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Antioxidant Properties of Active Fraction Extract Derived from Yellow-Red Pigment Produced by the Marine Sponge-Associated Bacterium *Bacillus haikouensis* AGS112 and Identification of Related Compounds

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ABSTRACT

Sponge-associated bacteria can produce bioactive compounds similar to the host. Here, the investigation of antioxidant properties of the yellow-red pigment produced by sponge-associated bacterium AGS112 was conducted using *in vitro* and *in vivo* analysis. The 16S rRNA gene sequence showed the sponge-associated bacterium AGS112 has the closest similarity with *Bacillus haikouensis* C-89 (99%). The crude pigment extract produced by *Bacillus haikouensis* AGS112 contained both phenolic and flavonoid. The peaks occurred at 412 nm and 664 nm, which indicated as the presence of carotenoids and flavonoids, respectively. The active fraction extract obtained through bio-autography TLC, and had more potent antioxidant activity against DPPH (2,2-diphenyl-1-picrylhydrazyl) compared to the crude pigment extract with an IC_{50} value of 68.62±0.59 µg/ml and 198.88±1.66 µg/ml, respectively. Moreover, the active fraction extract at the concentration of 35 µg/ml could better enhance the viability of *Schizosaccharomyces pombe* and achieve the highest expression of *sod1* and *ctt1* genes. Metabolite profiling using LC-MS analysis confirmed the active fraction extract contains eudesmin and artelastin that might be contributed as antioxidants. These results suggest that the active fraction extract of the yellow-red pigment produced by *B. haikouensis* AGS112 had potency to be used as candidate for natural antioxidants.

1. Introduction

Most of Indonesia's top death cases belong to degenerative diseases (WHO 2019). These diseases can cause by excessive Reactive Oxygen Species (ROS). In the cells, ROS from metabolism is stabilized by indigenous antioxidants such enzymatic antioxidants to keep the redox capacity in normal conditions. Unfortunately, bad habits, poor lifestyles, and environmental factors contribute to the increasing amount of ROS, leading to oxidative stress. This condition can contribute to the emergence of degenerative diseases. Besides indigenous antioxidants, dietary antioxidants intake may help maintain an adequate antioxidant status in the body (Lu *et al.* 2010). On the other hand, common-use synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butyl hydroquinone (TBHQ) are known to have toxicity and carcinogenic effect (Yang *et al.* 2018). These antioxidants are used in livestock feed and cosmetics but are not desirable as human food additives. Therefore, it is necessary to discover more non-toxic natural antioxidants such as from bio-pigment.

Pigments are secondary metabolites with various colors that have biological properties such as anticancer, antimicrobial, anti-inflammatory, and antioxidant. Plants and microbes like bacteria can produce pigment. However, producing and developing pigment from plants is costly and causes environmental destruction. Therefore, pigment from bacteria becomes one of the interesting objects to be investigated for its antioxidant properties. Pigments produced by bacteria are faster in production, and

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environmentally friendly for use as dyes, foodstuffs, pharmaceuticals, cosmetics, and other industrial purposes (Usman *et al.* 2017). Pigment-producing bacteria can be found in several habitats, including in marine. In habitats such as the ocean, intense selection pressure occurs as microbes compete for space and nutrients. Then, marine microbes can have natural products such as pigment in the form of secondary metabolites (Ibrahim *et al.* 2014). In addition, marine bacteria can form an association with other organisms, such as sponges. This association allows the bacteria to produce secondary metabolites similar to the host.

Previous studies have reported that pigments produced by sponge-associated bacteria possess biological properties such as antioxidant, antimicrobial, and anticancer properties. For example, a crude pigment extract of the sponge-associated bacterium *Zobellia laminarie* sp. 465 showed high antioxidant activity (Silva *et al.* 2019). The orange-pigmented sponge-associated bacterium *Paracoccus haeundaensis* SAB E11 had strong antioxidant activity (Abubakar *et al.* 2022). Antioxidant activity of pigment can be measured through *in vitro* analysis using DPPH assay, and *in vivo* analysis using model organisms such as yeast *Schizosaccharomyces pombe*. Therefore, this study aimed to investigate the antioxidant activity of the yellow-red pigment produced by sponge-associated bacterium AGS112 using these methods, and the metabolite profiling of related compounds that might contribute as antioxidant was analyzed to discover natural antioxidant properties.

2. Materials and Methods

2.1. Materials

The sponge-associated bacterium, coded as AGS112, isolated from the marine sponge *Agelas* sp. collected from Seribu Island, Indonesia (Maharsiwi 2020) was used in this study as a source of pigment. This bacterium was routinely cultured in Sea Water Complete (SWC) medium(5 g/L peptone, 1 g/L yeast extract, 15 g/L agar (Himedia, India), 3 ml/L glycerol (Merck, Germany), 750 ml of sea water and 250 ml of distilled water). Beside that, yeast *S. pombe* wild-type strain ARC039 (h −leu1-32 ura4-294), a culture collection from Astuti *et al.* (2021) was used to understand the antioxidant activity of the pigment extract at the cellular level. This yeast was routinely cultured in Yeast Extract Supplement (YES) medium (5 g/L yeast extract, 20 g/L agar (Himedia, India), 30 g/L glucose, 0.128 g/L histidine, 0.128 g/L leucine, 0.128 g/L adenine, 0.01 g/L uracil, and 0.128 g/L arginine).

2.2. Identification of AGS112 Isolate

Sponge-associated bacterium AGS112 was rejuvenated on SWC agar medium and incubated for 7 days at 27°C. The appearance of the colonies was observed. The purified isolate was re-cultured for Gram staining and hemolytic test.

