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Bacillus sp. SAB E-41-derived extract shows antiaging properties *via* ctt1-mediated oxidative stress tolerance response in yeast *Schizosaccharomyces pombe*

Muhammad Eka Prastya[1], Rika I. Astuti[1], Irmanida Batubara[2,3], Aris T. Wahyudi[1]✉

[1]*Department of Biology, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Bogor 16680, Indonesia*

[2]*Department of Chemistry, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Bogor 16680, Indonesia*

[3]*Tropical Biopharmaca Research Center, Bogor Agricultural University, Bogor 16128, Indonesia*

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ABSTRACT

Objective: To analyze potential activation of oxidative stress tolerance systems by SAB E-41 bacterial extract in promoting the life span of yeast *Schizosaccharomyces pombe*. **Methods:** *In vitro* analysis was done to assess antioxidant activity of SAB E-41 bacterial extract. Antiaging property of the particular extract was then assayed through spot test and chronological life span assays. Furthermore, *sty1* mitogen-activated protein kinase, *pap1* transcriptional factor of oxidative stress response and its downstream genes, *ctt1* were evaluated *via* real time PCR. The protein level of *ctt1* was then observed *via* Western Blot analysis. In addition, accumulation of reactive oxygen species and mitochondrial activity were conducted to understand the effect of SAB E-41 upon oxidative stress response systems *in vivo*. **Results:** The IC_{50} values of corresponding extract for antioxidant (DPPH; ABTS) and antiglycation were 402.40, 358.13 and 683.55 μg/mL, respectively. In addition, SAB E-41 extract (750 μg/mL) exhibited antiaging properties, which could be attributed to significant up-regulation of oxidative stress response genes, *sty1*, *pap1* and *ctt1*. Interestingly, SAB E-41 extract could enhance stress tolerance phenotype of *Schizosaccharomyces pombe* against H_2O_2-induced oxidative stress. These results were supported by increasing mitochondrial activity and reactive oxygen species intracellular levels. **Conclusions:** SAB E-41 extract could promote yeast life span likely *via* up-regulation of oxidative stress responses in yeast. Our results suggest that adaptive response *via* up-regulation of oxidative stress transcriptional factors, and its downstream gene, *ctt1*, as well as mitochondrial activity contributes in combating oxidative stress thus promoting yeast life span.

1. Introduction

Aging is a highly complex biological phenomenon that occurs in all living organisms. Oxidative stress, caused by the excess amount of reactive oxygen species (ROS), and accumulation of advanced glycation end products (AGEs) are called as the main factors caused aging[1,2]. Both of molecules may damage DNA, lipids, and also have been implicated in numerous diseases, thus proposed as a

✉Corresponding author: Aris T. Wahyudi, Department of Biology, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Bogor 16680, Indonesia.
Tel: +62-251-8622833
Fax: +62-251-8622833
E-mail: aristri2011@gmail.com

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molecular cause of aging[1,3]. ROS, including hydrogen peroxide (H_2O_2), superoxide anions (O^-) and hydroxyl radicals (OH^-), are generated by the chemical reduction of oxygen by a variety of cellular enzymes or by incomplete reduction of oxygen to water in the mitochondrial respiratory chain. However on the mild level, ROS are employed some signalling pathways involved in stress response, proliferation and inducing the adaptive response mechanisms[3]. On the other hand, AGEs are cellular molecules that are formed by glycation process through maillard reaction between proteins and reducing sugars. These processes result in formation of Amadori products leading to the formation of the toxic AGEs molecules[4]. In line with that, it can be suggested that anti-ROS and anti-AGEs effects of compounds that are implicated with antioxidant and antiglycation activities are possible to be used as one of the actions to counteract aging *in vitro*.

In this study, we used SAB E-41 bacteria isolated from marine sponge *Jaspis* sp. taken from Waigeo Island, Raja ampat, Indonesia[5]. Crude extract of this bacteria was reported to have antibacterial activity. In addition, this isolate exhibited Ketide Synthase and Adenylation domain of encoding bioactive compound genes and was identified as *Bacillus* sp. based on 16S rRNA[6]. Interestingly, corresponding extract showed antioxidant and anticancer activities against human cervix HeLa cells[7]. In the present study, the potential antiaging properties of the particular SAB E-41 extract are studied. In doing so, we have investigated the potential antiaging properties of corresponding extract by using yeast *Schizosaccharomyces pombe* (*S. pombe*) as model organism correlated with H_2O_2 induced oxidative stress.

