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Antibacterial Activity of Yellow Pigment from *Micrococcus flavus* AK 11 Isolates of the Coral *Porites* sp. from Gosong Beach, Central Java, Indonesia

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ABSTRACT

Multidrug-resistant (MDR) infections in the wound have significant concern. Yellow pigmented marine bacteria, strains AK 11, were isolated from the coral *Porites* sp. on Gosong Beach, Rembang, Central Java, Indonesia, and cultured in the laboratory. This study aimed to determine the bacterial symbionts and the type of pigment generated and ascertain the antibacterial effectiveness of yellow pigment against bacteria that cause wound infections. The extraction process was performed using three different methods, each distinguished by the solvent and evaporation process. The antibacterial activity test was carried out by the diffusion method using the test bacteria of *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*. The results showed that the most suitable pigment extraction method was Method III, with methanol as solvent and drying technique using N2. The antibacterial activity of the yellow pigment extract against *S. aureus* ATCC 6538 and *S. aureus* strain MDR exhibited an inhibition zone diameter of 35± 1.08 and 25± 1.06mm. At the same time, *P. aeruginosa* and *E. coli* bacteria did not demonstrate any antibacterial activity. The results also disclosed that the bacterial symbionts were *Micrococcus flavus* using 16S rRNA gene sequencing, which produced carotenoid pigments. In conclusion, the yellow pigment extract has the potential to be antibacterial, particularly against *S. aureus*. It is advised that future research continue to concentrate on how antibacterial action operates *in vivo*.

INTRODUCTION

It is estimated that 1- 2% of the population in developing countries will experience chronic wounds during their lifetime **(Gottrup, 2004)**. Wound care must be carried out properly and correctly. Improper handling of wounds can result in skin wounds getting wider and taking a long time to heal. Saprophytic microorganisms of normal flora generally contaminate all wounds on the skin with varying types and amounts that can

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lead to infection. Bacterial species that commonly appear in the identification of wound swabs include *Staphylococcus aureus* (37%), followed by *Pseudomonas aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%), and *Corynebacterium* sp. (5%) **(Bessa *et al*., 2015)**.

Treatment of wound infections generally uses antibiotics. The uncontrolled use of antibiotics in the community has resulted in the emergence of resistant strains that continue to grow and become a new problem with significant development of cases. Bacterial resistance can also develop not only to one type of drug but also to several types of antibiotic drugs, known as multi-drug resistance (MDR) **(CDC, 2019)**. The MDR bacterial infections constitute a serious challenge to global healthcare systems **(Prastiyanto *et al*., 2024a)**, especially in the context of wound infections. Therefore, alternative antibacterials from natural sources are needed. Numerous research studies explained the potential of natural resources as alternative antibiotics such as natural antibacterial resources comprising plasma jet **(Darmawati *et al*., 2019)**, lactic acid bacteria **(Lestari *et al*., 2019)**, marine microorganisms **(Sibero *et al*., 2019; Prastiyanto *et al*., 2022; Prastiyanto *et al*., 2024b)**, seeds **(Prastiyanto *et al*., 2020a; Prastiyanto, 2021)**, mushroom **(Prastiyanto *et al*., 2020b)**, latex **(Prastiyanto *et al*., 2020c)**, and fruit **(Prastiyanto *et al*., 2020d)**. Marine sources are a significant source of potential as antibacterial agents **(Radjasa *et al*., 2023, 2013)**. It is necessary to use natural component antibiotics as a backup to treat bacterial infections in light of these circumstances. One of them comes from the marine bacteria.

Indonesia is a country that is rich in sources of biodiversity, including marine microorganisms that can be used as a source of new antibacterial drugs **(Setiyono *et al*., 2020)**. One type of biodiversity that is often found in the Indonesian marine is the coral reefs. Among these is the coral *Porites* sp., which is often found on Gosong Beach, Rembang, Central Java. Research conducted with samples of the coral *Porites* sp. from Gunung Kidul, Yogyakarta, showed that the active compounds present in the symbiont of the coral *Porites* sp. microorganisms had antibacterial activity against *Staphylococcus* sp. and *E. coli* **(Madilana *et al*., 2018)**. The marine bacterial community is considered to have great potential to produce bioactive such as pigments that can be used as antibacterial, antifungal, and antiviral agents **(Ramesh *et al*., 2019)**.