The molecular identification was conducted based on 16S rRNA gene. DNA isolation was performed using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) based on the manufacturer's protocol. The DNA genome was amplified using Polymerase Chain Reaction (PCR) with targeted fragment ±1,300 bp using 63F and 1387R primer (Marchesi *et al.* 1998). The total volume of the PCR reaction was 50 µL and consisted of 25 µL kit PCR MyTaq Red Mastermix 2x, 5 µL (10 pmol) primer F and R respectively, DNA genome 5 µL (~100 ng/µL) and nuclease-free water (NFW) 10 µL. PCR conditions are pre-denaturation (94°C, 5 mins), denaturation (94°C, 30 seconds), annealing (58°C, 45 seconds), elongation (72°C, 1 minute 10 seconds), and post-elongation (72°C, 10 mins) with 35 cycles. Separation of PCR products is performed using electrophoresis with 1% agarose at 75 V for 30 mins. The PCR product was sequenced in First Base (Selangor, Malaysia). The DNA sequences were aligned to the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool Nucleotide (BLAST.N) software. The phylogenetic tree was constructed using MEGA 11 software with Neighbor Joining method (1,000× bootstraps).

2.3. Pigment Production, Extraction, and UV-Vis Analysis

One liter of *B. haikouensis* AGS112 culture was incubated in a shaker incubator at 120 rpm for 7 days at 27°C. The culture was centrifuged at 6,000 rpm for 15 mins at 25°C. The medium was removed. The cells were added with chloroform to extract the pigment and subsequently heated in the water bath at 50°C for 10 mins until the cells became colorless. It was then centrifuged at 6,000 rpm for 15 mins at 4°C and evaporated at 40°C to obtain the crude pigment extract. UV-Vis analysis was conducted using 20 mg of crude pigment extract that dissolved in 5 ml of chloroform. The maximum wavelengths absorption

of the pigment was analyzed at 200-800 nm using a visible spectrophotometer (UV-Vis Hitachi U-2800) (Abubakar *et al.* 2022).

2.4. Thin Layer Chromatography (TLC) Analysis

The silica gel 60 F_{254} TLC plates (10 cm × 2 cm with 0.2 mm thickness, Merck, Germany) were used as the stationary phase. About 3% of *B. haikouensis* AGS112 crude pigment extract was diluted into chloroform and then spotted as bands using Camag Linomat 5 applicator (Muttenz, Switzerland). Nine solvents including methanol, ethanol, butyl alcohol, isopropyl alcohol, ethyl acetate, dichloromethane, chloroform, n-hexane, and acetone, were used to find the best eluent indicated by many well-separated bands when observed under 254 and 366 nm. The combination of 2 best eluents was used for the optimization step with ratios 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 (v/v). The bio-autography TLC analysis was conducted by directly spraying the TLC plates with 5 mM DPPH solution to get the active fraction indicated by yellow bands formation after 30 mins incubation in dark conditions. These yellow bands were isolated by using preparative TLC with 10% concentration extract applied on TLC plates. The yellow bands were scraped, collected, and re-evaporated to obtain the active fraction extract.

2.5. Total Phenolic, Total Flavonoid, and Antioxidant Activity Assay

The total phenolic and flavonoid compounds were measured following the method described by Pudjas *et al.* (2022). The antioxidant assay of extracts were measured through DPPH assay, which previously described by Batubara *et al.* (2009). A hundred µL DPPH solution (125 µM diluted in methanol) was mixed with 100 µL of crude methanol pigment extract solution with various concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 0 µg/ml). The blank was made with 100 µL methanol mixed with 100 µL DPPH solution. The standard was made using ascorbic acid as a positive control. Measurements of absorbance reduction at wavelengths of 517 nm were performed using an ELISA microtiter plate reader (EPOC, USA) in triplicate after 30-minute incubation in dark conditions at room temperature. The scavenging activity was calculated using the formula:

$$\% \text{ inhibition } (\%) = \left(1 - \frac{\text{Sample's absorbance} - \text{Control's absorbance}}{\text{Blank's absorbance} - \text{Control's absorbance}}\right) \times 100\%$$

The percentage of inhibition was used to determine the IC_{50} value of each sample using a linear regression model.

2.6. Oxidative Stress Response Assay

Oxidative stress response was carried out using *S. pombe* as *in vivo* model organism qualitatively through the spot assay method (Prastya *et al.* 2022). As the culture starter, *S. pombe* cultured in YES broth medium at room temperature for 24 hours. The starter was then re-inoculated into 3 ml of YES broth medium, which was added by several concentrations of crude pigment extract and active fraction extract based on IC_{50} value from DPPH assay with an initial OD_{600} 0.05, respectively. Ascorbic acid (5 µg/ml) was used as a positive control, and 10% dimethyl sulfoxide (DMSO) was used as the negative control. Each culture was incubated at room temperature for 24 hours. Spot assay then began with an initial OD_{600} 0.1 of each culture followed by serial dilution (10^{-1}–10^{-4}). Each culture (3 µL) was spotted from left to right on YES agar medium, which has been added some concentration of H_2O_2 (0, 0.5, 1, and 2 mM), respectively. Incubation was carried out for 3 days at room temperature. The density of spot in each extract treatment was observed.

