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Effect of Storage of Yellow Pigment from Halophilic *Bacillus clausii* J1G-0%B on Antioxidant Activity

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Abstract

Oxidative stress is a state of excess free radicals in the body, which results in increased oxidation processes in the body's cells and causes damage. In previous studies, one way to neutralize this situation was found, namely with halophilic bacteria. Halophilic bacteria can live at a salt content of 2% to 30%. These bacteria can produce various kinds of pigments for self-defense from extreme environments, which function as immunomodulatory compounds and have antioxidant activity. The antioxidant activity of the yellow pigment halophilic bacteria *Bacillus clausii* J1G-0%B using the DPPH method showed that the effect of light and air on antioxidant activity was 17.88% inhibition in the crude extract and 14.24% inhibition at 1600 ppm. Antioxidant activity decreased by 17.13% under the influence of air, 1.89% under the influence of light, and 28.58% under the influence of air and light. FTIR analysis under the influence of air experienced a decrease in the peak of the CO carbonyl group of 1739.29 cm^{-1} and an increase in the alkane C-H group of 1369.52 cm^{-1}, while under the influence of light and open conditions, the carbonyl CO group experienced an increase of 1739.50 cm^{-1} and 1739.46 cm^{-1} and the increase in alkane C-H groups 1371.41 cm^{-1} and 1369.53 cm^{-1}.

1. Introduction

Free radicals are produced daily in this fast-paced modern period from a variety of sources, including cigarette smoke, air pollutants, and pesticide-contaminated food [1, 2]. Free radicals can cause oxidative stress on the body. Oxidative stress is when the number of free radicals exceeds the body's capacity to neutralize them, causing the oxidation process in normal body cells to increase and cause damage [3]. Free radicals that the body cannot handle are one of the main causes of chronic diseases such as cancer and cardiac ischemia [4].

One of the factors that can prevent the accumulation of free radicals is increasing the number of antioxidants in the body. Antioxidants can protect cells from the harmful effects of free radicals and can prevent oxidation reactions of molecules that can produce free radicals [5]. Antioxidants can donate electrons to free radical species so that they become neutral molecules. Antioxidants can reduce the amount of reactive oxygen in the body and restore the physiological systems of the human body [6].

Natural antioxidants can be found in vegetables, fruits, woody plants, algae, fungi, and bacteria [7, 8]. Currently, many researchers use microorganisms as a source of antioxidants. Utilizing bacteria is preferable since it grows more quickly, is independent of weather, is environmentally friendly, and uses resources more effectively in terms of both area and cost. Halophilic bacteria are bacteria capable of producing antioxidant compounds [9].

Halophilic bacteria are bacteria that live in environments with high salt levels. Based on the requirement of salt content, halophilic bacteria are classified into three, namely mild, moderate, and extreme halophilic [10]. Halophilic bacteria can be isolated from seawater, brine lakes, sea salt evaporation ponds, and foods preserved by salting. Halophiles can grow well in environmental conditions with salt levels between 2-30% [11]. In health, halophilic bacteria can

produce secondary metabolites in the form of pigments that have biological activity, one of which is carotenoid pigments [9].

Because of their extreme sensitivity, antioxidant pigments react quickly with airborne oxygen and free radicals. Additionally, exposure to light can lessen the antioxidant activity of pigments, particularly carotenoids. Carotenoids can undergo degradation due to light exposure, disrupting the structural stability of carotenoid compounds [12]. The pigment molecule's double and unsaturated bonds' structural stability is easily broken due to oxidative degradation by chemicals, oxygen, light, and temperature.

This research is a continuation of [13], namely determining the effect of storage variations on the antioxidant activity of the halophilic yellow pigment compound *Bacillus clausii* J1G-0%B. The influence of storage was carried out with various experimental conditions, namely the effect of air, light, and normal conditions (closed from light and air).

2. Methods

2.1. Materials

The materials used were yellow-pigmented halophilic bacterial cell stock 5% NaCl, 70% alcohol, pro analysis alcohol, distilled water, halophilic media, tryptone, yeast extract, KCl, $MgSO_4.7H_2O$, NaCl, Trisodium citrate, $FeCl_3$, agar pack, DPPH, organic solvent methanol, HCl. The instruments were an incubator, incubating shaker, UV-VIS spectrophotometer, and FTIR spectrophotometer.

