

MIKROBIOLOGICAL TESTING OF CAROTENOID PIGMENT EXTRACT AGAINST THE PATHOGENIC BACTERIUM *Vibrio harveyi* IN SHRIMP PONDS

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ABSTRACT

Shrimp is one of the key fishery commodities for the national economy. The main problem in shrimp farming is infection by the bacterium *Vibrio harveyi*, which is commonly controlled using conventional antibiotics. Uncontrolled antibiotic use can lead to bacterial resistance; therefore, it is necessary to identify alternative antibacterial compounds that are more environmentally friendly, such as carotenoid pigment extracts from marine microorganisms. This study aims to analyze the antibacterial activity and determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of carotenoid pigment extracts against *V. harveyi*. Carotenoid pigment analysis was performed using UV-Vis spectrophotometry, while antibacterial activity was tested using the disk diffusion method (Kirby-Bauer). MIC and MBC were determined using the liquid dilution and pour plate methods. The extract concentrations used were 12,5%, 25%, 50%, and 100%. The results showed that the extract exhibited a maximum absorbance at 490 nm, with a total carotenoid content of 14.56 µg/mL. In addition, the carotenoid pigment extract from isolate LD02 inhibited the growth of *V. harveyi*, with inhibition zones ranging from 1.41 to 3.58 mm. The MIC was 14.56 µg/mL, while the MBC was >14.56 µg/mL, indicating that the extract exhibits bacteriostatic activity against *V. harveyi*.

Keywords: Antibacterial, Bacteriostatic, Carotenoids, *Vibrio harveyi*

1. INTRODUCTION

Shrimp is one of the key fishery commodities for the national economy. However, efforts to increase shrimp production still face various challenges, particularly disease outbreaks caused by pathogenic bacteria. One bacterium that frequently causes losses in shrimp farming is *Vibrio harveyi*, the causative agent of vibriosis, which can lead to reduced productivity and even mass mortality in shrimp¹.

Control of *V. harveyi* infections is generally carried out using antibiotics. However, uncontrolled use of antibiotics can trigger bacterial resistance, leave residues in farmed organisms, and negatively impact the aquatic environment². Therefore, safer and

more environmentally friendly antibacterial alternatives are needed.

One potential alternative is carotenoid pigments produced by certain marine microorganisms, such as marine photosynthetic bacteria. Carotenoids are known to possess antioxidant and antimicrobial activities and to inhibit the growth of various pathogenic bacteria. Additionally, marine photosynthetic bacteria offer advantages such as stable productivity, short generation times, and high biomass³.

2. RESEARCH METHOD

Time and Place

This research was conducted from August 2025 to March 2026 at the Marine Microbiology Laboratory, Faculty of

Fisheries and Marine Sciences, Universitas Riau, and the Marine Chemistry Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Riau.

Method

The study employed a one-factor completely randomized design (CRD), with the independent variable being the concentration of carotenoid pigment extract. The treatments consisted of four concentration levels (12.5, 25, 50, and 100% v/v), a positive control (chloramphenicol), and a negative control (methanol), each with three replicates. The research stages included bacterial culture, biomass production, carotenoid pigment extraction, pigment analysis using UV-Vis spectrophotometry, antibacterial activity testing using the disk diffusion method (Kirby-Bauer), and determination of MIC and MBC using the liquid dilution and solid dilution (pour plate) methods.

Procedures

Source of Isolate

The pigmented marine bacterial isolate LD02 used in this study was derived from the research of Batubara et al.⁴, which was isolated from the waters of Dumai and collected at the Marine Microbiology Laboratory of the Faculty of Fisheries and Marine Sciences, Universitas Riau. The test bacterium, *V. harveyi*, was obtained from the culture collection of the Central Brackish Water Aquaculture Research and Development Center (BBPBAP), Jepara, Central Java, and was derived from shrimp infected with vibriosis and identified as *V. harveyi*.

