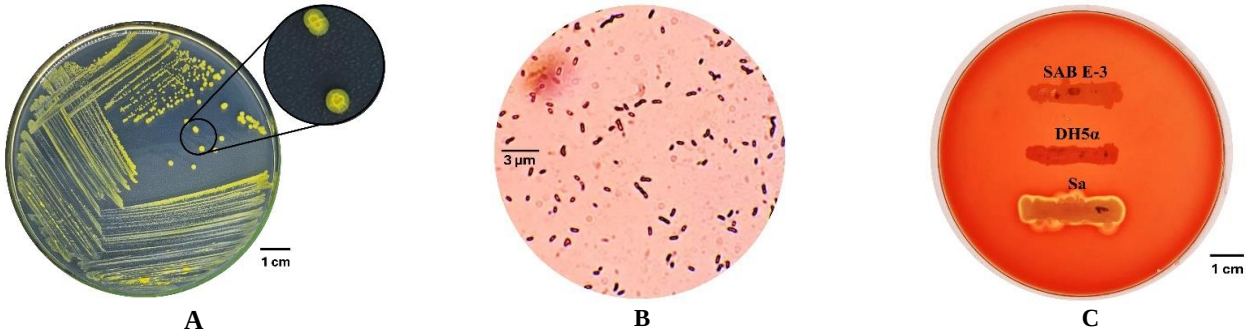


**Figure 1.** Characteristic (A) colonies of SAB E-3 on SWC medium after five days of incubation; (B) SAB E-3 Gram staining showed red cells indicating Gram-negative bacteria; (C) Hemolytic assay of SAB E-3 bacterium with no hemolytic zone observed, *Escherichia coli* DH5α as negative control and *Staphylococcus aureus* (Sa) as a positive control with the hemolytic zone (yellowish)

**The antioxidant activity and its correlation with the total phenolic (TPC) and flavonoid content (TFC)**

Based on the TPC and TFC results, the phenolic and flavonoid compounds found in the yellow pigment extract of *P. oryzihabitans* SAB E-3 in each solvent were believed to contribute to the antioxidant activity of the extract. The TPC values in each extract solvent ranged from 11.76-30.03 mg GAE/g, and TFC values ranged from 0.44-5.24 mg QE/g (Table 1). The methanol pigment extract exhibited the highest TPC and TFC, 30.03 mg GAE/g and 5.24 mg QE/g, respectively. Furthermore, the pigment extracts from each solvent were tested for *in vitro* antioxidant activity to determine their ability to reduce DPPH and ABTS radicals. The antioxidant activity of the pigment extract was represented by the $IC_{50}$ value, which is the concentration of the extract that can reduce radicals by 50%. Based on these results, the different polarities of the four solvents used to extract the pigments from *P. oryzihabitans* SAB E-3 also resulted in different antioxidant activities (Figure 4). However, the crude pigment extract extracted with methanol showed the highest antioxidant activity against both radicals. Therefore, the methanol crude pigment extract was selected for further analysis.

Pearson's correlation test was carried out to determine the relationship between the total phenolic and flavonoid content of the extracts of the yellow pigments and their antioxidant activity. In this study, TPC and TFC values were negatively correlated with DPPH and ABTS radicals (Table 2). These strong negative correlations suggest that high levels of flavonoids and phenolic compounds are associated with lower $IC_{50}$ values, indicating better antioxidant activity. A coefficient value close to -1 indicates that the phenolic and flavonoid compounds present in the yellow pigment extract of *P. oryzihabitans* SAB E-3 play a significant role in scavenging DPPH and ABTS radicals, contributing to the antioxidant activity of the extract.

**Maximum absorbance by UV-Vis spectrum analysis**

UV-Vis analysis was used to characterize pigment groups based on their maximum absorbance. The yellow pigment from methanol produced by *P. oryzihabitans* SAB E-3 had a maximum absorbance of 453 nm (Figure 5).

**Thin-layer chromatography (TLC) and bioautography analysis**

Optimization of the eluent conditions for TLC analysis revealed that chloroform:ethyl acetate mixture at a ratio of 9:1 (v/v) was the most effective eluent, as it produced 17 well-separated bands under UV light at 254 and 366 nm. TLC bioautography was used to evaluate the antioxidant activity of each band, which was achieved by spraying a 5 mM DPPH solution onto the TLC plate. The bioautography results indicated the presence of five active fractions (F1, F2, F7, F15, and F16) within the pigment extract that possessed distinct antioxidant properties. The active fractions were subsequently isolated using preparative TLC (Figure 6).