2.7. Anti-oxidative Gene Expression Analysis

The best concentration of the crude pigment extract and the active fraction extract from spot assay were supplemented onto YES broth medium after the addition of *S. pombe* inoculum at an initial OD_{600} of 0.05, respectively, and 10% DMSO was used as the negative control. All treatments were incubated for 24 h, and 1 mM of H_2O_2 was added an hour before the cell was harvested. RNA was extracted from the yeast cells using TRIsure™ Kit. Reverse transcription was conducted using ReverTraAce™ qPCR RT Master Mix Kit to obtain cDNA. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using QuantStudio 5 instrument and Thunderbird SYBR qPCR master mix (Toyobo, Japan) as the fluorescent reporter. The primer sequences of target

genes used in this study are as follows: *sod1* (F5'-CGATCAAAACTCTCAAGTTTCTGTG-3',R5'-AAACC ACGCTTGGCATTAGC-3'),*ctt1*(F5'-TCGTGACGGCCCTA TGAATG-3',R5'-AGCAAGTGGTCGGATTGAGG-3'), *act1* (F5'-CGGTCGTGACTTGACTGACT-3', R5' ATTTC ACGTTCGGCGGTAGT-3') genes and the thermal cycling parameters used were 40 cycles, 95°C (15 seconds), 55°C (30 seconds), and 72°C (30 seconds) (Astuti *et al.* 2021). To measure the relative expression level, the cycle threshold (Ct) values of *sod1* and *ctt1* genes were normalized to the Ct of housekeeping gene *act1*.

2.8. Liquid Chromatography-Mass Spectrophotometry (LC-MS) Analysis

The antioxidant active fraction chemical composition was analyzed by Ultra-Performance Liquid Chromatography (UPLC) coupled to a Xevo G2-S QTOF-MS (Waters, USA) using an electrospray ionization. The mobile phases used were water + 5 mM Ammonium Formic (A) and Acetonitrile + 0.05 % Formic acid (B). A total of 5 µL of antioxidant active fraction extract was injected into LC column High Strength Silica (HSS) with type ACQUITY UPLC® HSS C18 (1.8 µm 2.1 × 100 mm). The mass spectrophotometry was determined with two generation quadrupole time-of-flight mass spectrophotometry in positive ion mode. The result was analyzed using Masslynx 4.1 software.

3. Results

3.1. Isolate Identification

The colonies appearance of AGS112 were yellow-red with 0.5-3.0 mm (small-moderate) in size, glistening, convex, curled, and circular on SWC agar medium and confirmed as Gram-positive bacteria. The isolate did not exhibit hemolytic activity compared to the positive control *Staphylococcus aureus* ATCC 6538 (Figure 1). Based on 16S rRNA gene, AGS112 had 99% similarity with *Bacillus haikouensis* C-89 (E-value 0.00; Query cover: 100%) (Figure 2). In addition, the DNA sequences of *B. haikouensis* AGS112 have been submitted to the NCBI GenBank with accession number OP04178.

3.2. Pigment Production, Extraction, and UV-Vis Analysis

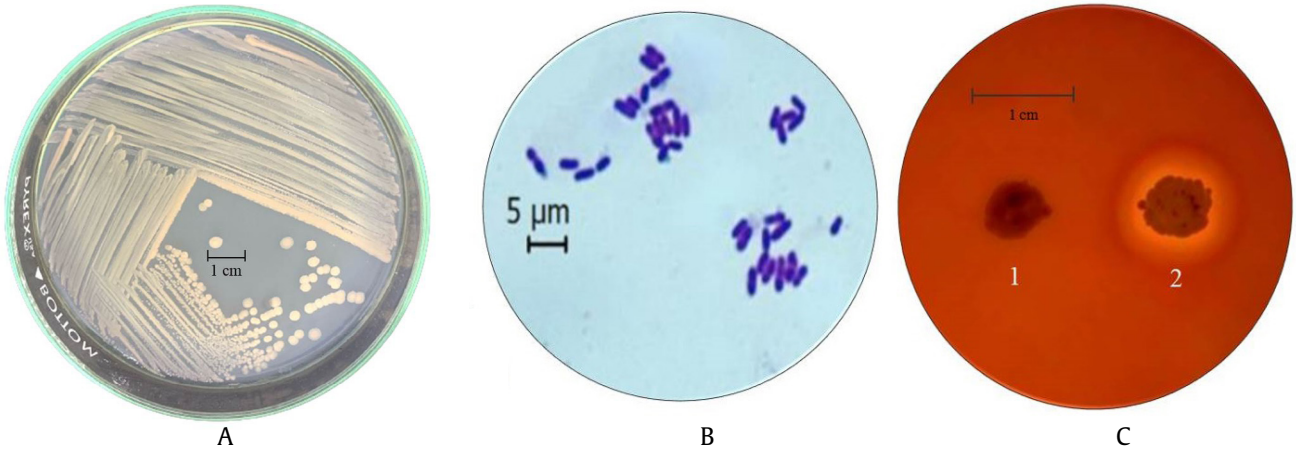
The culture of *B. haikouensis* AGS112 was yellow-red after 7 days of incubation at 27°C in SWC broth medium. The crude pigment extract obtained in this study appeared yellowish and dry, yielding of 12.57% (w/v) (Figure 3). It performed two peaks at 412 nm and 664 nm in UV-Vis analysis (Figure 4).

3.3. TLC Analysis

The mobile phase optimization in TLC analysis revealed dichloromethane and chloroform combination with a ratio of 1:9 (v/v) was the best eluent, giving 11 well-separated bands. The best eluent was then used for bio-autography and preparative TLC. The yellow band with the best antioxidant activity from bio-autography TLC was selected to be continued to preparative TLC using a larger TLC plate (Figure 5).