2.2. Refreshment of *Bacillus clausii* J1G-0%B

Refreshment of the *Bacillus clausii* J1G-0%B was performed by planting one inoculation loop of bacteria from the agar slant stock culture in 50 mL of liquid halophilic media. Then the cultures were incubated in an incubator shaker at 37°C at 150 rpm for 84 hours. Furthermore, the halophilic cultures grown on liquid media were isolated by planting them on solid media to obtain a single isolate [13].

2.3. Multiplication of Bacterial Cells

The bacterial starter was prepared by transferring a loopful of a single isolate from the surface of the agar slant into a liquid halophilic nutrient medium and incubated in an incubating shaker at 150 rpm at 37°C for 84 hours [13]. As much as 1% halophilic bacterial starter was inoculated each into 4 Erlenmeyer containing 50 mL of halophilic liquid media and incubated in an incubating shaker at 150 rpm, 37°C, for 84 hours. The selection of the optimal time of 84 hours is based on the results of Pratiwi [13], which is the mid-stationary phase where the highest number of cells and the highest pigment antioxidant activity, as shown in Table 1. The pellets formed were then separated from the liquid medium using a centrifuge at 2000 rpm for 20 minutes [13].

Table 1. Antioxidant activity of yellow pigments produced by *Bacillus clausii* J1G-0%B [13]

Halophilic *Bacillus clausii* J1G-0%B (hours)	Growth phase	Percent inhibition (%)
36	Middle logarithmic	12.60
48	Stationary start	22.66
84	Mid stationary	34.84
120	Early death	31.73
156	Mid death	27.20

2.4. Extraction of Yellow Pigment

The pellets were extracted with 1 M methanol-HCl (24:1) using a centrifuge at 6000 rpm for 30 minutes until the pellets turned white. The resulting extract was evaporated to remove the remaining solvent, and a dark yellow viscous pigment extract was produced [13].

2.5. Antioxidant Activity of Yellow Pigment

Initially, 10,000 ppm of pigment extract stock solution was prepared (by weighing 0.1 g pigment extract) in 10 mL of methanol as solvent. The stock solution was diluted to obtain a yellow pigment solution (1600 ppm) for antioxidant activity assay using 0.1 mM DPPH.

The antioxidant activity of the yellow pigment extract was analyzed using the DPPH method. Yellow pigment extract was made in a ratio of 1:3 with DPPH solution. As well as a negative control made with methanol with a ratio of 1:3 with DPPH solution and a positive control made with β-carotene with a ratio of 1:3 with DPPH solution. Samples and controls were kept for 30 minutes. Absorbance measurements were carried out using a UV-Vis spectrometer at 517 nm.

Storage variations of yellow pigment under different conditions, such as normal, the influence of air, the influence of light, and the influence of air and light, were analyzed for their antioxidant activity using the DPPH method. Each storage condition used yellow pigment extract and DPPH solution with a ratio of 1:3 which was made in triplicate. The negative control was made by mixing methanol and DPPH solution (1:3), and the positive control was made by mixing β-carotene and DPPH solution (1:3). All samples and controls were allowed to stand for 30 minutes and then were measured using a UV-Vis spectrophotometer to determine the antioxidant activity of the yellow pigment extract. Details of the differences in each variation of storage conditions can be seen in Table 2.

Table 2. Variation of yellow pigment storage conditions

Storage condition	Description
Normal sample	Closed vial cap, aluminum foil-wrapped bottle
Sample-1 (+air)	Opened vial cap, aluminum foil-wrapped bottle
Sample-2 (+light)	Closed vial cap, aluminum foil-unwrapped bottle
Sample-3 (+air and light)	Opened vial cap, aluminum foil-unwrapped bottle

2.6. FTIR Characterization

FTIR was employed to identify the functional groups present in a 1600 ppm yellow pigment extract which was put into vials and allowed to stand for 30 minutes at each variation of storage conditions. A detailed description of each variation of storage conditions is the same as in Table 2.