Bacteria can produce various types of pigments as bioactive compounds, such as red pigment or prodigiosin, purple pigment or violacein, and yellow pigment or carotenoids **(Ramesh *et al*., 2019)**. Carotenoids are natural pigments with yellow, orange to red colors, resulting from secondary metabolites of bacterial species. An earlier study reported that carotenoid has an anticancer activity **(Avila-Roman *et al*., 2021)**. Lately, we isolated one marine bacterial strain symbiont coral *Porites* sp. that has characteristics of yellow pigment. The strains were designated as *Micrococcus flavus* AK 11 according to a 16S rRNA sequence analysis phylogenetic analysis. In the current study, the

researchers extracted and identified the yellow pigment from marine and evaluated its antibacterial properties against the MDR bacteria.

MATERIALS AND METHODS

Marine coral *Porites* sp. sample collection

Coral samples were obtained from the Gosong Beach, Rembang, Central Java, Indonesia at 6°38'57.7"S 111°26'06.4"E (Fig. 1). Samples collected at a depth of 1- 5 meters were placed in plastic ziplock bags and stored temporarily in a cool bag. Corals were identified and categorized at the Diponegoro University Fisheries and Marine Laboratory in Semarang, Indonesia. Coral samples were identified as the *Porites* sp.

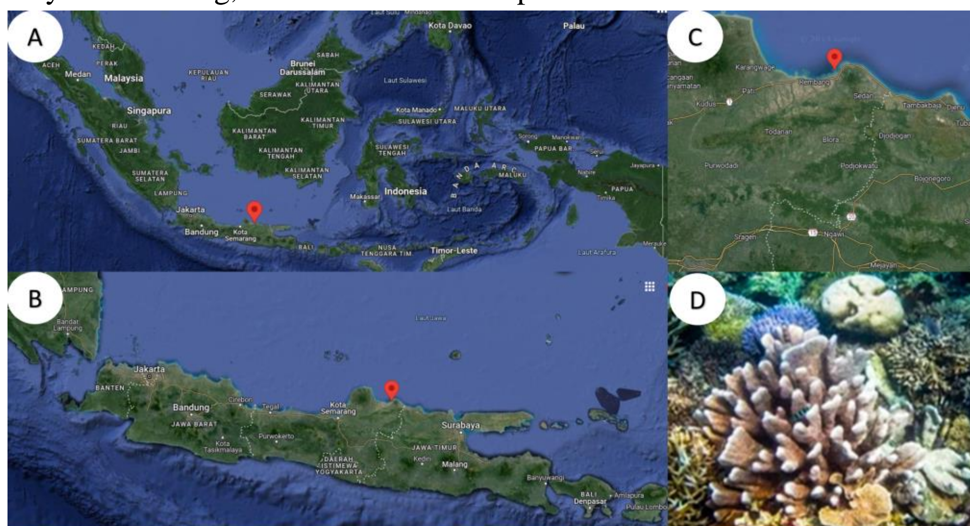

Fig. 1. Sampling location of the coral *Porites* sp. in Gosong Beach, Rembang, Central Java, Indonesia. **A- C** the sampling location, and **D** the sample of the *Porites* sp. taken from the location point

Bacterial AK 11 Isolation

The isolates of AK 11 were collected from the coral *Porites* sp. The isolation was carried out using Zobell Marine Agar (ZMA) 2216E medium and cultured for 48 hours at 35± 2°C to produce isolates of the coral *Porites* sp. symbiont bacterium. The growth colonies were circular with a yellow pigment (Fig. 2A).

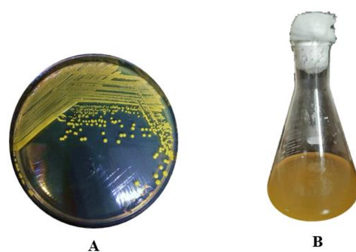

Fig. 2. **(A)** Macroscopic observation of AK 11 isolates on ZMA; **(B)** AK 11 isolates inoculation on ZMB