3.4. Total Phenolic, Total Flavonoid, and Antioxidant Assay

The total phenolic and flavonoid measurements showed the crude pigment extract contained both



A B C

Figure 1. AGS112 colonies appearance on SWC agar medium after incubated at 27°C for 7 days (A), Gram staining result with 1,000× magnification (B) and hemolytic test of AGS112 (C1) compared to *Staphylococcus aureus* ATTC 6538 (C2)

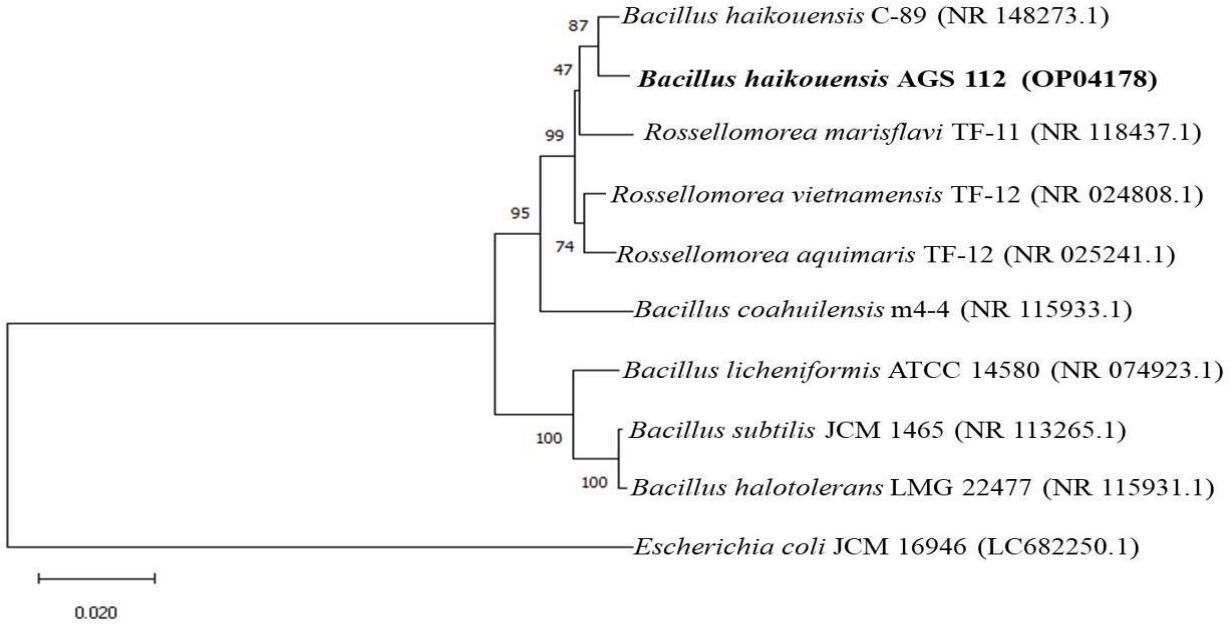
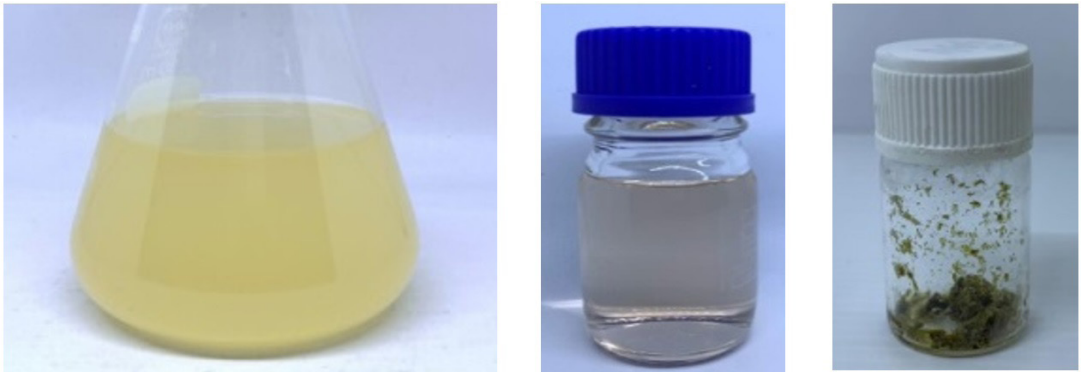


Figure 2. Phylogenetic tree of *B. haikouensis* AGS112 and its related species was constructed by a neighbor-joining model with 1,000× bootstraps



A B C

Figure 3. *B. haikouensis* AGS112 culture on SWC broth medium after incubated at 27oC for 7 days (A) pigment extraction using chloroform (B) and crude pigment extract (C)

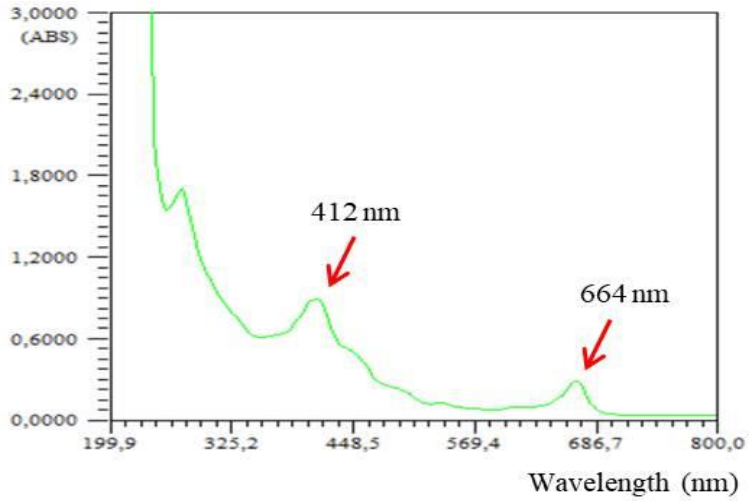


Figure 4. Wavelength scan of *B. haikouensis* AGS112 crude pigment extract showed the presence of two peaks at 412 nm and 664 nm