3. Results and Discussion

3.1. Effect of Storage Conditions (Air and Light) on Antioxidant Activity of Yellow Pigment

Antioxidant activity was tested on three different treated yellow pigments to determine the effect of storage conditions. The third treatment included samples of yellow pigment stored under conditions influenced by air (samples in dark and open bottles), light (samples in bright and closed bottles), and air+light (samples in bright and open bottles). The comparison samples used were crude extracts and samples stored under normal conditions (in dark and closed bottles). The results of testing the antioxidant activity at different storage conditions are presented in Table 3.

Table 3. Antioxidant activity of the yellow pigment produced by *Bacillus clausii* J1G-0%B under different storage conditions

Storage condition	Antioxidant activity				
	I	II	III	Average	% Inhibition
Control negative (DPPH)	0.737	0.737	0.737	0.737	-
Crude extract	0.598	0.597	0.596	0.597	17.88%
Normal sample	0.622	0.638	0.638	0.632	14.24%
Sample-1 (+air)	0.648	0.651	0.651	0.650	11.80%
Sample-2 (+light)	0.639	0.635	0.630	0.634	13.97%
Sample-3 (+air and light)	0.649	0.685	0.652	0.662	10.17%

The results of measuring the absorbance of the sample at 517 nm obtained absorbance values in the range of 0.622-0.685. This absorbance value is lower than the negative control absorbance value (DPPH), so a positive value is obtained in the calculation of % inhibition, indicating the presence of antioxidant activity from the four yellow pigment samples.

Based on the results of the analysis of the data in Table 3 shows that the storage conditions influenced by air experience a greater decrease than those affected by light. This indicates that the air content, especially oxygen, will oxidize the yellow carotenoid pigment compounds [13, 14] to their inactive form. The conjugated double bond will break in carotenoid compounds during oxidation processes, forming the reduced structure of the molecule. In their reduced state, Carotenoids cannot reduce DPPH compounds, decreasing their antioxidant activity, as shown in Figure 1.

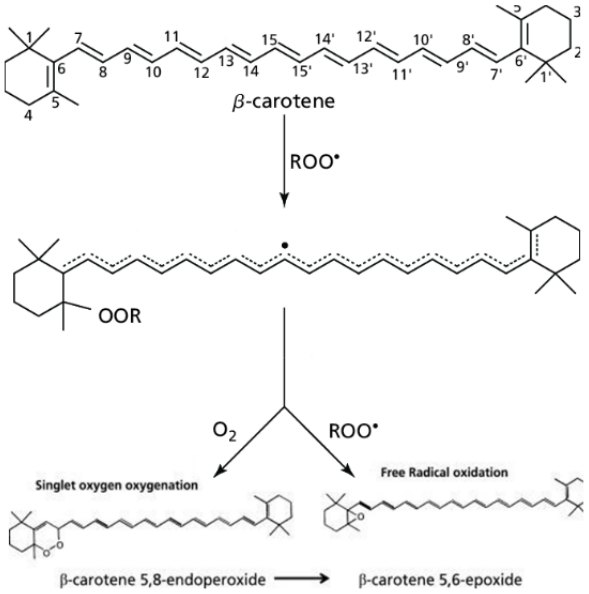

Figure 1. The radical scavenging process by carotenoids [15]

The influence of light in reducing antioxidant activity is because the carotenoids in the yellow pigment undergo degradation due to light radiation. Degradation of the carotenoid molecular structure can be caused by several factors, namely oxidative chemicals, oxygen, light, and temperature, as shown in Figure 2.