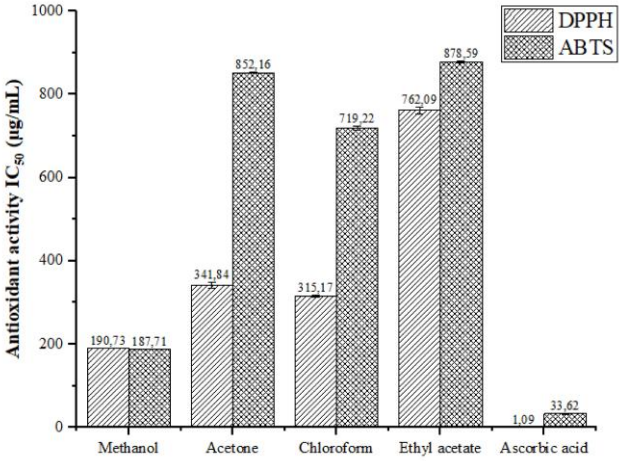


**Figure 4.** Antioxidant activity of crude extract pigments of *P. oryzihabitans* SAB E-3 from methanol, acetone, chloroform, and ethyl acetate solvents against DPPH and ABTS radicals

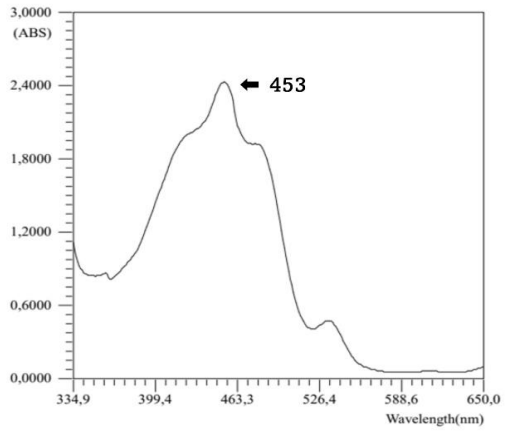


**Figure 5.** The yellow pigment of *P. oryzihabitans* SAB E-3 UV spectra. The maximum absorbance is indicated by black arrows

**Table 1**. Total phenolic and flavonoid contents of *P. oryzihabitans* SAB E-3 yellow pigment extract

| Solvent | Yield (%) | Total phenolic content (mg GAE/g) | Total flavonoid content (mg QE/g) |
|---|---|---|---|
| Methanol | 5.83 | $30.03 \pm 2.64^{a}$ | $5.24 \pm 0.57^{a}$ |
| Acetone | 4.84 | $20.56 \pm 2.29^{b}$ | $4.30 \pm 0.17^{b}$ |
| Chloroform | 6.41 | $17.62 \pm 0.19^{b}$ | $1.27 \pm 0.36^{c}$ |
| Ethyl acetate | 1.02 | $11.76 \pm 0.33^{c}$ | $0.44 \pm 0.2\ 9^{c}$ |

Note: Mean values and standard deviation (n=3) in the same column, followed by different superscript letters, mean significantly different based on the results of Duncan's test (p-values <0.05)

**Table 2.** Correlation coefficients of TPC and TFC with antioxidant activity of *P. oryzihabitans* SAB E-3 yellow pigment extract

| | TPC | TFC | DPPH | ABTS |
|---|---|---|---|---|
| TPC | 1 | | | |
| TFC | 0.845 | 1 | | |
| DPPH | -0.837 | -0.754 | 1 | |
| ABTS | -0.862 | -0.645 | 0.673 | 1 |