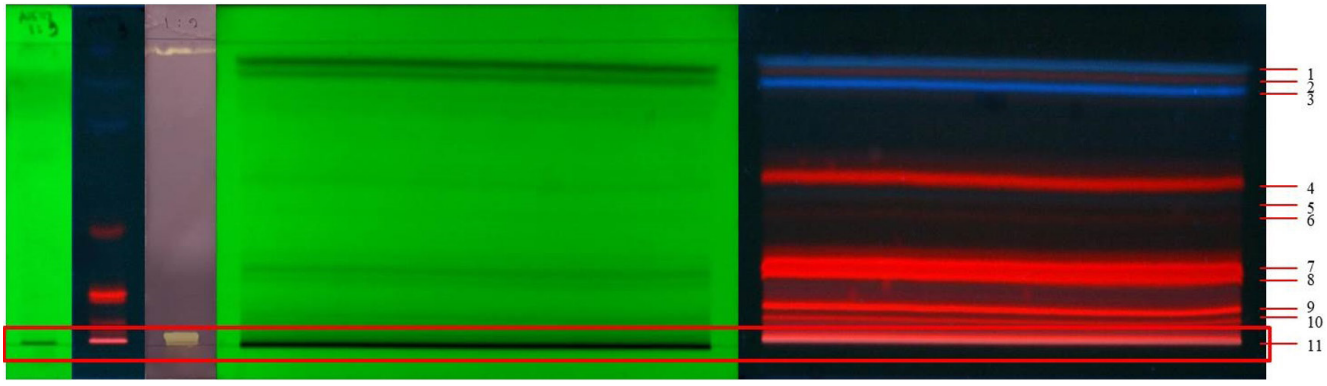


Figure 5. Dichloromethane:chloroform (1:9 v/v) obtained 11 bands observed under UV light 254 (A) and 366 nm (B), bio-autography TLC showed the presence of yellow bands (C), Preparative TLC observed under UV light 254 (D) and 366 nm (E)

phenolic and flavonoid of 54.70±0.32 EAG/g and 7.21±0.11 EQ/g, respectively (Table 1). The *in vitro* antioxidant activity of the crude pigment extract and the active fraction extract were analyzed through DPPH assay. Based on the result, the active fraction extract had stronger antioxidant activity compared to its crude pigment extract with an IC_{50} value of 68.62±0.59 µg/ml and 198.88±1.66 µg/ml, respectively. Each extract treatment had significantly differed with ascorbic acid, which had the IC_{50} value up to 4.3±0.01 µg/ml (pointed out by different letters).

3.5. Oxidative Stress Response Assay

At the cellular level, spot assay showed both the crude pigment extract and the active fraction extract could better maintain the viability of *S. pombe* cells compared to the negative control using 10% DMSO. The negative control showed the cells growth only up to 10^{-2}. The crude pigment extract treatment (100 µg/ml) could maintain the viability of the cells up to 10^{-3}. Meanwhile, the active fraction extract at a lower concentration (35 µg/ml) could maintain and enhance the viability of *S. pombe* up to 10^{-4} in 2 mM H_2O_2 treatment. The result also showed the active fraction extract and positive control (5 µg/ml ascorbic acid) treatment had a similar result. On the other hand, the higher concentration of the crude pigment extract could reduce the viability of *S. pombe* which showed by the cells growth only up to 10^{-2} at the concentration of 500 µg/ml in 2 mM H_2O_2 treatment, in which this result was similar to negative control result (Figure 6).

3.6. Anti-oxidative Gene Expression Analysis

The *in vivo* analysis then continued to anti-oxidative gene expression analysis. The target gene expressions in the extract treatments were up-regulated when added by 1 mM H_2O_2. The *sod1* expressions in the crude pigment extract and the active fraction extract treatment were up to 2 and 3 fold, respectively. Moreover, the *ctt1* expressions were up to 5 and 16 folds, respectively. The active fraction treatment achieved the highest fold expression of *sod1* and *ctt1* genes at 35 µg/ml (Figure 7).

3.7. Liquid Chromatography-Mass Spectrophotometry (LC-MS) Analysis

On *in vitro* and *in vivo* results, the active fraction extract showed better antioxidant activity compared to the crude pigment extract. Therefore, the active fraction extract was further analyzed using LC-MS and obtain several compounds (Table 2), and pointed out by red arrow (Figure 8).

4. Discussion

The isolate identification result in this study corresponded to Li *et al.* (2014) who first reported the identification of *B. haikouensis* C-89 from paddy soil in Haikou Province, Republic of China. In this study, *B. haikouensis* AGS112 was isolated as marine-sponge associated bacterium and produced the yellow-red pigment. The yellow-red pigment produced does not diffuse into the medium, which refers to intracellular pigment. This intracellular pigment was extracted

Table 1. Antioxidant activity, total phenolic and flavonoid content

Sample		IC_{50} (µg/ml)	mg phenolic (EAG/g)	mg flavonoid (EQ/g)
B. haikouensis AGS112	Crude pigment extract	198.88±1.66^{c}	54.70±0.32	7.21±0.11
	Active fraction extract	68.62±0.59^{b}	*nd	*nd
	Ascorbic acid	4.3±0.01^{a}	*nd	*nd

*nd: not determined

The values listed in the table are the means and standard deviations in triplicates. Statistically significant differences were determined by one-way ANOVA followed by the Duncan test (p values < 0.05). Value with the different superscript letters is significantly different