Preparation of Media and Bacterial Cultures

All equipment and media used were sterilized in an autoclave at 121°C for 15 minutes. Isolate LD02 was cultured on mineral salt medium consisting of 2.5 g Na-acetate, 1.25 g NH₄SO₄, 0.9 g K₂HPO₄, 0.6 g KH₂PO₄, 0.5 g yeast extract, 0.2 g MgSO₄·7H₂O, 0.07 mg CaCl₂, FeSO₄·7H₂O

0.003 mg, and EDTA 0.002 mg dissolved in 1 L of distilled water³. In contrast, *V. harveyi* was cultured on Thiosulfate Citrate Bile Salts Sucrose (TCBS) medium. The LD02 culture was incubated at room temperature under a 40 W incandescent light for 5–7 days, while the *V.harveyi* culture was incubated at 30°C for 24 hours before being used in the testing phase.

Biomass Production and Carotenoid Pigment Extraction

Biomass production was carried out by inoculating a loopful of LD02 culture into a glass bottle containing 1 L of liquid mineral salt medium, which was then incubated at room temperature under a 40 W incandescent light for 24–48 hours. The cell biomass was harvested by centrifugation at 4,500 rpm for 20 minutes to obtain the cell pellet. The cell pellets were then extracted using a dichloromethane: methanol (7:3) solvent mixture with a biomass-to-solvent ratio of 1:1 (v/v)⁵. The suspension was vortexed until homogeneous and centrifuged again at 4,500 rpm for 20 minutes. The supernatant containing carotenoid pigments was then collected and used as the pigment extract for further analysis.

Analysis of Carotenoid Pigments

Carotenoid pigment analysis was performed using a UV-Vis spectrophotometer by placing the pigment extract in a quartz cuvette and measuring the absorbance spectrum in the wavelength range of 350–650 nm⁶. The presence of carotenoids was identified based on the maximum absorption peak (λ_{max}) formed in the absorption spectrum. Total carotenoid content was determined using a standard astaxanthin calibration curve with concentration variations of 0, 5, 10, and 20 ppm. The relationship between standard concentration and absorbance values was used to establish a linear regression equation, which was subsequently used to calculate the total carotenoid content in the extract samples⁷.

Antibacterial Activity Test

The antibacterial activity of carotenoid pigment extracts against *V. harveyi* was tested using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (MHA). The treatments consisted of four extract concentrations: 12.5%, 25%, 50%, and 100% (v/v). A suspension of *V. harveyi* was evenly inoculated onto the surface of the MHA medium. Then three paper discs, each containing 20 μ L of extract at the same concentration, were placed as replicates. Chloramphenicol and 99.8% methanol were used as positive and negative controls, respectively. The plates were incubated at 37°C for 24–72 hours. Antibacterial activity was determined based on the diameter of the inhibition zone formed around the discs and classified according to Clinical and Laboratory Standards Institute (CLSI) standards⁸.

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The determination of the Minimum Inhibitory Concentration (MIC) of the carotenoid pigment extract against *V. harveyi* was performed using the liquid dilution method in Mueller-Hinton Broth (MHB). A total of 0.1 mL of bacterial suspension standardized to a McFarland turbidity of 0.5 was added to 3.5 mL of MHB, followed by the addition of carotenoid pigment extract at concentrations of 12.5%, 25%, 50%, and 100% (v/v). Chloramphenicol (30 μ g/mL) and methanol were used as positive and negative controls, respectively. Initial absorbance (OD_0) and absorbance after 24-hour incubation at 37°C (OD_{24}) were measured using a UV-Vis spectrophotometer at a wavelength of 600 nm. The MIC value was determined as the lowest concentration yielding a negative absorbance difference ($\Delta OD = OD_{24} - OD_0$) and was corroborated by observations of media turbidity⁹.

MBC determination was performed by subculturing 0.1 mL of a sample from each

MIC tube onto Mueller-Hinton Agar (MHA) medium, followed by incubation at 37°C for 24 hours. The MBC value was defined as the lowest extract concentration that did not show bacterial colony growth on the agar medium after the incubation period¹⁰.

Data Analysis

This study employed a completely randomized design (CRD) with four treatments of carotenoid pigment extract concentrations (12.5%, 25%, 50%, and 100%) and three replicates. Data on inhibition zone diameters were analyzed using one-way ANOVA at a 95% confidence level. Data on maximum wavelength (λ_{max}), total carotenoid content, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) were analyzed descriptively.