Yellow pigment extract

In the extraction process of the yellow pigment extract, experiments were carried out three times with different methods until an extract was obtained that could inhibit the test bacteria. In the first method, the AK 11 isolates of coral *Porites* sp. symbionts were cultured in Zobell Marine Broth (ZMB) medium, and then incubated for 48 hours at 37°C (Fig. 2B). The growing bacterial cells were then transferred into a conical tube and centrifuged at 6,000rpm for 15 minutes at 4°C. The pellets obtained were then macerated into a solution of methanol-acetone (7:3) with a ratio of 0.1g/ mL mixture. The mixture was vortexed five times, with each lasting for 1-minute, followed by 1 minute left on ice. It was then sonicated for 10 minutes, followed by the addition of $CaCO_3$ and an ascorbic acid to prevent pigment degradation due to oxidation. Centrifugation was carried out to obtain pellets at a speed of 6.000rpm for 15 minutes at 4°C. The pellets were then evaporated at 35°C until dry, and stored at -30°C until used **(Setiyono *et al*., 2020)**. The second method used the same procedure as the first method. However, the maceration solution was replaced using an ethyl acetate solution. A fermentation process was carried out using a buffer solution by inoculating the AK 11 isolates sample into ZMB medium, incubated at 37°C for 48 hours, and centrifuged at a speed of 5000rpm for 5 minutes. Subsequently, the supernatant was removed, and the pellet was soaked using a solubilizing buffer with PBS for 24 hours. Then, the sample was soaked with ethyl acetate maceration solution, dissolved with mortar and pestle, evaporated, and obtained crude pigment, stored in a sterile vial tube **(Umadevi & Krishnaveni, 2013)**. The third method involved using methanol solvent. The pellet was soaked until the maceration solution turned yellow, and the cell pellet became pale. Extraction was carried out using methanol, and the pigment extract obtained was then evaporated using nitrogen gas **(Wiguna *et al*., 2016)**.

Bacterial Tested

The test bacteria consisted of Gram-positive and Gram-negative bacteria. *S. aureus* isolates ATCC 6538 and MDR *S. aureus* were obtained from wound infection samples at Tugurejo Hospital Semarang, Central Java. The MDR bacteria were inoculated into a blood agar plate (BAP) medium at 37°C for 24 hours. Separate colonies were taken using a sterile inoculation needle and then gram staining was performed. Furthermore, the sensitivity test on antibiotics (oxacillin, vancomycin, nitrofurantoin, streptomycin, ciprofloxacin, and tigecycline) was carried out. The Gram-negative bacteria used consisted of the MDR *P. aeruginosa* and MDR *E. coli* isolates obtained from wound infection samples available at the Microbiology Laboratory Universitas Muhammadiyah Semarang. Then, they were inoculated into MacConkey (MC) medium at 37°C for 24 hours. After a separate colony with a sterile inoculation needle, Gram staining and biochemical tests were carried out. Furthermore, sensitivity tests were

carried out on the following antibiotics: aztreonam, cefotaxime, ceftazidime, amikacin, gentamicin, meropenem, and tigecycline.

Molecular identification of bacterial symbiont in the coral *Porites* sp.

The process described in the experiment involved using the PrestoTM Mini DNA Bacteria Kit to extract the DNA from bacterial cells following the manufacturer's instructions. A final elution volume of 50µL was used. The DNA was stored at 4°C until being required for the PCR. In this investigation, 50ng/ µL of bacterial DNA was utilized, and a 16S rRNA gene primer was mixed with 2µL of the bacterial DNA. With a final primer concentration of 10µM, primers 27F '5'-AGAGTTGATCMTGGCTCAG-3' and 1492R '5'-CGGTTACCTTGTTACGACTT-3' were used. As per the protocol for PCR amplification, the Taq polymerase enzyme was activated at 95°C for four minutes. The next 35 cycles involved denaturation (30 seconds at 95°C), primer annealing (30 seconds at 57°C), extension (2 minutes at 72°C), further extension (10 minutes at 72°C), and chilling (10 minutes at 4°C). After the PCR results were separated on a 1% agarose gel, distinct DNA bands were identified using Flourovue. Genetica Science Tangerang used the PCR product sequencing to analyze the 16S rRNA gene sequences. After that, the study was conducted using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) database service **(Rahman *et al*., 2011)**.

Phylogenetic analysis

The method used the MEGA X, a phylogenetic analysis tool. The data from the 16S rRNA gene sequencing were aligned using ClustalW. Using the Tamura-Nei model, neighbor-joining was utilized to generate phylogenetic trees. To build the trees, a non-parametric bootstrapping method was carried out on 1000 datasets collected from 16S rRNA gene sequences. These evolutionary links showed the linkages between closely related strains **(Kumar *et al*., 2018)**.

