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Molecular analysis and bioactive compounds of symbiont bacteria isolates from *Polycarpa aurata* in Makassar City

Gemini Alam[1*], Sartini[2], Abdul Rahim[3], Herwin[4], Ayyub Harly Nurung[4], Amirullah[4], Nasrul Haq[5]

[1] Laboratory of Phytochemistry, Department of Pharmaceutical Sciences and Technology, Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia
[2] Laboratory of Microbiology, Department of Pharmaceutical Sciences and Technology, Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia
[3] Laboratory of Pharmacognosy, Department of Pharmaceutical Sciences and Technology, Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia
[4] Laboratory of Microbiology, Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar, 90231, Indonesia
[5] Laboratory of Pharmaceutical, Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar, 90231, Indonesia
* Corresponding author's e-mail: daengta007@yahoo.com

ABSTRACT

Symbiont bacteria are microbes that live in a mutualistic relationship with their hosts, produce secondary metabolites such as adenine riboside, polycarpaurine which act as bioactive compounds. This study aims to isolate and analysis bioactive compounds of symbiont bacterial isolates form *Polycarpa aurata* based on different pH and sea depths in Barrang Lompo Island, Makassar city as antibacterial. 34 isolates of symbiont bacteria coloured-blue *Polycarpa aurata* is a active isolate were isolated based on pH variation and sea depths. The result of isolate at pH 6 with sea depth 5 and 15 meters = 4 isolate, 5 isolates at sea depth 10 meters, pH 7 with sea depth 5 metes = 4 isolates, 3 isolates at sea depth 10 and 15 metes, pH 8 with sea depth 5 and 15 metes = 4 isolates at and 3 isolate at sea depth 10 meters. Antagonist test of 17 isolates as pure isolate, obtained 13 pure isolate inhibition against 10 pathogenic bacterial. Growth optimization revealed that the optimal growth times symbiont bacteria isolates, namely AQB10-1, AQB10-2 isolate = 22 hours, AQB10-3 isolate = 34 hours and AQB10-5 isolate = 32 hours with the nutritions used in AQB10-1 and AQB10-2 isolate is yeast extract, AQB10-3 is meat, and AQB10-5 isolate is triptone. Molecular analysis was performed through 16S rRNA sequencing, identified as *Bacillus licheniformis* strain (AQB10-1, AQB10-2, AQB10-3 isolate), *Paenibacillus alvei* strain (AQB10-5 isolate) and *Pseudomonas aeruginosa* strain (ALB10-5). Antibacterial activity of symbiont bacteria isolates *Polycarpa aurata*, obtained the largest inhibition zone diameter in AQB10-2 isolate against *Vibrio cholerae* ATCC 25175. The bioactive compounds of AQB10-2 isolate containing 1,4-dimethyl-, butanoic acid, azulena, 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a] pyrazine-1,4-dione, cyclo(L-prolyl-L-valine), hexadecanoic acid, cis-13-Octadecenoic acid, lycopersen, dodec-11-enylbenzene, and 1,2-Benzenedicarboxylic acid compound.

Keywords: *Polycarpa aurata*, symbiont bacteria, molecular analysis, bioactive compounds, antibacterial.

INTRODUCTION

Indonesia is a prominent maritime nation with a diverse range of marine biota that may harbor unique bioactive chemicals not found in terrestrial organisms. Approximately 75% of Indonesia's territorial area consists of maritime environments, resulting in a rich and diverse range of biodiversity. As a result, Indonesia is sometimes referred to as the country with the highest level of diversity in the world, also known as "Mega diversity in the World" (Mulawarmanti, 2019). The wide range of marine organisms has the capacity to serve as a fundamental component for the development of medications, including those obtained from sponges, molluscs, bryozoas,

tunicates, and other organisms. Tunicate, a marine invertebrate belonging to the subphylum Urochordata, plays a crucial role in maintaining the stability of marine ecosystems. It serves as a bioindicator for evaluating water quality, occupies a position in the food chain, serves as prey for various marine animals, and contributes to the composition of coral reefs, which are part of the marine biotope (Rompas et al., 2022; Ayuningrum et al., 2019). Tunicate is the most prevalent marine ecosystem, consisting of over 3,000 species that inhabit diverse habitats, ranging from shallow to deep seas. These organisms possess a wide range of bioactive and characteristic compound (Casertano, 2020). Biodiversity provides an opportunity to utilize bioactive compounds from marine biota as a source of treatment such as the treatment of infectious diseases. The current knowledge of marine biodiversity is incomplete, particularly in the deep sea (Kadarusman, 2019). The marine material that serves as a source of bioactive compounds might originate from the organisms themselves or from the interactions between symbiotic bacteria and the organisms. These interactions are crucial in the production of medicinal materials used to treat various diseases (Cragg et al., 2013).