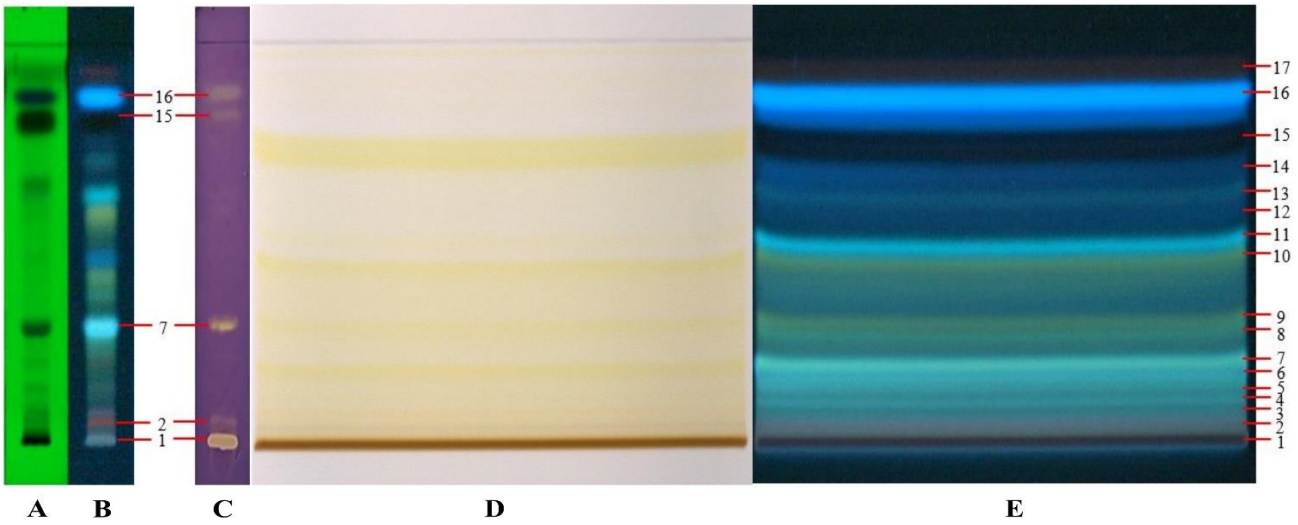


**Figure 6.** TLC chromatogram of *P. oryzihabitans* SAB E-3 pigment extract observed under UV light at (A) 245 nm, (B and E) 366 nm, and (D) visible light. (C) The yellow band indicates bioautography TLC results for the five active fractions

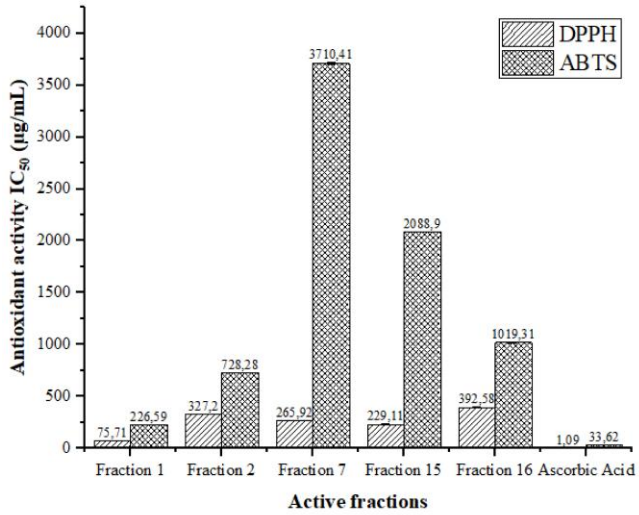


**Figure 7.** The antioxidant activities of the active fractions of *P. oryzihabitans* SAB E-3

## Antioxidant activities of the active fraction

The active fractions obtained from preparative TLC were re-evaluated using DPPH and ABTS assays (Figure 7). Among these fractions, fraction 1 of the yellow pigment extract from *P. oryzihabitans* SAB E-3 exhibited the strongest antioxidant activity with a value of 75.71 µg/mL against DPPH radical. In the ABTS radical assay, fraction 1 of the extract displayed lower antioxidant activity, with a value of 226.59 µg/mL, compared to the crude pigment extract, but surpassed the activity of the other fractions. Consequently, fraction 1 of the yellow pigment extract of *P. oryzihabitans* SAB E-3 was selected as the most promising active fraction for further analysis.

## Effect of pigment extract and fractions as antioxidants at the cellular level

In the *in vitro* antioxidant activity test, the methanol crude pigment extract and fraction 1 of *P. oryzihabitans* SAB E-3 were able to scavenge radicals better than the other extracts. Crude pigment extract and fraction 1 were selected for further analysis at the cellular level using the spot method as an initial test. In this assay, different extracts (based on $IC_{50}$ values) were used to determine the optimal concentration of both extracts in regulating the oxidative stress response in *S. pombe* ARC039 yeast cells. The results demonstrated that the crude pigment extract and the selected active fraction derived from *P. oryzihabitans* SAB E-3 effectively provided tolerance, maintaining cell viability under $H_2O_2$ stress compared to the negative control (DMSO) (Figure 8). Specifically, treatment with the lowest concentration of crude yellow pigment extract (50 µg/mL) maintained cell viability at $10^{-4}$, while higher concentrations showed reduced cell viability up to $10^{-3}$ at 2 mM $H_2O_2$. Furthermore, treatment with active fractions at all concentrations maintained and increased *S. pombe* ARC039 cell viability up to $10^{-4}$ at 2 mM $H_2O_2$. However, treatment with the lowest fraction concentration (18.75 µg/mL) showed better cell viability at $10^{-3}$ compared to other fraction concentrations. These findings showed that the active fraction extract resulted in better cell viability patterns than the positive controls (ascorbic acid at 5 µg/mL and caloric restriction (CR) 0.3%).