Antibacterial activity of yellow pigment extract against wound infection bacteria: Agar well diffusion test

The antibacterial activity of the chloroform extract of rambutan peel was evaluated using the diffusion method. Diffusion method using the well (hole) method, using test bacteria standardized to the 0.5 McFarland standard were inoculated on Mueller Hinton Agar (MHA) medium and then streaked over the entire surface of the agar medium using a sterilized cotton swab. After being allowed to stand for 10 minutes, wells were made using a sterilized cork borer with a diameter of 0.6cm. Each well was filled with 10mg/ mL of the yellow pigment extract. All plates were then incubated at 37°C for 24 hours.

Identification of pigment groups with UV-Vis spectrophotometry

The yellow pigment was measured for its wavelength optimization using the UV-vis spectrophotometry. It was to determine the most optimal wavelength range, with the most optimum absorbance peak on the wavelength optimization graph to be compared with the wavelengths of the pigment compounds that have been identified.

RESULTS AND DISCUSSION

Identification of AK 11's morphology

The result of the AK 11 identification shows that it is a Gram-positive bacteria that is close to the *Micrococcus* sp. (Tables 1, 2). *M. flavus* is a bacterium with a round shape, yellow color, and non-motile morphology **(Kocur *et al*., 1972)**. Previous studies have shown that this species of bacteria is commonly found in bioreactor mud **(Liu *et al*., 2007)**. The *Micrococcus* genus commonly found in symbionts with marine corals is *M. flavus*, which is found in marine coral organisms of the *Acropora* sp. **(Wilson *et al*., 2012)**. *M. flavus* is a bacterium that was found to be able to symbiose with marine corals of the coral *Porites* sp. These bacteria are usually found in the form of a microscopic tetrad. *M. flavus* has a yellow pigment that is difficult to dissolve in water. Table (1) presents the identification of the colony morphology. This is in line with previous research reports **(Rakhashiya *et al*., 2016)**. The *M. flavus* and *M. luteus* bacteria could not ferment carbohydrates but could grow well in a medium with 5- 15% salt content.

Table 1. Identification of colony morphology

Isolate	Shape	Color	Size	Edge	Elevation	Consistency
AK 11	circular	yellow	0.5-1 mm	Entire	Convex	Smooth

Table 2. Identification of cell morphology

Isolate	Shape	Gram staining	Oxidase test	Catalase test	Mannitol salt agar
AK 11	Coccus	Gram-positive	Positive	Positive	Negative

Molecular identification

The bacterial isolates were extracted with DNA, quantified for further electrophoresis, and then read on 1% agarose. The wavelength used in the process of reading the concentration of nucleic acid, in this case, was DNA, which was 260nm. In comparison, the wavelength of 230nm was used to measure the total protein concentration in the DNA extraction sample. The DNA concentration measurement results were obtained at 1.86, indicating that the DNA was pure (Table 3). In line with this, **Alperovich *et al* (2023)** stated that a good DNA purity is close to 1.8- 2.0, and the use of the kit method can produce a DNA with fairly high purity. After that, amplification was carried out by the PCR and followed by electrophoresis of 1% agarose and obtained

a product with a size of 1500bp (**Alperovich *et al.*, 2023**). The 16S rRNA gene sequences of strains AK 11 contained 1437bp. The nucleotide BLAST program (NCBI) for highly similar sequences was used to evaluate the similarity between these bacteria's 16S rRNA genes. A comparative analysis of the 16S rRNA gene sequences revealed that *M. flavus* NR_043661 and strain AK 11 shared 100% of the query cover. *M. flavus* NR_043661 and strain AK 11 were grouped in the neighbor-joining tree phylogeny (Fig. 3).