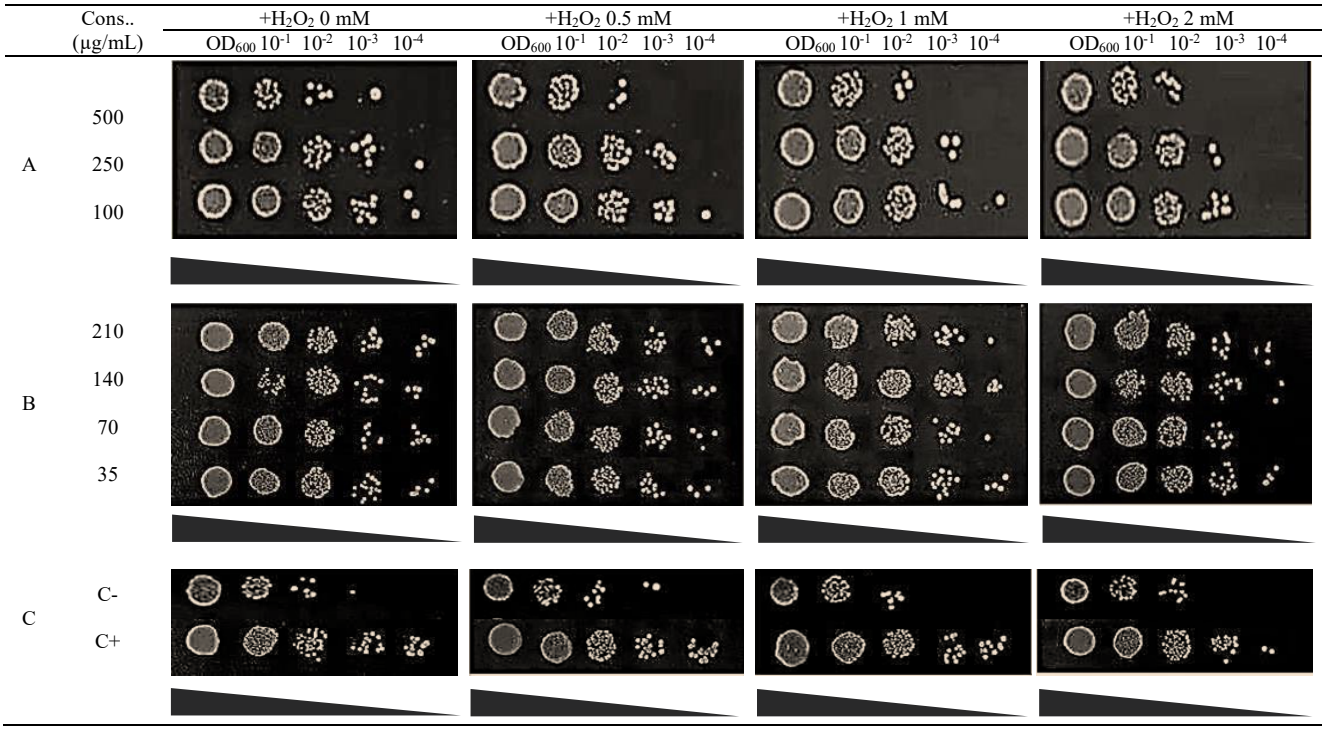


Figure 6. Spot assay using *S. pombe* with several concentrations of crude pigment extract of *B. haikouensis* AGS112 (100, 250, and 500 µg/ml) (A) and the active fraction extract (35, 70, 140, 210 µg/ml) (B) compared to 10% DMSO (C-: negative control) and 5 µg/ml ascorbic acid (C+: positive control)

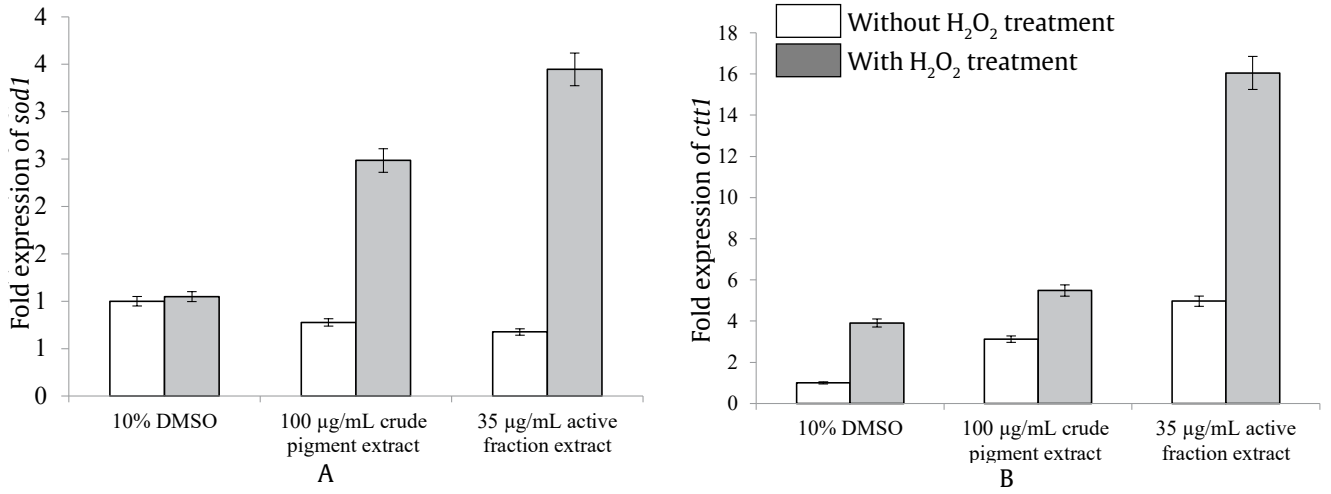


Figure 7. Antioxidant-related gene expressions *sod1* (A) and *ctt1* (B) of *S. pombe* cells treated with crude pigment extract (100 µg/ml) and the active fraction extract (35 µg/ml) on Reverse Transcriptase PCR analysis. 10% DMSO treatment was used as a control