## Effect of pigment extract and fraction on mitochondrial activity

Based on the results of the previous step, the crude yellow pigment extract (50 µg/mL) and fraction 1 (18.75 µg/mL) increased mitochondrial membrane potential, as indicated by the strong fluorescence intensity observed in yeast cells. The positive control treatments with ascorbic acid and CR produced similar results, whereas the DMSO treatment showed no fluorescence (Figure 9).

**LC-MS/MS analysis**

Analysis of fraction 1 was carried out using LC-MS/MS, which revealed the presence of several compounds exhibiting antioxidant properties, as previously reported (Table 3). Six dominant compounds were putatively identified and classified as stilbenes (piceatannol, resveratrol, and isorhapontigenin), flavonoids (isoliquiritigenin and liquiritin), and terpenes (2-O-methylisohemigossylic acid lactone) (Figure 10; Table 3).

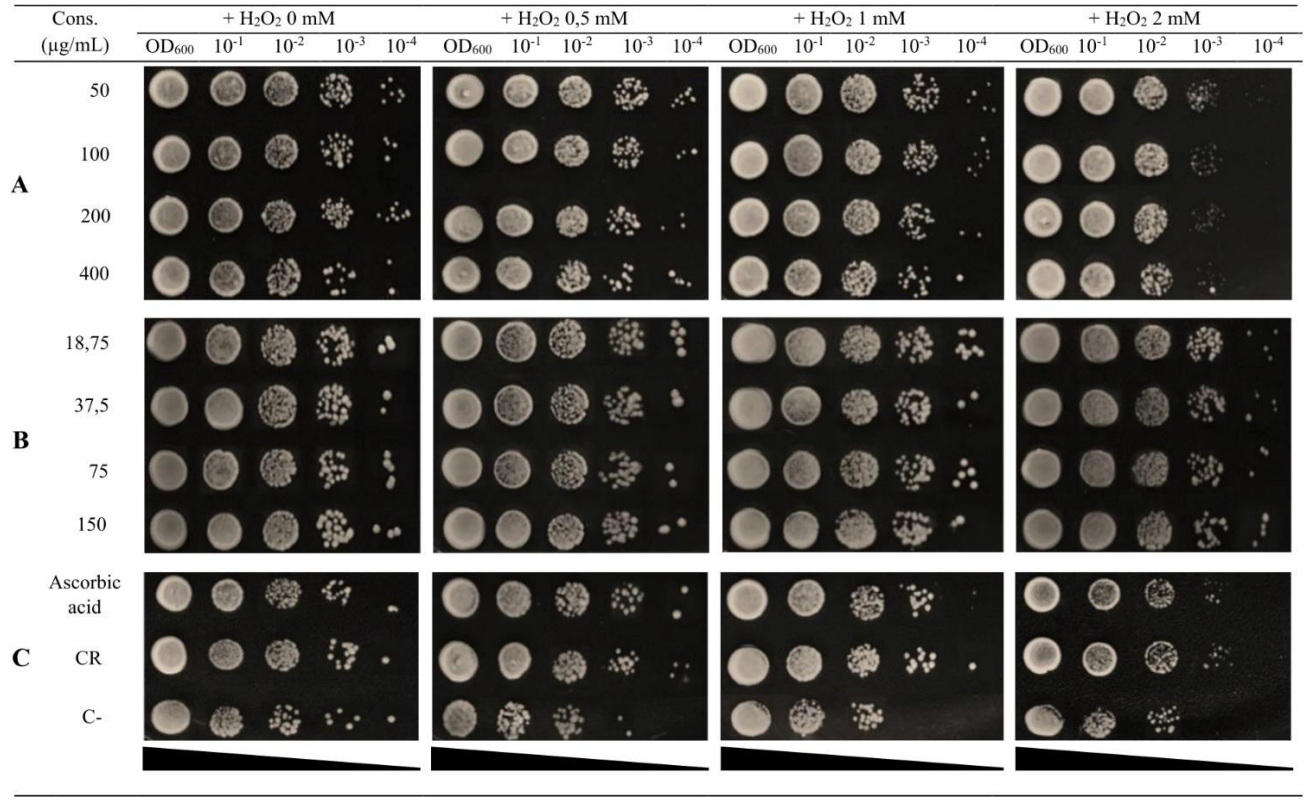


**Figure 8.** Oxidative stress response test using *S. pombe* ARC039 with several concentrations of *P. oryzihabitans* SAB E-3: A. Crude pigment extract (50, 100, 200, and 400 µg/mL), B. Selected active fractions (18.75, 37.5, 75, and 150 µg/mL), and C. 5 µg/mL ascorbic acid, 0.3% Calorie Restriction (CR), and DMSO (C-: negative control)

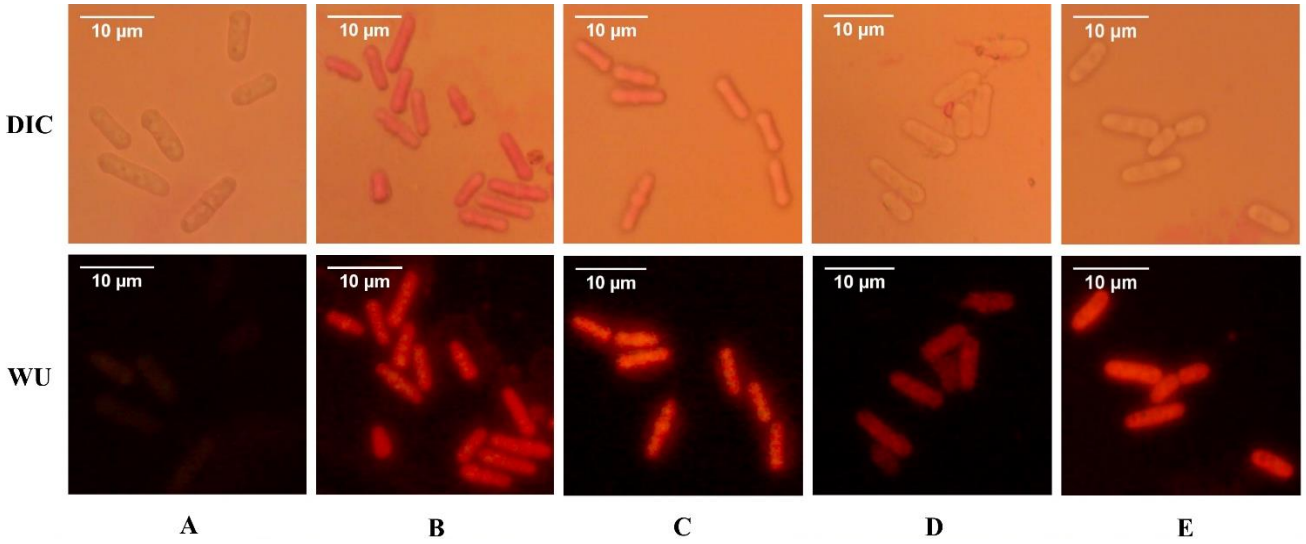


**Figure 9**. Effect of crude pigment extract and selected active fractions on the mitochondrial activity of *S. pombe* ARC039. (A) negative control (DMSO), (B) 0.3% calorie restriction (CR), (C) positive control (5 µg/mL ascorbic acid), (D) crude pigment extract (50 µg/mL), and (E) selected active fraction (18.75 µg/mL). DIC: Differential Interference Contrast. WU: Ultraviolet Fluorescence