3. RESULT AND DISCUSSION

Cultivation of Marine Photosynthetic Bacteria and Production of Carotenoid Pigments

The marine photosynthetic bacterial isolate LD02, when cultured on a solid mineral salt medium, exhibited colony growth accompanied by a red color change after approximately 5 days of incubation under a 40-watt incandescent light. This color change indicates the accumulation of intracellular pigments, which are suspected to be carotenoids. The yellow color formed is characteristic of carotenoid compounds and indicates the potential of isolate LD02 as a source of carotenoid pigments for further testing (Figure 1). Carotenoids are natural pigments commonly produced by marine microorganisms and are characterized by yellow, orange, and red hues¹¹. The formation of these pigments is linked to the metabolic activity of photosynthetic bacteria, which utilize light energy to support pigment biosynthesis¹².

The resulting biomass is then extracted using a mixture of dichloromethane and methanol (7:3), yielding a yellow supernatant. The use of this solvent combination is based on the lipophilic nature

of carotenoids, which makes them easily soluble in organic solvents. A combination of solvents with different polarities can enhance pigment extraction efficiency¹³.

The color change from red biomass to a yellow supernatant indicates that the pigments have been extracted into the solvent phase.

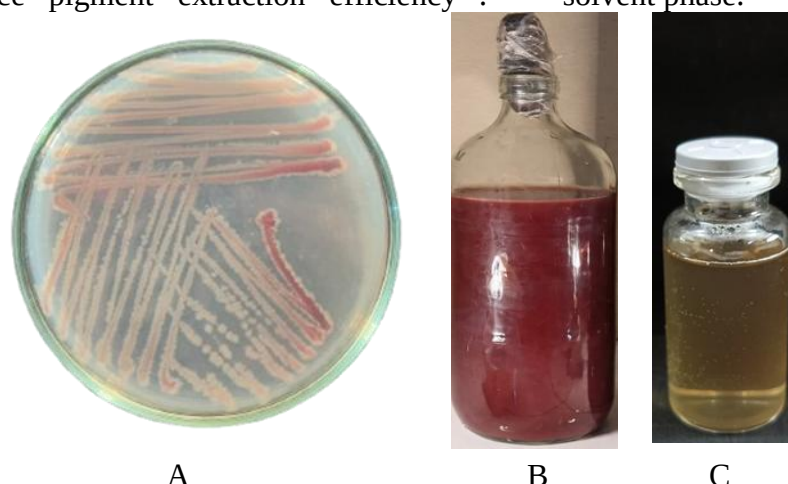


Figure 1. Cultivation of the marine photosynthetic bacterium LD02 and production of carotenoid pigments. (A) Growth of the isolate on solid mineral salt medium, (B) Biomass culture showing the formation of red pigments, (C) Supernatant from the extraction

Detection of Carotenoid Pigments

The detection of carotenoid pigments in the LD02 extract was performed using UV-Vis spectrophotometry in the wavelength range of 450–600 nm.

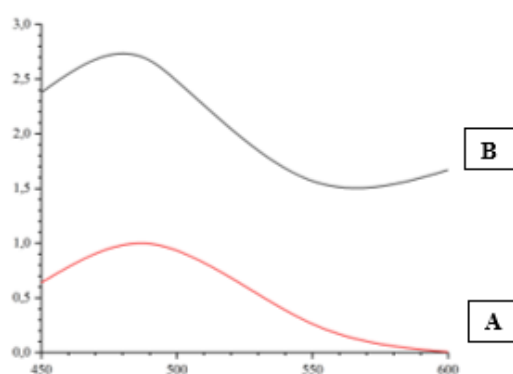


Figure 2. UV-Vis absorption spectra. (A) Standard solution, (B) LD02 extract

Based on the absorbance spectrum profile shown in Figure 2, the LD02 extract exhibited a maximum absorbance peak at a wavelength of 490 nm with an absorbance value of 2.670. This wavelength falls within the characteristic absorption range of carotenoids, namely 400–500 nm, which is attributed to the presence of a conjugated double bond system in the carotenoid

molecular structure¹⁴. The similarity in spectral patterns between the extract and the standard solution indicates that the pigment successfully extracted from the LD02 isolate is likely a carotenoid compound.

Quantification of total carotenoid content yielded the regression equation $y = 0.1776x + 0.0834$ with a coefficient of determination (R^2) of 0.9957, indicating a very strong linear relationship between concentration and absorbance. Based on this regression equation, the total carotenoid content of the LD02 extract was determined to be $14.56 \pm 0.006 \mu\text{g/mL}$. This value indicates that LD02 can produce sufficient carotenoids, making it a potential source for testing antibacterial activity against *Vibrio harveyi*.