Table 2. Compounds found in the active fraction extract of *B. haikouensis* AGS112 pigment by LC-MS

Peak number	Molecular formula	Proposed metabolite	[M+H]+, m/z	RT (mins)	Type of compounds	Biological activity	Ref
1	$C_{22}H_{26}O_6$	Eudesmin	387.1801	10.13	Lignin	Antioxidant	Jung *et al.* (2018)
2	$C_{17}H_{37}NO_2$	Sphinganine	288.2899	10.81	Sphingolipid	Anticancer	(Xu and Thornalley 2000)
3	$C_0H_{33}N7$	2-(Aminomethyl)-1,4,7,10,13,16-hexaazacyclooctadecane	288.2901	11.10	-	-	-
4	$C_{19}H_{41}NO_2$	3-(Hexadecylamino)-1,2-propanediol	316.3216	12.26, 12.53	-	-	-
5	$C_{30}H_{32}O_6$	Artelastin	489.2266	14.29	Prenylated flavone	Antioxidant, anticancer	(Bailly 2021)

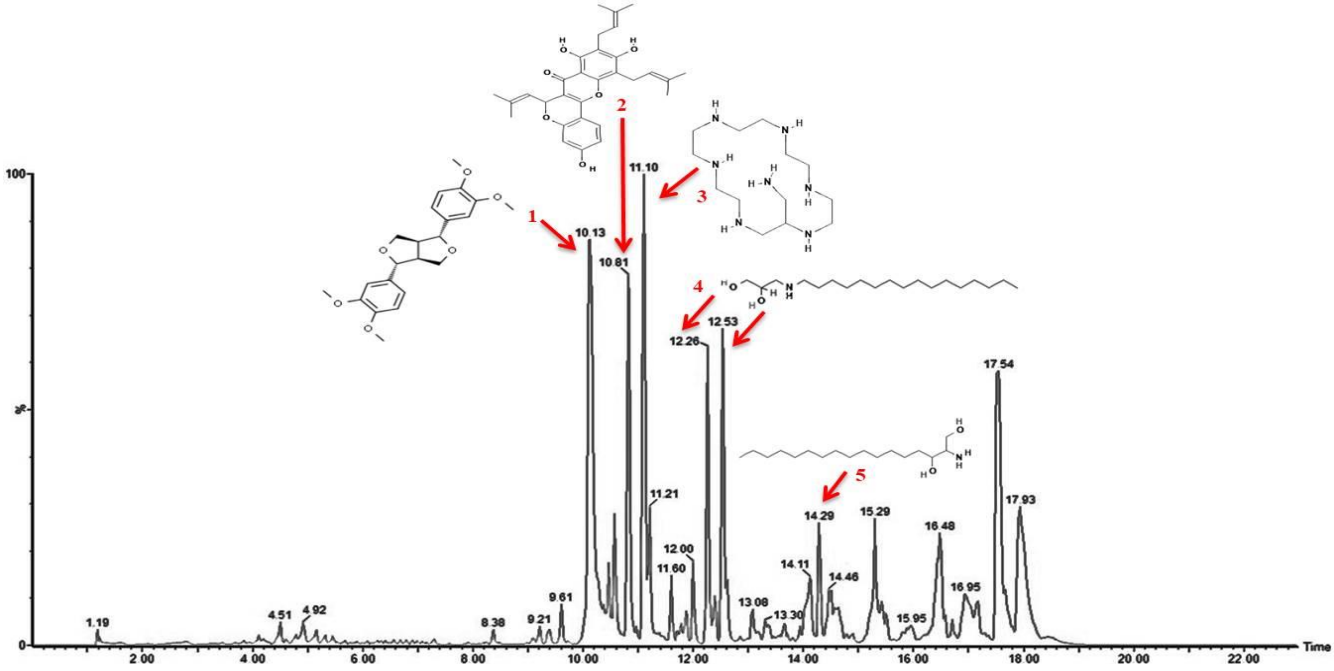


Figure 8. LC-MS chromatogram of *B. haikouensis* AGS112 active fraction extract. Peaks are labeled as follow, 1: eudesmin, 2: sphinganine, 3: 2-(Aminomethyl)-1,4,7,10,13,16-hexaazacyclooctadecane, 4: 3-(Hexadecylamino)-1,2-propanediol, 5: artelastin

using chloroform that has good affinity to pigment and can penetrate cells. This function is essential to release the pigment to the solvent through the plasma membrane (Saini and Keum 2018).

The highest peak of the crude pigment extract at 412 nm (Figure 4) suggested as the presence of carotenoids pigment group. Sharma and Ghoshal (2020) stated the 400-500 nm wavelengths known as carotenoids fingerprint regions. Meanwhile, another peak at 664 nm was suggested as the presence of flavonoids (Omara *et al.* 2022) that confirmed by the total flavonoid measurement (Table 1). The presence of carotenoid, phenolic and flavonoid indicated these compounds might play a role as antioxidants. The correlation of phenolic and flavonoid content with the antioxidant activity of an extract already reviewed (Sariwati *et al.* 2019; Pudjas *et al.* 2022). Phenolic and flavonoids are known as radical scavengers, metal chelators, reducing agents, and hydrogen donors. It could react with hydroxyl radicals, superoxide radical anions and known to protect DNA from oxidative damage (Senthilkumar *et al.* 2013; Chandra *et al.* 2014; Lefahal *et al.* 2018). The lower total flavonoid in this study might cause by the presence of other group of phenolic compounds,

such as phenolic acids and tannins (Suleria *et al.* 2020).