The pursuit of metabolites as beneficial substances is motivated by the prevalence of bacterial infectious illnesses in the community. The condition can be caused by several antibiotic-resistant bacteria that infiltrate the body through both systemic and topical routes. The disease is attributed to bacterial infections, including *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and others (Bayat, 2011; Boss, 2016, Wimmerstedt, 2008)**.** The mortality rate from infectious diseases is still relatively high, both in the world and in Indonesia (WHO, 2014). In 2019, bacterial antimicrobial resistance was estimated to be directly responsible for 1.27 million deaths worldwide, and approximately 4.95 million deaths per year are associated with bacterial resistance and predicted to increase 10 million in 2050, if not addreassed properly (Murray et al., 2022). The management of infectious disorders can be achieved through the administration of antibiotic medications. However, the improper and unregulated utilization of antibiotics might result in the emergence of antibiotic-resistant bacterial strains (Murti et al., 2012)**.**

Addressing antibiotic resistance is a major societal challenge that requires urgent resolution. Antibiotic resistance arises when the bacteria are not susceptible to the drug's effects, preventing it from effectively killing or inhibiting them. Antibiotic resistance is increasing and endangering all parts of the world (Murray et al., 2022; O'Neill, 2016). The World Helath Organization (WHO) released a list of pathogenic bacteria that are resistance to antibiotic drugs, so that research and development of new drugs is needed (WHO, 2017). Antimicrobial resistance significantly threatens of human health (Dhingra et al., 2020)**.** The growth and spread of pathogenic organisms that develop resistance to existing antibiotics provide a complicated worldwide public health concern. There is no straightforward method that can effectively tackle this issue (Widayati et al., 2015). Due to the problem of antibiotic resistance, it is necessary to search of novel antibiotics derived from marine biota, such as of the *Polycarpa aurata* species. *Polycarpa aurata* contains novel alkaloid chemicals, including policarpaurin A, B and C, tetracyclic pyridoacridine, pentacyclic pyridoachridines, segoline A, tetrahydro--carbolines, N-methyl – carbolinium, cyclic peptides, depsipeptides and various types of aromatic alkaloids which serve (Menna et al., 2011; Dou et al., 2019). Secondary metabolite activity of *Polycarpa aurata* ethyl acetate extract originating from Barrang Caddi Island, South Sulawesi, has antibacterial activity against *Methicillin-resistant Staphylococcus aureus* (MRSA), *Bacillus cereus, Salmonella typhi* and *non-Multidrugs Resistent* (non-MDR) *Escherichia coli* at lowest concentrations of 0.5 mg/ml but has no antifungal effect on *C. albicans* and *T. rubrum*. The raw extract exhibits low antioxidant activity at a concentration of 634.60 ppm and moderate cytotoxicity against murine leukemia p388 cancer cells with an IC_{50} value of 0.08 μg/mL (Sibero, 2022). The methanol fraction of *Polycarpa aurata* has activity against bacteria *Eschericia coli* and *Staphylococcus aureus* at a concentration of 150 μg/disk (Ayuningrum, et al., 2019). The results of the isolation of associated bacteria in tunicates of the *Polycarpa aurata* type yield the best activity against Gram (+) MDR bacteria *Bacillus cereus* and MRSA, MDR *B. cereus*, and Gram (-) MDR *E. coli* pathogenic bacteria (Rompas, et al.,2022).

Tunicate, *Polycarpa aurata* can also produce secondary metabolites of symbiont microorganisms which possess therapeutic potential like antitumors with mechanisms of action.

(apoptosis, antiangiogenesis, dan antiproliferative). The *Ecteinascidia turbinate* has been developed as an anti-cancer drug and has been approved by the FDA under the trademark Yondelis®. The other *Aplidium albicans* containing the dehyrodidemnin B compound and plitidepsin have been manufactured under the trade name Aplidin® (Watters, 2018). The tunicate of the *P. aurata* species can be studied to discover bioactive compounds that exhibit biological activity. These compounds can serve as a potential substitute for antibiotics derived from symbiotic bacteria.