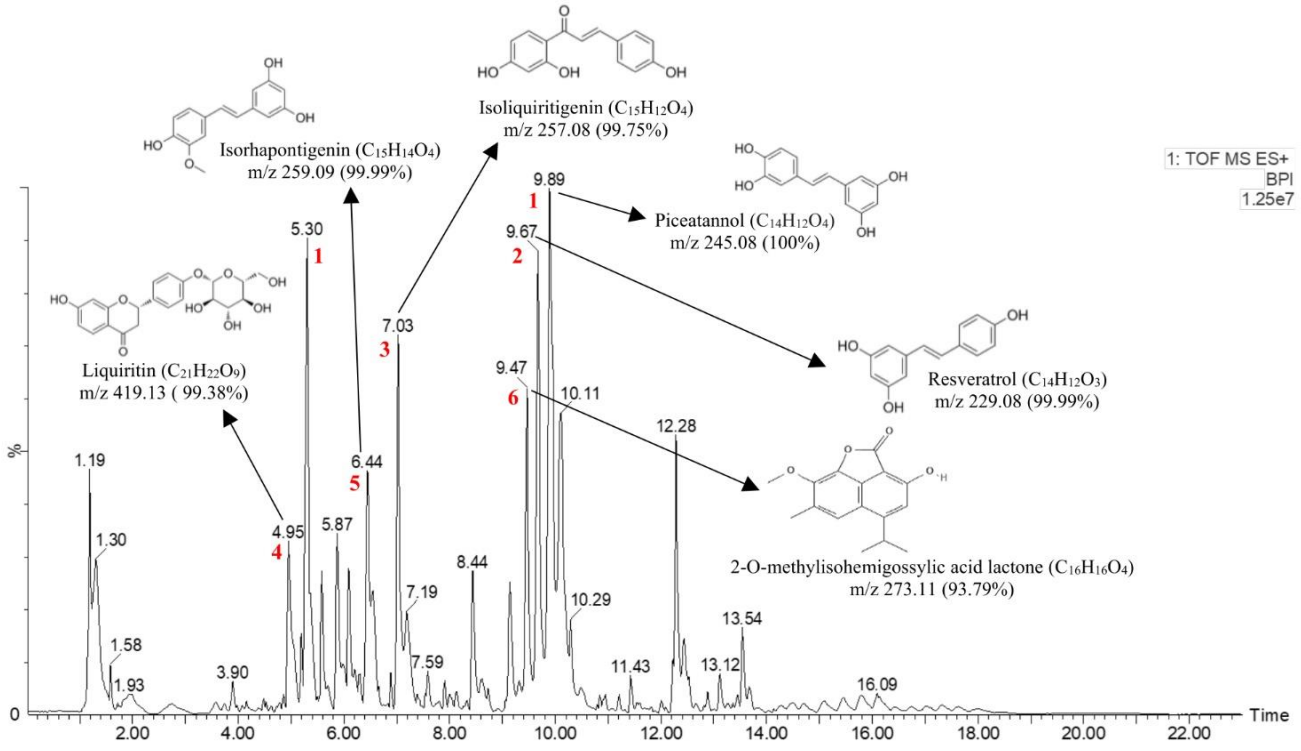


**Figure 10.** LC-MS/MS analysis of active fraction 1 from *P. oryzihabitans* SAB E-3 pigment extract

**Table 3**. Compounds found in active fraction 1 of *P. oryzihabitans* SAB E-3.

| Compound name | Compound group | Biological activities | References |
|---|---|---|---|
| Piceatannol | Stilbenes | Antioxidant | (Lu et al. 2017) |
| Resveratrol | Stilbenes | antioxidant | (Li et al. 2021) |
| Isoliquiritigenin | Flavonoid | antioxidant, neuroprotective | (Shi et al. 2020) |
| Liquiritin | Flavonoid | antioxidant | (Nakatani et al. 2017) |
| Isorhapontigenin | Stilbenes | antioxidant | (Lu et al. 2017) |
| 2-O-methylisohemigossylic acid lactone | Terpene | antioxidant | (Hibasami et al. 2004) |

## Discussion

The sponge-associated bacterium, SAB E-3, was identified as *P. oryzihabitans*. This bacterium produces an intracellular yellow pigment that does not diffuse into its growth medium. Therefore, various solvents with different polarities have been tested for pigment extraction. Polar to semi-polar solvents are effective for pigment extraction, whereas non-polar solvents, such as n-hexane, are ineffective. It may be due to the polarity of the compounds or pigments in cells. The extraction of chemical compounds from a sample using organic solvents is influenced by the solubility of the solvent and the nature and characteristics of the compounds present (Dai and Mumper 2010).

Phenolic and flavonoid compounds are commonly found in plants and contribute to their antioxidant activity. There is evidence that microorganisms can produce these compounds as secondary metabolites (Prastya et al. 2019; Cahlia et al. 2023; Marchut-Mikołajczyk et al. 2023). Notably, *P. oryzihabitans* SAB E-3 produced phenolic compounds and flavonoids, as indicated by the high TPC and TFC values obtained from the methanol pigment extract. The high solubility of the phenolic compounds and flavonoids in methanol may account for these results. Previous studies have suggested that the more polar nature of methanol compared with other solvents may affect the content of these compounds (Mehmood et al. 2022).