In order to obtain more specific compounds that act as antioxidants, the crude pigment extract was fractionated using TLC. In bio-autography TLC, two yellow bands formed were indicated as the active fraction extract. However, the upper yellow band (Figure 5C) did not showed the strong antioxidant activity after tested using DPPH assay (data does not sound), so the lower yellow band with red mark in Figure 5C was then selected to be used for further steps in this study. The yellow spots on a purple background of TLC plate indicated as antioxidant compounds that could scavenge DPPH radicals (Belaqziz *et al.* 2017).

The higher antioxidant activity of the selected active fraction extract corresponded to Prastya *et al.* (2022), who reported that an active fraction extract of *Pseudoalteromonas flapivulchra* STILL-33 extract had a better antioxidant activity than its crude extract. Nevertheless, the crude pigment extract showed a better antioxidant activity compared to a previous study described by Kusmita *et al.* (2017), who reported that four yellow pigment extracts produced by bacteria associated with soft-coral *Sarcophyton* sp. had an antioxidant activity with an IC_{50} value >2,000 mg/L against DPPH. At the cellular level, the active fraction extract at the concentration of 35 µg/mL could better maintain and enhance the viability of *S. pombe* similar to positive control treatment compared to other treatments (Figure 5).

The compounds contained in the active fraction extract that have been screened through bio-autography TLC suggested to be more specifically act as antioxidant compared to the compounds contained in the crude pigment extract. On the other hand, the higher concentrations of the crude pigment extract gave the lower effect of cells' viability. This result corresponded to Fauzya *et al.* (2019), who described the clove leaves extract at lower concentration was better in increasing the viability of *S. pombe*. The effect of the extract was unlikely to occur in a dose-dependent manner in promoting oxidative stress response in *S. pombe*. In addition, several compounds such as catechin (epigallactechin gallat) in certain concentrations could inhibit catalase and induce the increase of ROS and leading to apoptosis (Pal *et al.* 2014). Therefore, some compounds in the crude pigment extract might cause a cytotoxic effect in higher concentrations.

This spot assay result contrasted by anti-oxidative gene expression result. The highest up-regulation of *sod1* and *ctt1* achieved in the active fraction extract treatment (35 µg/ml), and might occur through the mitogen-activated protein kinase (MAPK) pathway, which is induced by 1 mM H_2O_2, then activates the transcription factor pap1 in *S. pombe*. The most important MAPK pathway is *sty1*, a stress-activated kinase. *Sty1* triggered the activation of *atf1* and *pap1* transcription factors. Both *sty1-atf1* and *pap1* were reported as redox sensor that is directly activated by increasing H_2O_2 levels (Kudo *et al.* 1999). Then, these transcription factors trigger the expression of stress response genes including *sod1* and *ctt1* (Toone *et al.* 1998). These genes are essential to raising oxidative response, direct ROS scavenging, and promotion of redox homoeostasis in *S. pombe* (Papadakis and Workman 2015). The *sod1* encoded superoxide dismutase and *ctt1* encoded catalase, in which these enzymes are essential in ROS stabilizing (Nimse and Pal 2015). The *sod1* and *ctt1* are regulated by the same key transcriptional factors gene *pap1* (Astuti *et al.* 2021). Therefore, the up-regulated expression of the two genes might coincide. The *ctt1* gene was reported to have promoter containing both *atf1* and *pap1* transcription binding sites. Thus, *ctt1* could express by using different transcription factors depending on the H_2O_2 level, in which *pap1* will be active at a lower dose (<1 mM), while *sty1* will be activated at higher dose (>2 mM) of H_2O_2 (Quinn *et al.* 2002).

The LC-MS analysis result revealed the active fraction extract was rich in eudesmin, following by four other compounds (Table 2). Eudesmin reported to be found in Magnolia flos methanol extract (Jung *et al.* 2018). Artelastin was successfully isolated from *Artocarpus elasticus* (Bailly 2021), and sphinganine is including as sphingolipids group (Huang *et al.* 2018). These 3 compounds reported to have biological activities. Meanwhile, the biological activity report of 3-(Hexadecylamino)-1,2-propanediol and 2-(Aminomethyl)-1,4,7,10,13,16-hexaazacyclooctadecane are not available, in which these compounds reported to be found in *Marcelia crenata* leaves extract (Ma'arif *et al.* 2019) and in tannin fraction of *Piper crocatum* leaves (Safithri *et al.* 2016), respectively. Therefore, eudesmin and artelastin here might have essential role as antioxidants.

Throughout previous studies, there is not report yet about the potency of *B. haikouensis* in the

pharmaceutical application and its antioxidant activity. Therefore, it becomes very interesting to found the novel potency of the yellow-red pigment produced by *B. haikouensis* AGS112 as antioxidant in this study. Thus, this study result could be one of the scientific reports regarding to the potency of this species for further studies.

In conclusion, the yellow-red pigment produced by the sponge-associated bacterium *Bacillus haikouensis* AGS112 showed antioxidant activity against DPPH. It contained both phenolic and flavonoid, and the UV-Vis analysis indicated the presence of carotenoids. The active fraction extract had stronger antioxidant activity than its crude pigment extract. Moreover, it could better enhance the viability of *S. pombe* cells and achieve the highest expression of *sod1* and *ctt1* genes. Based on metabolite profiling using LC-MS analysis, the active fraction extract contained eudesmin and artelastin that might have essential role as antioxidants. Thus, this study reported the potency of the yellow-red pigment produced by *B. haikouensis* AGS112 as antioxidant to be prospective candidates for natural antioxidants.

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