The purpose of this study is to optimize, analyze the antibacterial activity, and perform molecular analysis of symbiont bacteria isolates obtained from *Polycarpa aurata* (Quoy & Gaimard 1834) located on Barrang Lompo Island, Makassar. Besides, it aims to identify and characterize bioactive compounds that could potentially as novel therapeutic agents against pathogenic bacteria.

MATERIAL AND METHODS

Material

The materials used in this research were *Polycarpa aurata* (yellow color, white color and blue color), nutrien agar medium (Merck granuCult®, 500 g), maltose broth (Comedco peru SAC, 500 g), muller hinton agar (Merck KGsA 54271, 500 g), (Himedia, 500 g); tryptone (Himedia, 500 g); yeast extract (Merck KGsA 64271, 500 g), and meat extract (Merck VM209979, 500 g), violet crystal (ACS Reag. Ph Eur, C.I 42555), lugol solution (Reagensia 2,100 mL), alcohol acetate (Emsure, ACS ISO Reag. pH Eur, 2.5 L), alcohol, test bacteria (*Escherichia coli* ATTC 25922, *Salmonella thypi* NCTC 786, *Vibrio cholerae* ATCC 25175, *Shigella dysenteriae*, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 14990, *Bacillus subtilis* ATCC 6633, *Propionibacterium acnes*, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175). And the tools used disc blanc, UV-Vis spectroscopy (Thermo scientific), microscope (Fluoresen Trinocular Digital BestScoep BS-2048FT), laminar air flow (Mycolab, Type HLM120), Rotary shaker incubator (Argo Lab. thermostatic shaking incubator).

Preparation and determination of samples

The sample used was *Polycarpa aurata* originating from the waters of Barrang Lompo Island located on the northwest side at a distance of ± 11 km from the town of Makassar, South Sulawesi province. After collecting samples through snorkeling and skin diving, they were placed in a container filled with seawater and thoroughly rinsed by washing with running water. *Polycarpa aurata* samples were determined at the Laboratory of Environmental and Marine Sciences, Department of Biology, Faculty of Mathematics and Natural Sciences of Hasanuddin University Makassar.

Isolation and purification of *Polycarpa aurata* symbiont bacteria

The surface contamination of the wet-sorted *Polycarpa aurata* sample was treated by applying 70% ethanol for 1 minute, followed by 5.25% sodium hypochlorite for 1 minute. The sample was then rinsed three times with sterile Aquadest for 1 minute each time. The *Polycarpa aurata* specimen was dehydrated and finely chopped into little pieces measuring around 1 cm, then the symbiont bacteria were isolated using Nutrient Agar (NA) medium with seawater as a solvent dan NA medium with destilated water as a solvent. The isolation of symbiont bacteria is carried out aseptically, where small pieces of *Polycarpa aurata* were placed on top of the NA medium in a sterile petri dish, then incubated at a temperature of 37 °C for 1-3 days depending on the growth rate of the symbiont bacteria (Krishnan et al., 2012, Hidayat et al., 2018).

The symbiotic bacteria were purified and isolated by aseptically taking 1 ounce using a sterile wire, and then a part of the bacteria was spread over the surface of the NA medium in a sterile petri dish. Each different isolate was purified using the quadran grinding method, followed by incubation at a temperature of 37 °C for 24 hours. The pure insulate was prepared by inoculating on NA medium and transferred into sterile reaction tubes as stock (Marzuki et al., 2018).

Antagonist test

Antagonist test of symbiont bacteria isolates was carried out in a densely diluted way in which all the isolates of *Polycarpa aurata* bacteria that grew on the NA medium were blocked

by cutting them using sterile stainless cylinder shrinkers. The pieces of the bacterial isolate were placed in a petri dish containing a sterile Muller Hinton Agar (MHA) medium with a suspension of the test bacteria (*Escherichia coli* ATTC 25922, *Salmonella thypi* NCTC 786, *Vibrio cholerae* ATCC 25175, *Shigella dysenteriae ATCC 13313*, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 14990, *Bacillus subtilis* ATCC 6633, *Propionibacterium acnes NCTC 737*, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175), then incubated at a temperature of 37 °C for 24 hours. Each isolation was observed by visually examining the formation of a lymphatic zone around the isolation piece and measuring the diameter of the barrier zone (Rante et al., 2022; Alghazeer et al., 2024).

Morphological examination

The morphological examination was conducted macroscopically (to observe colony shape, edge shape, elevation, colony color) and microscopically (to determine Gram-negative or Gram-positive bacteria). Each individual symbiotic bacterium was examined macroscopically. It was then placed on a sterile axis and inoculated onto the surface of sterile MHA medium. The medium was incubated at a temperature of 37 °C for 1x24 hours and observed the morphology of bacteria colony (Yetti et al., 2015).