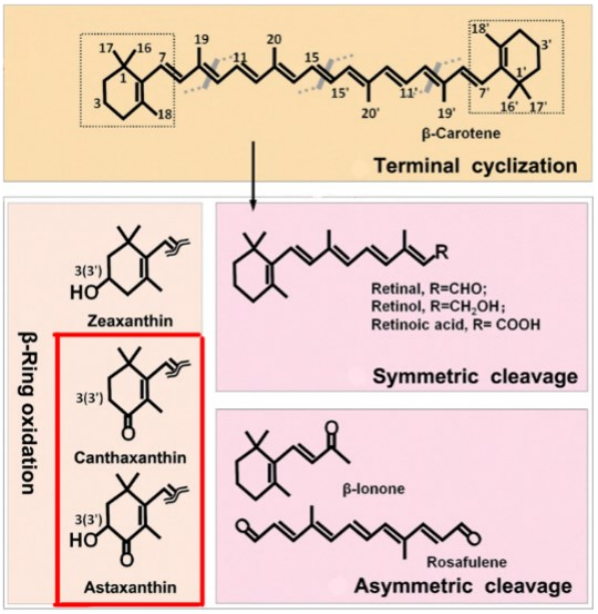

Figure 2. Interaction of carotenoids and air [16]

The influence of air on antioxidant activity is greater than the effect of light. This is because antioxidants can attach to oxygen and free radicals in the air, which causes a reduction in antioxidant levels. Meanwhile, antioxidants are degraded into their isomers when exposed to light, although some can be re-bonded to prevent the number of antioxidants from drastically diminishing. The combination of the effects of air and light can cause a more significant reduction because antioxidants that are degraded into their isomers can directly bind to oxygen in the air, which causes a more

significant decrease in the number of antioxidants, as shown in Figure 3.

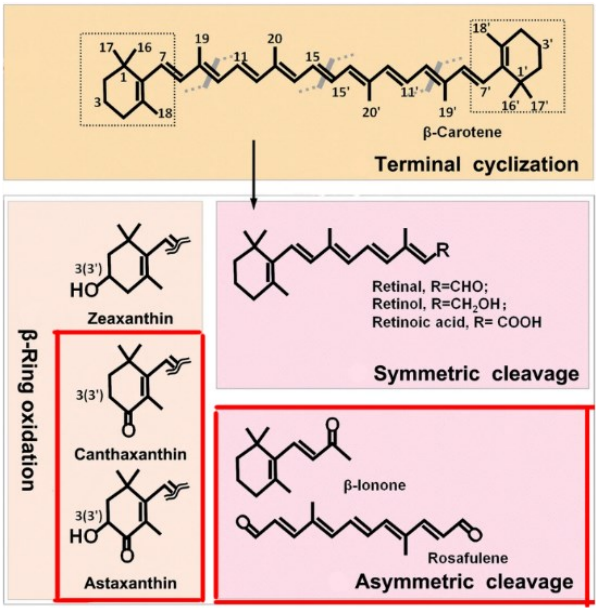

Figure 3. The interactions between carotenoids and air and light [16]

Free radicals in DPPH will be inhibited by antioxidants in pigments, resulting in the neutralization of free radicals. The conjugated double bond in the pigment will bind to the O-radical in DPPH, making DPPH no longer a free radical. The degree to which DPPH was inhibited reveals the potency of the antioxidant activities. Percent inhibition can be calculated by the absorbance of the control minus the absorbance of the sample, divided by the absorbance of the control, times one hundred percent.

3.2. FTIR Analysis

The FTIR spectra of the pigments from various conditions (Figure 4) did not show a significant difference. The pigment FTIR spectra showed a high absorption peak at wave number ~1027 cm^{-1} for the four circumstances, which is indicated by an alkoxy C-O stretching vibration. A similar peak also appears at ~1216 cm^{-1} as an absorption vibration of the C-O alcohol [17]. Strong absorption peaks also appear at ~1739 cm^{-1} which are indicated as C=O stretching absorption [18]. At ~1370 cm^{-1}, the two investigated pigments showed a vibrational mode due to symmetric deformation δ_{sym} (CH_3), while the deformation vibrations of δ (CH_2) were observed at near ~1450 cm^{-1} [19]. Asymmetric and symmetric C-H sp^3 stretching vibration absorptions also appear at ~2956 cm^{-1} and 2833 cm^{-1} [20]. The broad absorption peak also appears at ~3325 cm^{-1} which is indicated as an absorption of O-H stretching vibrations [21]. There are differences in pigment peaks between conditions of air influence and other conditions. the N-H absorption peak (~3667 cm^{-1}) did not appear in the pigment under air conditions [22].