*In vitro* antioxidant assays on crude extracts of *P. oryzihabitans* SAB E-3 pigments extracted using different solvents showed different antioxidant activities. The methanol extract of the yellow pigment showed the strongest antioxidant activity against DPPH ($IC_{50}$ 190.73 µg/mL) and ABTS ($IC_{50}$ 187.71 µg/mL) radicals compared with pigment extracts from other solvents (Figure 4). It may be due to the higher content of antioxidant compounds in methanol than in different solvents. This result is supported by previous studies, which showed that methanol is the most efficient solvent for the extraction of such compounds (Boeing et al. 2014; Taghavi et al. 2023). In addition, the methanol pigment crude extract contained the

highest levels of phenolic and flavonoid compounds, which may contribute to its antioxidant activity. Therefore, this study selected crude pigment extract of methanol for further analysis. In addition, the results of this study also showed higher antioxidant activity than those reported by Patkar et al. (2021), where the yellow pigment extract of *Halomonas* spp. displayed antioxidant activity against DPPH radicals of 46.8% at 10 mg/mL and ABTS of 75.37% at 5 mg/mL. Furthermore, Mesrian et al. (2021) reported an orange methanol pigment extract from *Micromonospora tulbaghiae* SCA54.P2 exhibited an $IC_{50}$ value of 369.30 µg/mL against DPPH radicals. Dawoud et al. (2020) reported that the yellow pigment extract produced by *Bacillus* sp. DBS4 has antioxidant activity of 75.125 µg/mL against ABTS radicals. Pearson correlation analysis revealed a strong negative correlation between phenolic compounds in yellow pigment extracts against DPPH and ABTS radicals. It indicates that phenolic and flavonoid compounds are the main compounds contributing to the antioxidant activity (Muflihah et al. 2021). It was consistent with the LC-MS/MS analysis results, which identified stilbene (phenolic) and flavonoid compound groups in one of the fractions from the crude pigment extract. These compounds have been widely reported to exhibit antioxidant activities (Table 3).

UV-Vis analysis was conducted on the methanol pigment extract, which showed the highest antioxidant activity. The yellow pigment extract of *P. oryzihabitans* SAB E-3 showed maximum absorbance at 453 nm. Based on these findings, the pigment is considered to belong to the carotenoid group of pigments, as characterized by a yellow-to-red color spectrum. It is consistent with the report by Ashenafi et al. (2023), which states that carotenoid pigment compounds exhibit high molar absorptivity coefficients ($\varepsilon$) in the wavelength range of 400-500 nm.

The crude extract with antioxidant activity was extracted using TLC to separate the compounds from other impurities. Notably, out of the 17 bands detected, five displayed yellow bands identified as active fractions and believed to possess antioxidant activity (Figure 6). Fraction 1 exhibited stronger antioxidant activity against DPPH radicals than the crude pigment extract and other active fractions. Therefore, fraction 1 was selected as the active fraction for further analysis. However, it showed lower ABTS radical reduction activity than the crude extract. This difference is attributed to different types of radicals triggering different radical degradation mechanisms (Platzer et al. 2021). Furthermore, Asati et al. (2021) indicated that crude extracts of *Cyamopsis tetragonoloba* and *Prosopis cineraria* exhibit better radical-reducing potential than their fractions. This difference can be attributed to the many active antioxidant compounds in the crude extracts, which may have been eliminated during the fraction separation process.

Cellular level antioxidant activity was performed using yeast oxidative stress response assay. The results showed that the yellow pigment extract and active fractions effectively preserved the viability of *S. pombe* ARC039 cells against $H_2O_2$ stress (Figure 8). At low concentrations, crude pigment extracts and fractions effectively maintained yeast cell viability up to $10^{-4}$, while at higher concentrations, they could only maintain cell viability up to $10^{-3}$. It suggests that such extract activity is unlikely to be concentration-dependent, as higher concentrations showed the opposite effect. It is consistent with previous reports that certain or high concentrations of compounds exhibit potent antioxidant or pro-oxidant effects (Nowak et al. 2022). The findings of this study are similar to those of Cahlia et al. (2023), who reported that a low concentration (100 µg/mL) of crude yellow-red pigment extract produced by *Bacillus haikouensis* AGS112 was more effective than higher concentrations in maintaining cell viability. Fauzya et al. (2019) also reported that the lowest concentration of clove leaf extract was more effective in increasing yeast cell viability than other concentrations. The extract and fraction treatments were also more effective in maintaining cell viability than CR and ascorbic acid (positive control) treatments. CR and ascorbic acid protect cells against damage caused by ROS (Branduardi et al. 2007; Dilova et al. 2007). In contrast, yeast cultured on high-glucose YES medium (3%) without extract or fraction treatment exhibited mechanisms different from those cultured under CR and ascorbic acid conditions. It suggested that the extracts and fractions likely mimicked the effects of ascorbic acid and CR treatment, even under $H_2O_2$-induced oxidative stress and non-CR conditions.