Antibacterial Activity of Carotenoid Pigment Extracts

The antibacterial activity of the LD02 carotenoid pigment extract against *V. harveyi* was assessed using the Kirby–Bauer disk diffusion method. Based on the test results presented in Table 1, the LD02 carotenoid pigment extract exhibited antibacterial activity against *V. harveyi*. The

resulting inhibition zone diameters ranged from 1.41 to 3.58 mm; these values increased with higher extract concentrations and were directly proportional to the amount of bioactive compounds that inhibited *V. harveyi* growth.

According to the CLSI classification, all inhibition zone diameters obtained are still classified as weak. The low antibacterial activity is suspected to be related to the

characteristics of *V. harveyi* as a Gram-negative bacterium with an outer membrane consisting of a lipopolysaccharide (LPS) layer, which can inhibit the penetration of antibacterial compounds into the cell¹⁵. Additionally, the hydrophobic nature of carotenoids and the use of crude extract are suspected to limit the diffusion of compounds in the agar medium, resulting in relatively small inhibition zones.

Table 1. Results of the antibacterial activity test of LD02 carotenoid pigment extract

Test Bacteria	Concentration (%)	Concentration (µg/mL)	Inhibitory Zone Diameter (mm) ±SD	Microbial Index	Category
<i>V. harveyi</i>	12,5	1,82	1,41±0,52	0,03	Weak
	25	3,64	1,98±0,83	0,05	Weak
	50	7,28	2,61±1,24	0,07	Weak
	100	14,56	3,58±1,84	0,09	Weak
	+	30	37,06±1,29	-	Strong
	-	-	0,00±0,00	-	Weak

Notes: (+) Positive Control (Chloramphenicol), (-) Negative Control (Methanol), (%) Dilution of stock solution, (µg/mL) concentration conversion of extract from standard solution

According to the CLSI classification (2024), all inhibition zone diameters obtained are still classified as weak. The low antibacterial activity is suspected to be related to the characteristics of *V. harveyi* as a Gram-negative bacterium with an outer membrane consisting of a lipopolysaccharide (LPS) layer, which can inhibit the penetration of antibacterial compounds into the cell¹⁵. Additionally, the hydrophobic nature of carotenoids and the use of crude extract are suspected to limit the diffusion of compounds in the agar medium, resulting in relatively small inhibition zones.

One-Way ANOVA analysis showed a significance value of 0.232 ($p > 0.05$), indicating that differences in extract concentration did not have a significant effect on the diameter of the inhibition zone. These findings suggest that the LD02 carotenoid pigment extract exhibits antibacterial activity against *V. harveyi*, although its inhibitory efficacy remains relatively low at the tested concentrations.

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

Minimum Inhibitory Concentration (MIC) testing was conducted to determine the lowest concentration of the LD02 carotenoid pigment extract that was still capable of inhibiting the growth of *V. harveyi*. Based on visual observation, the turbidity level of the medium decreased as the extract concentration increased, with the 14.56 µg/mL treatment showing the clearest condition compared to other concentrations (Figure 3). The decrease in turbidity indicates reduced bacterial growth due to the antibacterial activity of the compounds in the extract.

Optical Density (OD) measurements indicated that the 14.56 µg/mL concentration yielded a ΔOD of -0.015, whereas all lower concentrations showed positive ΔOD values (Table 2). A negative ΔOD value indicates a decrease in the number of bacterial cells during the incubation period; therefore, this concentration was established as the MIC

value for the LD02 extract. These results indicate that the carotenoid pigment extract inhibits the growth of *V.harveyi* at a concentration of 14.56 µg/mL. The inhibitory activity is thought to be related to carotenoid compounds' ability to disrupt cell membrane permeability, thereby inhibiting

bacterial metabolism and growth¹⁵. MIC results only indicate a compound's ability to inhibit bacterial growth; therefore, further testing using the MBC method is required to evaluate the extract's ability to kill bacteria completely.