The fission yeast *S. pombe* is a popular model organism to study aging as well as oxidative stress response mechanisms[8,9]. The conserved mRNA splicing process with metazoan indicates mitochondrial inheritance mechanism similar to mammalian cells[8]. In addition, *S. pombe* has the *sty1* mitogen-activated protein kinases pathway that contributed to the response of yeast cells against oxidative stress. The *sty1* pathway activates both transcriptional factors *pap1* and *atf1*, thus controlling the expression of some downstream antioxidative genes including *ctt1*, *gpx* and *tpx*. Mammalian homologs of these factors, Atf2 and cJun, are regulated by the JNK and p38 stress activated protein kinases[10]. However, oxidative stress response is essential for yeast survival since this process will activate yeast defense mechanisms to combat oxidizing agent[11].

2. Materials and methods

2.1. Extraction of bioactive compounds and phytochemical analysis

Briefly, about 1% of liquid bacterial cultures were re-inoculated on Sea Water Complete liquid medium and incubated for 3 d with shaking on 120 rpm at room temperature. Subsequently, bacterial cultures were mixed with ethyl acetate (1:1) and shaked as modification technique was performed at room temperature for 20 min[7]. The ethyl acetate layer was separated and evaporated by using heating temperature of 60 ℃ to collect bacterial crude extract. In addition, phytochemical screening was conducted to assess the corresponding compound groups of crude extract[7].

2.2. In vitro antioxidant and antiglycation assays

Briefly, 2,2-diphenyl-1-picryl-hydrazil (DPPH) solution (125 μM) was mixed with extract or standard (ascorbic acid) in 96-well plate. The mix was incubated on the dark at room temperature for 30 min and the optical density was measured at 515 nm[12]. In addition, ABTS solution (7 mM) was oxidized by potassium peroxide sulfate for 12-16 h. Furthermore, about ABTS radical was reacted with extract or standard (ascorbic acid) and incubated for 30 min at 96-well plate. The absorbance was measured at 734 nm[13]. Inhibitory concentration of 50% (IC_{50}) scavenging of radicals was determined.

For antiglycation assay, solution consisted of bovine serum albumin (20 mg/mL), glucose (235 mM), fructose (235 mM) and extract or positive control (Aminoguanidine) in phosphate buffer 0.2 M (pH 7.4)[14]. Solutions were incubated at 60 ℃ for 40 h, then the excitation fluorescence intensity was measured at 330 nm and the emission at 440 nm. Inhibitory concentration of 50% (IC_{50}) against the AGEs molecule was measured.

2.3. Yeast strains, plasmid and growth conditions

Yeast strain used in this study was wild type of *S. pombe* ARC039 (*h⁻leu1-32 ura4-294*). Genetic manipulation including epitope tagging on *ctt1* with green fluorescent protein (GFP) was performed by using chromosomal integration of PCR amplified cassettes of the targeted DNA-flanking regions[15]. pFA6a-GFPkanMX6 plasmid was used as a template to generate carboxyl-terminal GFP-tagged proteins cassettes. Subsequently, yeast transformation procedure was then performed to integrate the corresponding PCR cassettes into yeast cells[16]. For selective growth of the transformants, G418 (Santa Cruz Biotech) antibiotics were added to Yeast Extract with Supplement (YES) plates medium at concentration of 100 mg/mL.

2.4. In vivo antiaging activity and oxidative stress tolerance assay using spot test

Spot test was performed as the study described earlier with some modifications[17]. The inoculum was re-inoculated on 3 mL YES treatment culture (on 20 mL test tube) with an initial OD_{600} of 0.05. Extract diluted on DMSO was supplemented in treatment culture. Yeast cells on Edinburgh Minimal Medium (EMM) and YES liquid medium supplemented with DMSO were designed as positive and negative controls, respectively. Each culture was incubated with shaking at 300 rpm for 11 d at 30 ℃ and spot test was conducted on day 1, 5, 9 and 11. For oxidative stress tolerance assay, yeast cells were grown, diluted and spotted (as described above) on YES plates containing the various concentrations of H_2O_2.