CR conditions are known to extend yeast lifespan by inducing mitochondrial activity and activating mitochondrial adaptive ROS signaling (Li et al. 2011). Treatment with crude pigment extracts and fractions increased mitochondrial activity in the log phase, similar to the effects of CR and ascorbic acid treatment (Figure 9). Previous research has also shown that CR inhibits nutrient signaling pathways, such as Pka1 and Sck2 signaling (components of the TOR pathway), by inducing Sir2 histone/protein deacetylase signaling. As a result, the reduced activity of these nutrient signaling pathways causes yeast to adapt to new conditions, such as increased mitochondrial activity, which indirectly activates mitochondrial adaptive ROS signaling, thereby increasing cell tolerance to oxidative stress (Wang 2014; Leonov et al. 2017). It is supported by previous findings showing that yeast cells treated with extracts and fractions exhibit a phenotype tolerant to $H_2O_2$-induced oxidative stress (Figure 8). Furthermore, research has revealed that *Bacillus cereus* DCN1 extract can extend yeast lifespan by stimulating mitochondrial activity (Utami et al. 2023). In addition, treatment with *Bacillus* sp. SAB E-41 extract and gallic acid have also been reported to induce mitochondrial activity (Prastya et al. 2018; Lesmana et al. 2021).

LC-MS/MS analysis revealed the presence of several dominant compounds in the active fraction that have been reported to have antioxidant properties. Piceatannol was the most dominant compound in the active fraction, with retention times of 5.30 and 9.89. Previous studies have indicated that *Paracoccus haeundaensis* SAB E-11 and *Ensifer* sp. KSH1, and *Arthrobachter* sp. KSH3 can also produce piceatannol (Furuya et al. 2019; Abubakar et al.

2022). Piceatannol and isorhapontigenin were found to have antioxidant activity by reducing the free radicals, nitric oxide (NO·), and nitrogen dioxide ($NO_2$·) (Lu et al. 2017). Resveratrol is a stilbene (polyphenolic) compound, analogous to piceatannol, and is a typical compound widely produced by plant groups. There have been few reports on resveratrol compounds naturally produced by bacteria. However, in a previous study, this compound was found in the extract of *Streptomyces lanatus* AR2 (Riahi et al. 2019). This compound has exhibited antioxidant activity *in vitro* and *in vivo* in HepG2 cells by increasing enzymatic antioxidants and inhibiting ROS formation (Li et al. 2021). Isoliquiritigenin and liquiritin have been shown to have antioxidant and neuroprotective activities (Nakatani et al. 2017; Shi et al. 2020). In addition, the pigment fraction extract from *P. oryzihabitans* SAB E-3 is believed to contain a group of terpenoid compounds, as evidenced by the identification of a terpenoid compound group (2-O-methylisohemigossylic acid lactone) that also has antioxidant activity (Hibasami et al. 2004). This compound was identified with a fit confidence value of 93.79%, which requires further confirmation. These results indicated that the dominant compounds in fraction 1 have antioxidant activity at the *in vitro* and cellular levels by increasing the tolerance of yeast to oxidative stress. In addition, based on the results obtained, active fraction 1 showed better antioxidant activity than the crude pigment extract. However, further studies are required to elucidate the role of these compounds in yeast cells.

In conclusion, the methanol crude extract/active fraction of *P. oryzihabitans* SAB E-3 has potential as an antioxidant, both *in vitro* and at the cellular level, in *S. pombe* ARC039. Mitochondrial activity in yeast increased after treatment with the lowest concentration of crude pigment extract and the active fraction, which may induce oxidative stress tolerance. Thus, these results are important for developing bioactive compounds in the yellow pigment extract of *P. oryzihabitans* SAB E-3 as antioxidant pharmacological products.

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