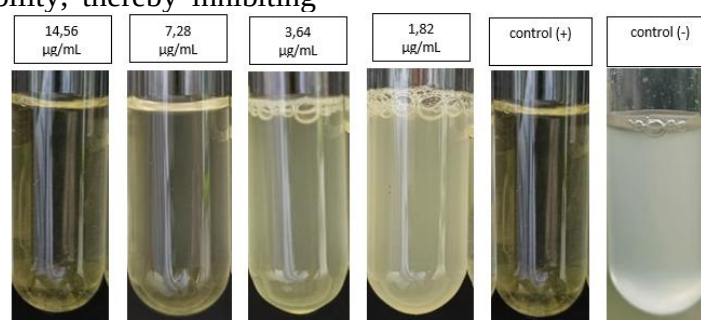


Figure 3. Results of the MIC Test of LD02 Extract on the Growth of *Vibrio harveyi*

Table 2. OD Measurement results in the MIC test of LD02 extract against *V. harveyi*

Test bacteria	Extract concentration (%)	Treatment Concentration (µg/mL)	OD Value		ΔOD
			Pre-incubation	Post-incubation	
<i>V. harveyi</i>	+	-	0,097	0,038	-0,059
	-	-	0,060	0,416	0,476
	100	14,56	0,093	0,078	-0,015
	50	7,28	0,090	0,241	0,151
	25	3,64	0,080	0,653	0,573
	12,5	1,82	0,054	1,322	1,268

Notes : (+) Positive Control (Chloramphenicol), (-) Negative Control (Methanol), (%) Dilution of stock solution, (µg/mL) concentration conversion of extract from standard solution

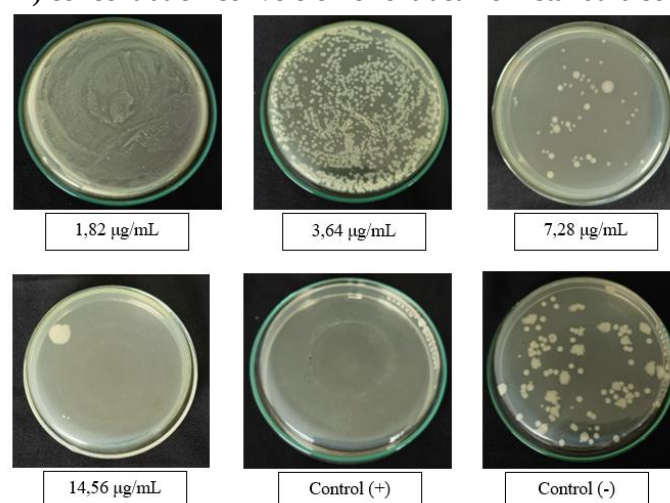


Figure 4. Growth of *V. harveyi* bacterial colonies from the MBC test of LD02 extract at various treatment concentrations after 24 hours

The results of the Minimum Bactericidal Concentration (MBC) test showed that the number of *V. harveyi*

colonies decreased as extract concentration increased (Figure 4). However, colony growth was still observed at the highest

concentration tested, which was 14.56 µg/mL. Therefore, the MBC value was determined to be greater than 14.56 µg/mL. These results indicate that the extract concentration used was insufficient to kill the bacteria, although it completely inhibited their growth.

The difference between the MIC and MBC values indicates that the LD02 carotenoid pigment extract is more likely to be bacteriostatic than bactericidal. A compound is classified as having bacteriostatic activity if it is capable of inhibiting bacterial growth at a certain concentration, but is not yet capable of causing complete cell death¹⁶. The failure to achieve a bactericidal effect in this study is likely due to the use of a crude extract, which still contains a mixture of various compounds, as well as the characteristics of *V. harveyi* as a Gram-negative bacterium with an outer membrane consisting of a

lipopolysaccharide (LPS) layer that can inhibit the penetration of antibacterial compounds into the cell¹⁵. Overall, these results indicate that the LD02 carotenoid pigment extract has potential as a natural antibacterial agent against *V. harveyi* through inhibition of bacterial growth.

4. CONCLUSION

The carotenoid pigment extract produced by the marine photosynthetic bacterial isolate LD02 exhibited antibacterial activity against *Vibrio harveyi*, as indicated by the formation of inhibition zones at all tested concentrations. The MIC was 14.56 µg/mL, while the MBC was >14.56 µg/mL. These findings indicate that the LD02 carotenoid pigment extract has the potential to be utilized as a source of natural antibacterial compounds against *V. harveyi*. However, the activity produced is still more bacteriostatic than bactericidal.

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