Table 3. Results of DNA quantification of AK 11 isolates

Sample code	Concentration (ng/L)	A260/280	Sample
AK 11 Isolates	461.173	1.86	DNA

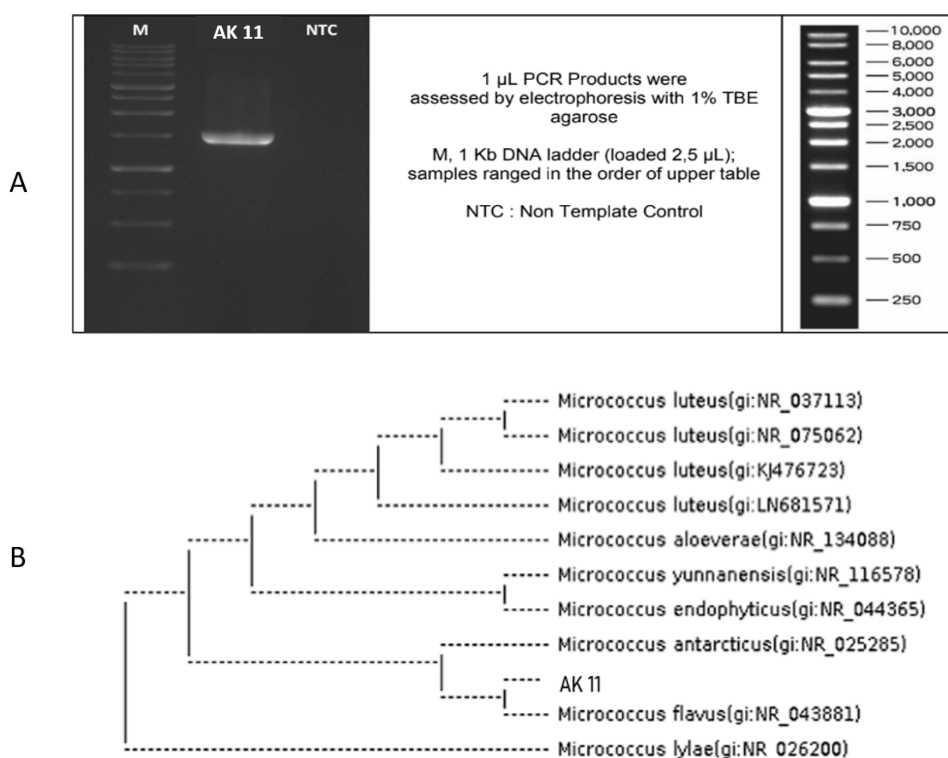

Fig. 3. (A) 16S rRNA gene PCR amplicons on 1% agarose gel of *M. flavus* AK 11. **(B)** Phylogeny of strains AK 11, the type strains of recognized species in the *M. flavus*, and representatives of related taxa

Yellow pigment extraction method

This study used three methods to determine the extraction method to obtain an extract that could inhibit the test bacteria (Table 4 & Fig. 4). The most appropriate maceration solution used in the carotenoid extraction process is methanol compared to acetone and n-hexane (**Papapostolou *et al.*, 2023**). In addition, extraction with methanol solvent produces the highest percentage of the antioxidant activity compared to other solvents. This method also uses a proper treatment by reducing the contact of the pigment

with light by covering the tube surface with aluminum foil and the process of transferring the solution and evaporation in a dark room. Adding solution and evaporation is better done in a room with red light. The use of N_2 gas aimed to remove residual solvents in the filtered extract. Spraying N_2 gas was chosen since N_2 gas is an inert gas that can replace air and reduce oxidation in the extract.

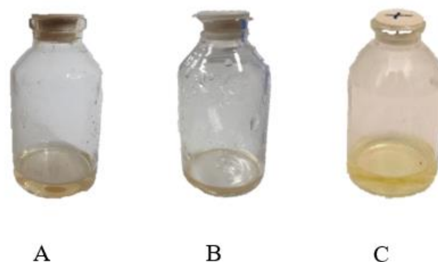

Fig. 4. Extraction results: **(A)** Method I; **(B)** Method II, and **(C)** Method III

Table 4. Extraction methods

No	Difference	Method I	Method II	Method III
1.	Maceration Solution Type	Methanol: Acetone (7:3)	Ethyl Acetate	Methanol
2.	Maceration Solution Comparison	0.1g pellets/1 mL Solution	0.2g pellets/1 mL Solution	0.2g pellets/1 mL Solution
3.	Evaporation Method	Heating waterbath $<30^{\circ}$C	Heating waterbath $<30^{\circ}$C	N_2 3 L/m flow rate
4.	Treatment	Sonication process	Fermentation with PBS 1x24 hours before the maceration process	Covered with aluminum foil and minimal exposure to light
5.	Results obtained	light yellow compound	Dark yellow compound	Yellow compound
6.	Antibacterial Activity Test Results	No inhibition	No inhibition	Inhibition