2.5. Chronological life span assay

Briefly, inoculum was re-inoculated into 50 mL (on 250 mL flask) of YES liquid medium with an initial OD_{600} of 0.05 as a treatment culture then added with extract. Yeast cells on YES liquid medium and EMM liquid medium supplemented with DMSO were set as negative and positive controls, respectively. All cultures were grown till day 20 with shaking (120 rpm) at 30 ℃. Maximum cell density was achieved approximately after 24 hours of growth in YES liquid medium. One day after inoculation was used as day 1 of chronological

life span. Furthermore, cellular viability was conducted at day 1, 4, 7, 10, 13, 17 and 20 by using Total Plate Count method[17].

2.6. Real-time quantitative PCR analysis

Yeast cells were cultured in 3 mL YES liquid medium (on 20 mL reaction tube) with an initial OD_{600} of 0.05 after the administration with extract. Furthermore, treatment was incubated with shaking on 300 rpm at 30 ℃ for 24 h before cell harvested. In addition, 1 mM H_2O_2 treatment was conducted 1 h prior to cell harvested. RNA was extracted by using Rneasy Mini Kit (Qiagen). cDNA synthesis was conducted *via* reverse transcription reaction with random primer (Applied Biosystems) with RNA concentration of 2 μg. The *sty1*, *pap1*, *ctt1* and control *act1* were determined by using Light Cycler 96 Systems Roche and using SYBR green PCR Master mixed (Applied Biosystems) as a fluorescent reporter. Subsquently, the amounts of *sty1*, *pap1* and *ctt1* mRNA level were normalized to that of *act1*.

2.7. Catalase activity assay

Wild type of yeast cells was cultured on designated treatment conditions as mentioned above. Soluble protein extracts and catalase activity were determined by using previous method[18]. Briefly, soluble extract was added to 20 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0). H_2O_2 decomposition was monitored at 240 nm (Beckman Coulter DU 800) (ε_{240} = 43.6 M/cm). One unit of catalase activity was described as the capacity of corresponding enzyme to catalyze the degradation of 1 mmol of H_2O_2 per-min.

2.8. Immunoblot analysis

To collect whole cell extract, yeast cells treatment was designed for the qRT-PCR analysis (see above). Trichloroacetic acid extracts were prepared as described earlier[19]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by subjecting 5 μg of total protein to 7.5% of polyacrylamide gel. Subsequently, proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore) and probed with a mouse monoclonal anti-GFP antibody (12CA5, Roche Life Science) followed by a mouse IgG secondary antibody. Western blot signals were detected by using a chemiluminescence-based approach using Image Quant LAS 4000 (GE Healthcare Life Sciences).

2.9. Mitochondrial activity

Mitochondrial activity was monitored *via* a fluorescent dye Rhodamine 123 (Sigma Aldrich). Yeast cells were cultured in 3 mL YES liquid medium (on 20 mL reaction tube) with an initial OD_{600} of 0.05 after the administration with SAB E-41 extract. Cells were harvested after grow till log phase and further suspended in 50 mM sodium citrate buffer (pH 5.0) containing 2% glucose. Rhodamine 123 was added at a final concentration of 50 mM to the cell suspensions and stained cells were observed *via* fluorescent microscope Axiovert 200M (Carl Zeiss, Germany).

2.10. Cellular ROS measurement

Cellular ROS measurement was determined by using flow cytometry as described by previous study[20]. Briefly, yeast cells were grown on designated conditions as mentioned in mitochondrial activity (see above). Yeast cells were suspended in 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM of 2',7'-dichlorofluorescein diacetate (Ana Spec Inc). Positive control was conducted by using 1 mM H_2O_2 treatment to the culture 2 h prior to being harvested. The fluorescence intensity was quantified by using the BD Accuri C6 instrument (BD Biosciences, USA) with the FL1-H channels.