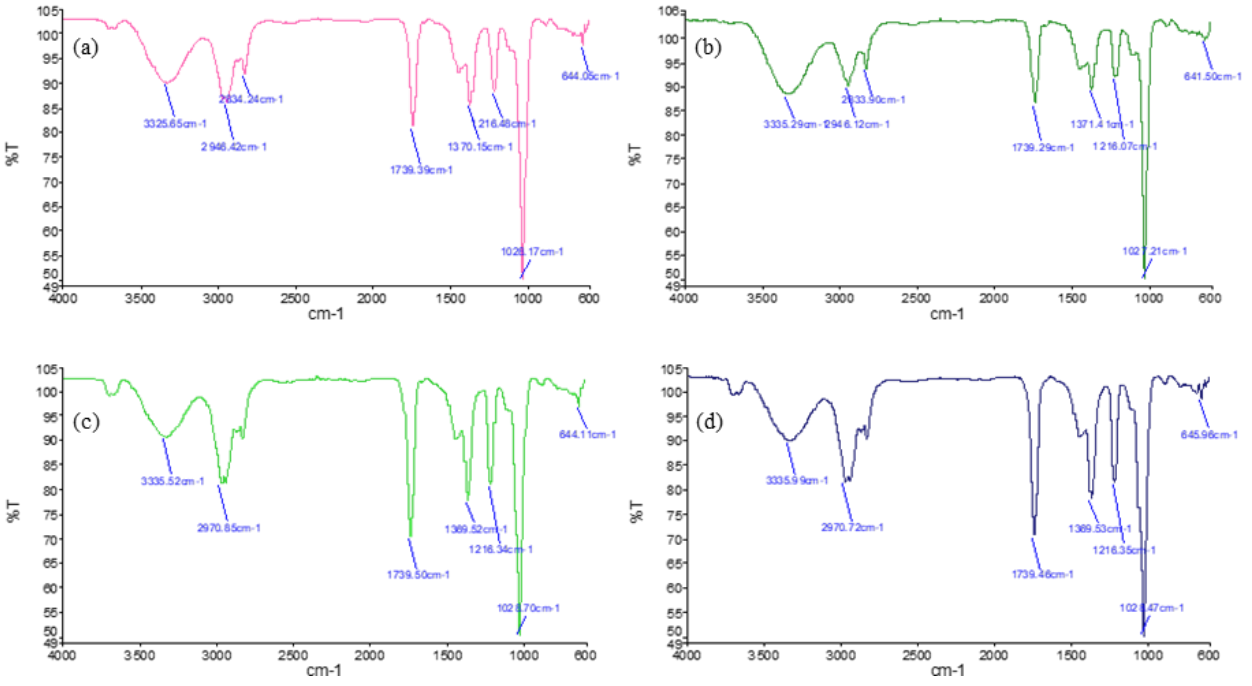

Figure 4. FTIR spectra of pigments (a) under normal conditions, (b) under the influence of air, (c) under the influence of light, and (d) under open conditions

4. Conclusion

The antioxidant activity of the yellow pigment halophilic bacteria *Bacillus clausii* J1G-0%B using the DPPH method obtained an inhibition value of 17.88% in the crude extract and 14.24% inhibition at 1600 ppm. The antioxidant activity of the halophilic yellow pigment *Bacillus clausii* J1G-0%B with the effect of storage using the DPPH method showed a decrease of 17.13% under the influence of air, 1.89% under the influence of light, 28.58% under the influence of air and light. The results of the FTIR analysis on the influence of air experienced a decrease in the peak on the carbonyl CO group of 1739.29 cm^{-1} and an increase in the alkane C-H group of 1369.52 cm^{-1}, while under the influence of light and open conditions, the carbonyl CO group experienced an

increase of 1739.50 cm^{-1} and 1739.46 cm^{-1} and the increase in the alkane C-H group 1371.41 $cm^{-1}$and 1369.53 cm^{-1}.

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