Extract yield

The yield was obtained by comparing the extraction results with the number of dry samples (pellets) extracted in percent (%). From a 500mL volume of ZMB medium, bacterial pellets weighing 1.19g were extracted, and 0.40g was obtained (1.6mg/ L). These results were calculated, yielding a value of 33.61%. The extraction was effective since the yield was >10%. The yield of yellow pigment in marine bacteria varies depending on the species and culture conditions. Previous studies have reported significant pigment yields from various marine bacteria. For example, the yield of yellow pigment from the *Citricoccus* sp. marine bacteria was optimized at pH 7, producing 9mg/

50ml, significantly higher than at pH 4- 6 and 8 (**Ezhil *et al*., 2022**). Another strain, SB2, yielded 7.81mg/ L of yellow pigment under optimized conditions, displaying antibacterial properties (**Aroumougame, 2022**). These findings highlight the diverse capabilities of marine bacteria in producing yellow pigments with potential biomedical applications. The yellow pigment yield results of the study showed a lower value than the previous study since in our study we did not optimize the production of curing pigment from *M. flavus* AK 11.

Antibacterial activity test

The antibacterial activity of the yellow pigment extract was evaluated using the agar well diffusion method against four test bacteria, consisting of the Gram-positive group: *S. aureus* ATCC 6538 and MDR *S. aureus*. In contrast, the Gram-negative group used the test bacteria MDR *P. aeruginosa* and *E. coli* with positive control in the form of an antibiotic compared to CLSI 2020. Tables (5, 6) show the results of the inhibition zone diameter of the antibacterial activity of the yellow pigment extract against Gram-positive and Gram-negative bacteria, respectively.

Table 5. Inhibition zone diameter of antibacterial activity of the yellow pigment *M. flavus* AK 11 extract against Gram-positive bacteria (mm)

		Concentration	*S. aureus* ATCC 6538	MDR *S. aureus*
Extract	Yellow pigment	10 mg/L	35±1.08	25±1.06
Antibiotics	oxacillin	1 µg	34 (S)[1]	0 (R)[2]
	tigecycline	15 µg	20 (S)[1]	28 (S)[1]
	nitrofurantoin	300 µg	25 (S)[1]	26 (S)[1]
	streptomycin	10 µg	19 (S)[1]	-
	vancomycin	30 µg	20 (S)[1]	25 (S)[1]
	ciprofloxacin	5 µg	25 (S)[1]	0 (R)[2]

CLSI Standard 2020, Notes: [1]S: Sensitive, [2]R: Resistant.

Table 6. Diameter of inhibition zone of antibacterial activity of the yellow pigment *M. flavus* AK 11 extract against Gram-negative bacteria (mm)

		Concentration	*P. aeruginosa*	*E. coli*
Extract	Yellow pigment	10 mg/L	0±0	0±0
Antibiotics	ceftazidime	30 µg	0 (R)[2]	0 (R)[2]
	tigecycline	15 µg	20 (S)[1]	20 (S)[1]
	aztreonam	30 µg	0 (R)[2]	0 (R)[2]
	cefotaxime	10 µg	0 (R)[2]	0 (R)[2]
	amikacin	30 µg	26 (S)[1]	12 (S)[1]
	meropenem	10 µg	32 (S)[1]	20 (S)[1]
	gentamicin	10 µg	20 (S)[1]	25 (S)[1]

CLSI Standard 2020, Notes: [1]S: Sensitive, [2]R: Resistant.

The results of the antibacterial activity test after incubation for 24 hours showed an inhibition zone on the *S. aureus* ATCC 6538 test bacteria with a clear zone around the well of 35± 1.08mm, while MDR *S. aureus* exhibited a clear zone around the well of 25± 1.06 (Table 6). Gram-negative test bacteria proved to have no clear zone around the well. The presence of an inhibition zone around the well indicated that the yellow pigment produced by *M. luteus* could be used as an antibacterial agent. These results align with those found by **Umadevi and Krishnaveni (2013)** reporting that the yellow pigment produced by *M. luteus* can inhibit the antibacterial activity of *S. aureus* and does not form an inhibition zone in the *E. coli* bacteria, forming a thin clear zone in *P. aeruginosa* **(Umadevi & Krishnaveni, 2013)**.