2.11. Statistical analysis

Statistical analysis was performed as mean ± SD from three independent replicates. One ways analysis of variance (ANOVA) was applied for comparison of the mean values with 95% and 99% confidence levels. Further analysis was performed by multiple Duncan test range test. Differences were significant as $P<0.05$ or $P<0.01$.

3. Results

3.1. In vitro activity of SAB E-41 extract

Based on phytochemical analysis, ethyl acetate-derived SAB E-41 extract contained some groups of bioactive compounds including alkaloid, flavonoid, steroid and terpenoid. Furthermore, for *in vitro* antioxidant and antiglycation assays, IC_{50} value indicated the ability of sample to stabilize 50% reactivity of the designated free radicals or to inhibit 50% production of AGEs molecules. Thus, low value of IC_{50} exhibited strong activity assays compared with positive controls. Antioxidant activity was assayed by using 2 different radicals including DPPH and ABTS and the obtained IC_{50} values were 402.40 and 358.13 μg/mL, respectively. The result for antiglycation showed that SAB E-41 extract had IC_{50} of 683.55 μg/mL (Table 1). The IC_{50} values of antioxidant and antiglycation activities indicate that SAB E-41 extract had low *in vitro* activity compared with positive control. However, the IC_{50} of corresponding extract was approximately 100, 17 and 33-fold higher than its positive control for DPPH, ABTS and antiglycation activity, respectively.

Table 1

Antioxidant and antiglycation activities of SAB E-41 extract.

Samples	DPPH IC_{50} (μg/mL)	ABTS IC_{50} (μg/mL)	Antiglycation IC_{50} (μg/mL)
Aminoguanidine	NA	NA	20.54 ± 0.58^a
Ascorbic acid	4.42 ± 0.02^a	21.04 ± 0.17^a	NA
SAB E-41	402.40 ± 16.55^b	358.13 ± 13.22^b	683.55 ± 0.87^b

Values with the same superscript letter have no significant difference ($P<0.05$); NA-Not applicable; IC_{50} indicates the ability of crude extracts to stabilize 50% reactivity of the designated free radicals or to inhibit 50% of AGEs molecules.

3.2. Antiaging effect and oxidative stress resistance induced by SAB E-41 extract on S. pombe

Antiaging activity of SAB E-41 extracts was performed by using spot test. We tested several concentrations of SAB E-41 extract on

YES medium and found that the concentration of 750 μg/mL could significantly increase life span of *S. pombe* till day 11 compared with controls and other extract concentrations (Figure 1A). We further sought to validate the effect of SAB E-41 extract (750 μg/mL) on yeast life span extension by determining their effect on the viability by measuring colony forming unit. In this case, corresponding extract caused increased longevity of yeast cells till day 20 that was comparable to that DMSO (YES) with longevity only till day 13 (Figure 2). Thus, concentration of 750 μg/mL was employed for next analysis. Interestingly, SAB E-41 extract treatment was exhibited to induce yeast tolerance against 0.75 mM H_2O_2 induced oxidative stress till day 11 (Figure 1B).