The symbiont bacteria were examined under a microscope using Gram staining. A sterile reproduction of the bacteria was taken, and a small amount was placed on a glass slide. Violet crystal droplets (Gram A) were added, and after 1-3 drops, the excess was removed. The slide was then washed with distilled water and dried. Next, a solution of lugol (Gram B) was added, again 1-3 drops were used, and after removal, the slide was washed and dried. Finally, the slide was treated with alcohol acetate (Gram C), using 1-3 drops, and after removal, it was washed with distilled water and dried. Subsequently, it is examined using a microscope with an objective lens magnification of 10x. Gram-negative bacteria are bacteria that do not retain purple methyl colour in the Gram colouring method. Gram-positive bacteria will retain dark purple metallic colour after being washed with alcohol (purple/blue bacterial cell) (Marzuki et al., 2018; Khalila et al., 2020).

Growth time optimization

A starter culture was made by auapending the symbiont bacteria colony in 100 mL of Maltose Broth (MB) medium, then incubated at a temperature of 37^0C for 1x24 hours. The symbiont bacteria culture is fermented by transferring 10 mL into a 100 mL MB medium to optimize the qgrowth time of the symbiont bacteria using a fermentor. The fermentation process was carried out at a speed of 150 rpm for 74 hours at 37^0C using 2-hour intervals (at 2', 4', 6', up to 74'). The fermentation fluid of 1 mL was taken to test the *optical density* (OD) value for each isolate, thus obtaining the optimal time for the growth of symbiont bacteria (Astuti et al., 2021; Risna, 2022).

Antibacterial activity

Based on nitrogen sources variations

The activity of the secondary metabolite produced by the symbiotic bacteria was tested by exposing a 6 mm diameter blank disc to various nitrogen nutrient variations. The disc was immersed in an insulated suspension and placed in a petri dish containing 10 mL of MHA medium. A 20 µL test bacterial suspension (with a density of $1x10^7$ cells/mL according to the McFarland standard) was added and the mixture was homogenized. The petri dish was then incubated in a 37 °C incubator for 1x24 hours. The diameter of the zone of inhibition formed was measured in millimeters (Suhendar et al., 2019; Hussein et al., 2024).

Based on optimization time variations

The optimal time for testing the activity of symbiont bacteria's secondary metabolites was determined by placing a 6 mm diameter blank disc in a suspension, which was then submerged in a petri dish containing 10 mL of MHA medium. A bacterial suspension of 20 µL (with a McFarland standard density of $1x10^7$ cells/mL) was added and the mixture was homogenized. The petri dish was then incubated at a temperature of 37 °C for 1x24 hours. After incubation, the diameter of the barrier zone formed was measured in millimetres (Suhendar, et al.; 2019, Army et al., 2023; Hussein et al., 2024).

Secondary metabolite production

The bacterial isolate intended for fermentation was cultured in a 10 mL MB medium and

incubated at a temperature of 37°C using the optimal growth time of each symbiont bacteria. It was then transferred to a fermentation medium with a modified substrate, specifically altering the nitrogen source to produce secondary metabolites. Various alternative nitrogen sources were used in this process. The additional source variation utilized consisted of peptone, tryptone, meat, and yeast extracts at a concentration of 0.3% (v/v). The incubation was carried out at a temperature of 37°C for 1x24 hours. The extraction outcome was impeded by the acquired extract weight (John et al., 2023).

Molecular analysis

The symbiotic bacteria AQB10-1, AQB10-2, AQB10-3, AQB10-5, and ALB10-5 were isolated by following the standard method of DNA genomics separation. It was done using QuickDNATM Magbead Plus kits (Zymo Research, D4082) until DNA products were obtained. The measurement of DNA products was conducted through electrophoresis of a 1% agarose gel in TBE solution, followed by amplification of the DNA product using 16S rRNA primers (forward: 5-GAGAGTTTGATCCTGG-3; reverse: 5-TACCGCGGCTGCTGGCAC-3). The Polymerase Chain Reaction (PCR) cycle involved pre-denaturation at 94 °C for 3 minutes, denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 2 minutes Astuti, 2021; Lee, 2023. The cycle was repeated 35 times, the post-extension phase was performed at 72^0C during 2 minutes. Sequencing in PT. Genetica Science Indonesia is performed using the BioEdit application, a program for scanning the sequence of nucleotides. Identification was done by BLAST analysis, which involved analyzing the results of BLAST, including parameters such as Query Coverage and Maximum Identity. A sequence with species identities ranging from 96–100% was found in symbiotic bacteria that closely matched sequences in the database. The phylogenetic tree uses the Neighbour-Joining algorithm method (Hidayat et al., 2018; Phongsopitanun et al., 2019).