This pigment compound was more effective at inhibiting Gram-positive than Gram-negative bacteria. The results show that there is no inhibition zone in *P. aeruginosa* and *E. coli* (Table 7). It was due to differences in the cell wall structure. Gram-negative has a lipopolysaccharide layer that can block the active substances entering the bacteria **(Basim *et al*., 2021)**. Similar results were also obtained by **Safari and Ahmady-Asbchin (2019)** indicating that the walls of Gram-positive bacteria coated with peptidoglycan have low permeability. Therefore, they are less effective in blocking the entry of compounds from outside the cell. Previous research has also shown that there was a relationship between antibacterial and antioxidant in which this antioxidant could inhibit the antibacterial in three ways, namely (i) impairing the permeability of the outer membrane: (ii) causing cytoplasmic leakage; and (iii) inhibiting the formation of nucleic acids **(Karpiński & Adamczak, 2019)**.

Pigment identification.

The extract was diluted with methanol, and the absorbance was measured using UV-vis spectrophotometry with a wavelength of 300- 800nm. The study shows that the optimal wavelength of the extract is λ350nm with an absorbance value of 0.578 (Fig. 5). These results align with the previous research report that the optimum wavelength of carotenoid compounds is λ350nm **(Majeed, 2017)**. Different results were found by **Basim *et al*. (2021)** that the optimum wavelength of carotenoid pigments was maximally capable of absorbing light at three peaks in the range of λ400- 600nm **(Basim *et al*., 2021)**. Carotenoid pigments are bacterial pigments with a color range of yellow, orange, to red **(Ram *et al*., 2020)**. The discrepancy in the graph of the wavelength optimization can be influenced by several factors, including the low concentration of the sample due to the low concentration. Characteristics of less stable carotenoids can also affect the absorbance measurement.

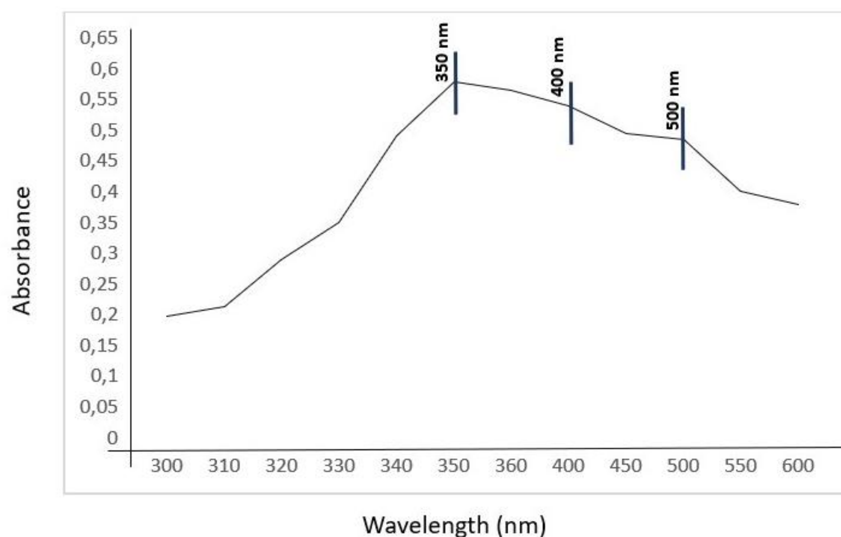

Fig. 5. Graph of the measurement of the wavelength optimization of the yellow pigment extract

CONCLUSION

One pigmented bacterium from an unidentified coral *Porites* sp. was isolated and identified as *M. flavus* AK 11. This isolate produced yellow pigment identified as carotenoid pigments. The result indicated that the most suitable pigment extraction method involved using methanol as a solvent and drying technique with N_2. Interestingly, only the crude extract from this method inhibited Gram-positive *S. aureus.*

Authors contribution

Each author played a significant role in the study's conceptualization and methodology. MEP role in the project included data curation, investigation, data visualization, and writing-original draft. SD, BSD, and ER coordinated the draft's study, conceptualization, and review. All authors contributed to the drafting, and revision of the manuscript, and approved the final version for submission.

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Conflict of interest

The author declares that there is no conflict of interest in this research.

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