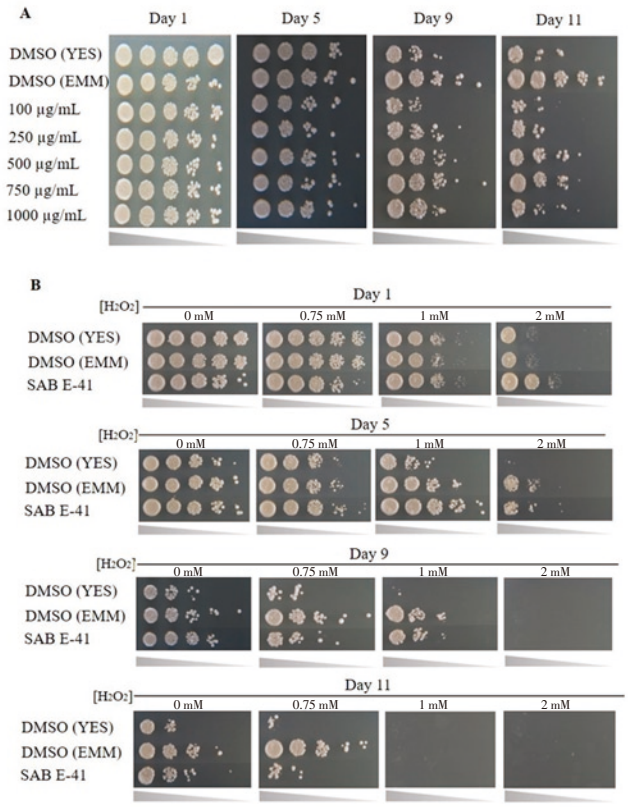

Figure 1. Chemical screening for life span-extending effect of SAB E-41 extract (different concentrations) in *S. pombe*.
(A) Life span extension was induced by SAB E-41 extract at the best concentration of 750 μg/mL. Spot on YES plates medium. (B) SAB E-41 extract at 750 μg/mL caused yeast cells more resistant to H_2O_2 oxidative-stress. DMSO was used as solvent control.

3.3. Molecular and cellular effects of SAB E-41 extract on S. pombe

Next, we measured the mRNA expression level of some genes correlated with antioxidant defence mechanism including *sty1* mitogen-activated protein kinase, transcription factor *pap1* and *ctt1* gene of yeast cells after being treated with SAB E-41 extract. Intriguingly, mRNA level of some targeted genes including *sty1*, *pap1* and *ctt1* was significantly increased after being treated with corresponding extract induced by H_2O_2 treatment (Figure 3). Based on these results, we subsequently used H_2O_2 treatment in the next experiment to understand fullly the effect of this extract. Furthermore, as shown in Figure 4, it was revealed that SAB E-41 extract increased *ctt1* mRNA level and catalase activity at the best concentration of 750 μg/mL. These results were supported with other results as shown in Figure 5 that extract treatment can increase catalase protein expression patterns based on western blot analysis, which was also supported by intracellular localization of catalase protein by detecting GFP signal. Subsequently, SAB E-41 extract treatment can induce mitochondrial activity on log phase, shown by bright fluorescent signal on yeast cells (Figure 6A). In line with that, the results indicated corresponding extract treatment could increase ROS intracellular level as shown in Figure 6B.

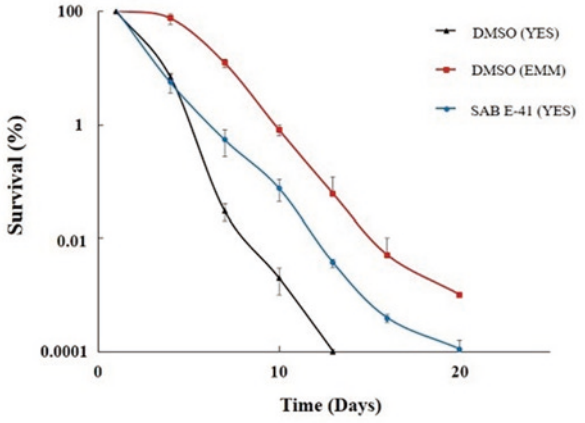

Figure 2. Life span-extending effect of SAB E-41 extract (750 μg/mL diluted on DMSO) on *S. pombe* cells based on standard chronological life span assay. Blue, black and red curves indicate SAB E-41 extract, DMSO on YES medium as negative control and DMSO on EMM medium as positive control, respectively.

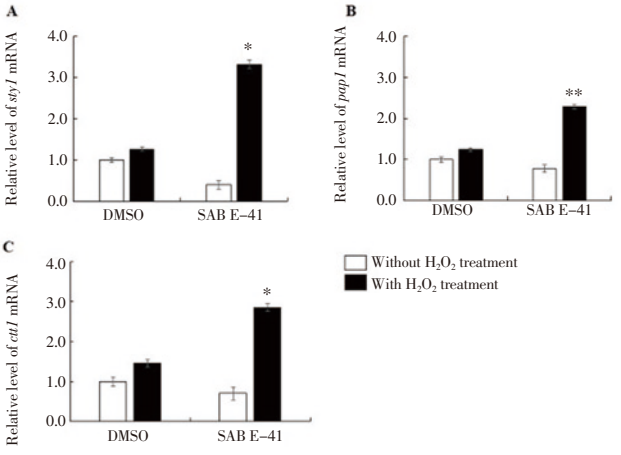

Figure 3. *sty1*, *pap1* and *ctt1* gene expressions (A, B, C) of *S. pombe* after administration with SAB E-41 extract (750 μg/mL diluted on DMSO).
Amplification of *act1* was used to normalize the relative levels of corresponding mRNA. One mM H_2O_2 was supplemented on yeast culture 1 h before cell harvested. $^{*}P<0.05$, $^{**}P<0.01$.