GC-MS analysis

The ethyl acetate extract obtained from the symbiotic bacterial isolate AQB10-2 associated with *Polycarpa aurata* was subjected to GC–MS analysis (Shimadzu QP 2010S instrument equipped with an Agilent 5973 inert MSD detector operating at 70 eV). Approximately 2 µL of the sample was injected into a Scientific ID DB-5MS capillary column (30 mm × 0.25 mm × 0.15 µm). Helium was utilized as the carrier gas at a flow rate of 1 mL/min with a split ratio of 1:10. The oven temperature program was initiated at 50 °C for 0.4 min, followed by an increase to 280 °C at a rate of 200°C/min, and subsequently held for 15 min. The injector temperature was maintained at 290 °C, while the interface temperature was adjusted to 230 °C. Identification of the chemical constituents was carried out using the Wiley database version 7.0 by comparing mass spectra and fragmentation patterns (Suhendar et al., 2019; Herwin et al., 2024; John et al., 2023).

RESULTS AND DISCUSSION

Polycarpa aurata was collected from Barrang Lompo Island, located in Makassar City (Figure 1). The sampling site was positioned at a longitude of 119˚19.516'E, a latitude of 5˚03.227'S and map scale 1:4,000. The samples were collected through snorkeling and skin-diving techniques from subtidal environments at depths ranging from 15 to 30 meters.

Polycarpa aurata is a marine biota classified under the tunicate category, sourced from Barrang Lompo Island in the city of Makassar. The sample consisted of three distinct color varieties of *Polycarpa aurata*, specifically yellow, white, and blue *Polycarpa aurata* **(**Figure 2).

The analysis of the sample confirmed that it belonged to the species *Polycarpa aurata*, with determination number 038/ILK.BIO/PP.13/06/2023. This species is characterized by its vase-shaped body and presence of two siphons, one on the top and the other on the side. This tunicate species has the ability to reach a size ranging from 5 to 15 cm. It possesses a rigid body and leads a solitary existence, remaining attached to the substrate. This particular species of tunicate has a combination of white, yellow, and blue colors on its body, along with yellow or orange patterns and purple on certain body sections. Additionally, its body is composed of either cellulose or tinicin. Tunics exhibit a transition from a coarse and abrasive texture to a smooth and muted one.