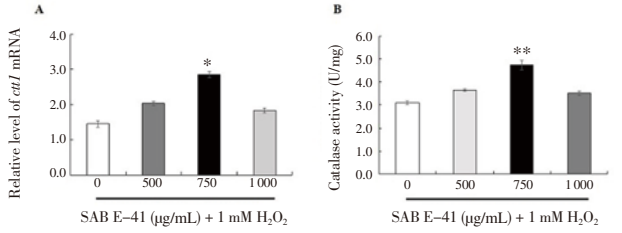

Figure 4. *ctt1* gene expression (A) and changes in catalase activity (B) of *S. pombe* after treatment with different concentrations of SAB E-41 extract ("0" indicates yeast cells supplemented with DMSO).

Catalase activity was assayed by using wild type of *S. pombe*. One mM H_2O_2 was supplemented on yeast culture 1 h before cell harvested. $^{*}P<0.05$, $^{**}P<0.01$.

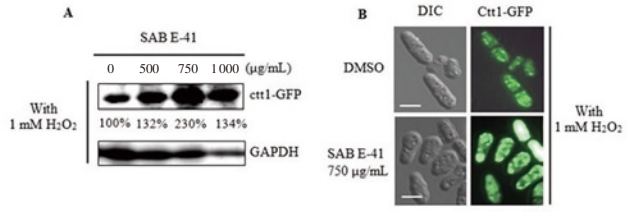

Figure 5. Catalase protein expression patterns after administration with various concentrations of SAB E-41 extract (diluted on DMSO) (A) and intracellular localization of catalase (B).

Bars represent 5 μm. "0" indicates yeast cells supplemented with DMSO.

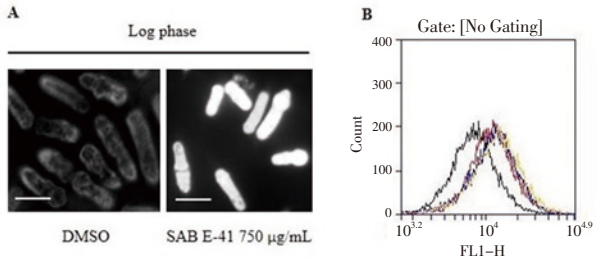

Figure 6. Mitochondrial activity after treatment with extract (A) and intracellular ROS level after treatment with extract (750 μg/mL) (B).

Bars represent 5 μm. Black, red, blue and yellow curves indicate DMSO, DMSO + H_2O_2, extract treatment and extract treatment + H_2O_2, respectively.

4. Discussion

The initial *in vitro* phytochemical analysis showed that SAB E-41 extract contained a group of alkaloids, flavonoid, steroid and terpenoid. This result was consistent with previous study that this extract contained some groups of compounds as mentioned above[7]. Generally, phenolic and flavonoid compounds were reported to possess high antioxidant, antiglycation and correlate with antiaging activity[21]. We furthermore report here that corresponding extract has low antioxidant and antiglycation activities compared with positive controls. In addition, previous study reported DPPH scavenging activity of compounds which were isolated from *Bacillus* sp. associated with sponge collected from Agatti Island, Maldives with IC_{50} value of 15.02 μg/mL[22]. On the other hand, only few study reported antiglycation activity of sponge-associated bacterial extract, which comes from plant extracts such as root crude extract from *Mangifera indica* with IC_{50} of 147 μg/mL[23]. Thus, SAB E-41 extract has the antioxidant activity with IC_{50} value of 23 until 26-fold higher than antioxidant activity of compounds isolated from *Bacillus* sp.[22]. In addition, corresponding extract showed the antiglycation activity with IC_{50} value of 4.6-fold higher than the same activity of root crude extract from *Mangifera indica*[23]. Based on these results, it can be known that SAB E-41 extract has low *in vitro* activities compared with previous research.

Surprisingly, our results showed that SAB E-41 extract can significantly increase yeast cells viability when cultured on the YES liquid medium. These suggest that SAB E-41 extract has potentially antiaging *in vivo* even low *in vitro* activity. In fact, life span extension effect of corresponding extract was shorter than that yeast viability on EMM medium. Notably, fission yeast was reported to have calorie restriction (CR) response while growing on low glucose medium such EMM. It was reported that CR can induce respiration of yeast cells rather than glucose fermentation since log phase affects high yeast mitochondrial activity, resulting in mild level of intracellular ROS and triggering adaptive response mechanism against oxidative stress[24]. CR also can inhibit nutrient signalling kinase such Pka1, Sck2 and Git3, which affects cells longevity[8,17]. In contrast, on high glucose medium such as YES, yeast cells showed the contradictory mechanism compare with CR condition. It was shown by the acceleration of aging process from yeast cells when cultured on YES medium. Thus, our results suggested that SAB E-41 extract may exhibit mimetics CR condition, in which this extract can increase yeast life span even on non CR condition.

Next, our results showed that extract treatment can increase expression level of *sty1* pathway, *pap1* transcriptional factor and *ctt1* gene compared to the untreated groups. Consistent with this, catalase activity and catalase expression patterns were also increased when treated with corresponding extract. Interestingly, SAB E-41 extract increases some targeted genes with H_2O_2 treatment and depending on the extract concentration. However, *sty1* is a mitogen-activated protein kinase cascade that phosphorylates and regulates the stability of transcription factor *atf1*. The *sty1-atf1* pathway is activated in response to multiple environmental stress including oxidative stress, osmotic stress, heat stress and heavy metal toxicity[9]. On the other hand, *pap1* is the transcription factor required for survival during oxidative stress by activating genes function in oxidant protection. Both of *sty1-atf1* and *pap1* were reported as redox sensor that is directly activated by increased H_2O_2 levels[25]. Interestingly, *ctt1* gene was reported as one of the genes and its promoter contains both of transcription binding sites. Thus, *ctt1* could express by using distinct transcription factor depending on H_2O_2 level, in which *pap1* will be active at low dose (0.025 - 1 mM), while *sty1* will be activated at acute dose (>1 mM of H_2O_2)[26,27]. Thus, it can be suggested that SAB E-41 extract may interfere with H_2O_2 treatment to activate *sty1* and *pap1* signalling pathway as well as increase *ctt1* expression. Up-regulation of *ctt1* may significantly reduce intracellular H_2O_2 radicals on stationary phase, causing beneficial effect on increasing yeast life span.

We furthermore report here that extract treatment can induce mitochondrial activity as well as increase ROS intracellular level

on log phase. One possibility is that corresponding extract likely increases the cellular ROS which leads to the modulation over some stress response genes, thus resulting in increased oxidative stress resistance and reduced cellular ROS level in stationary-phase. In line with that, previous result indicated that extract treatment could induce oxidative stress tolerance phenotype in yeast cells. Such results indicate that corresponding extract may act as pro-oxidant, as the extract could likely induce physiology mechanism of yeast cells to combat oxidative stress. Pro-oxidant mechanism was closely related with adaptive response which means that low dose of stress-inducing factors could lead to stimulatory response, whereas high doses could induce deleterious effect[28]. Previous studies reported the pro-oxidant mechanism of 3,3-diindolylmethane and Phloxin B on *S. pombe* on increase of yeast longevity[29,30].

In summary, our approach of using molecular and cellular aspects for identifying biological antiaging activity has proven potential mechanisms of compound isolated from sponge-associated bacteria. We show that SAB E-41 extract has low *in vitro* activity, but has some beneficial effects on increasing yeast life span *in vivo*. We reveal pharmacological induction of *ctt1* gene induced by H_2O_2, increasing mitochondrial activity and intracellular ROS level slows down aging process in *S. pombe*. Thus, we have proposed inducible as well as pro-oxidant mechanism of SAB E-41 extract to increase yeast longevity. However, further analyses are needed to determine the chemical compounds which are responsible for their promising activity.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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