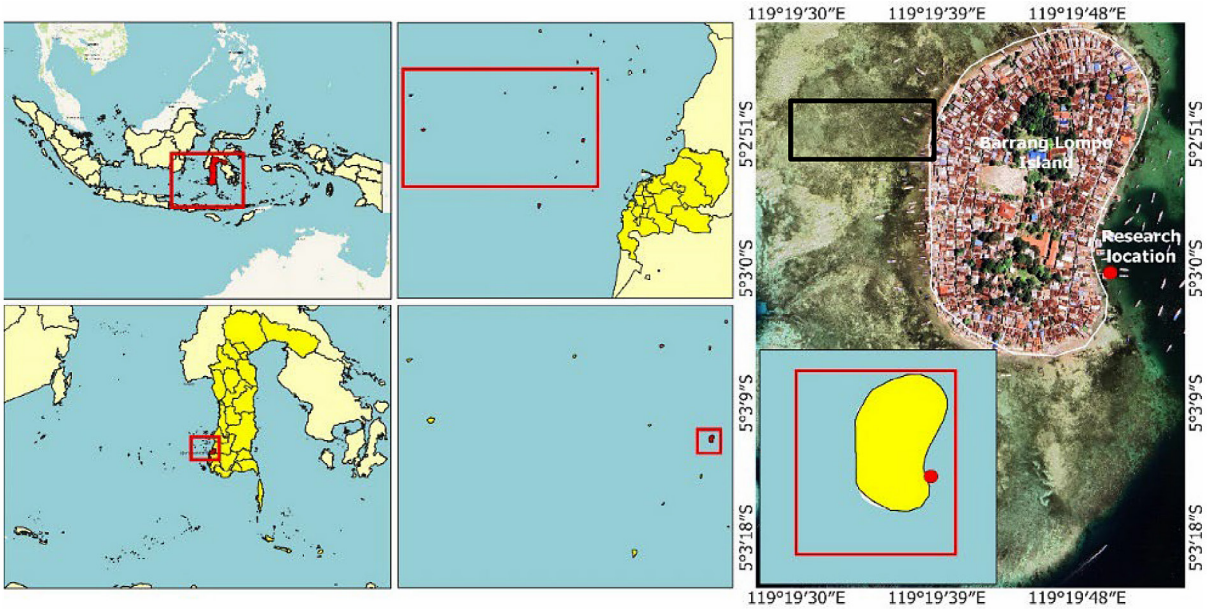

Figure 1. Location of *Polycarpa aurata* in Barrang Lompo Island, Makassar City, South Sulawesi

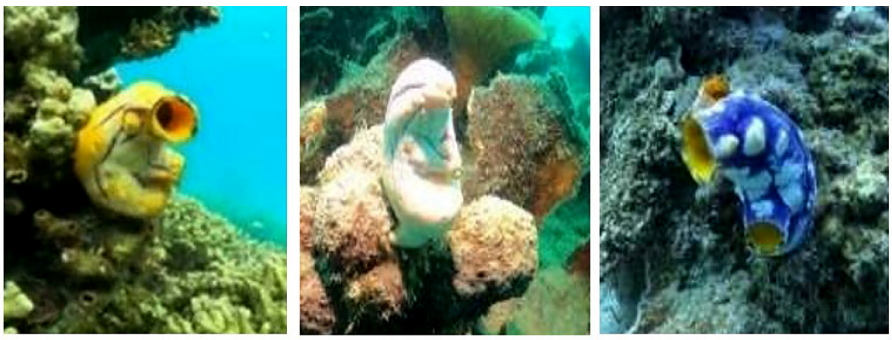

Figure 2. Tunicata type *Polycarpa aurata* from Barrang Lompo Island, Makassar City: (1) *Polycarpa aurata* is yellow, (2) *Polycarpa aurata* is white, (3) *Polycarpa aurata* is blue.

Isolation and purification

34 symbiont bacteria isolates were obtained by isolating bacteria from the tunicate marine biota using three different colors of *Polycarpa aurata* (yellow, white, and blue) on NA medium with aquadest solvent and sea water. 34 isolates of symbiont bacteria coloured-blue *Polycarpa aurata* were isolated based on pH variation and sea depths obtained pH 6 = 4 isolate at sea depth 5 and 15 meters, 5 isolates at sea depth 10 meters, pH 7 = 4 isolates at sea depth 5 meters, 3 isolates at sea depth 10 and 15 meters, pH 8 = 4 isolates at sea depth 5 and 15 meters and 3 isolate at sea depth 10 meters. The result of purification of isolate symbiont bacteria Polycarpa aurata obtained 17 pure isolate.

Isolated symbiont bacteria with a barrier zone diameter on the blue-coloured *Polycarpa aurata* sample was the best isolate compared to the yellow-coloured and white-coloured *Polycarpa aurata* isolated bacteria (eight from blue-coloured *Polycarpa aurata* inhibited the growth of the test bacteria, while four isolates from white-coloured *Polycarpa aurata* and one isolate from yellow-coloured *Polycarpa aurata* showed the same inhibitory effect). The Bacterial growth there are strongly influenced by encironmental factor, including physical, chemical, and biological factors. Physico-chemical factors include temperature, pH, aeration, agitaton, and growth media composition. Meanwhile, biological factors are competition and contamination (Phongsopitanun et al., 2019). These environmental parameters have an interrelated relationship with each other. Brigtness factors, depth, pH, temperature, salinity, time, and nutrient sources are factors that can

influence the activity of bacterial. Bacterial require a pH value ranging from 6.5-7.5 generally acids have a bad influence on bacterial growth (Ginting et al., 2019; Rocchetti et al., 2020). Temperature and salinity have a relationship with water depth, whereas the depth of a body of water increases, the salinity value increases, the salt content is high. Meanwhile, the temperature is also increasing (Ginting et al., 2019, Sidabutar et al., 2019). The nutritional factors greatly influence the weight of secondary metabolites products by each isolates of the symbiont bacterial *Polycarpa aurata*. Based on nutritional variations in meat, peptone, tryptone and yeast extract, obtained different weights extract of each isolates.

Antagonist test

The isolate symbiont bacteria of *Polycarpa aurata* was antagonized against 5 Gram-negative bacteria (*Escherichia coli* ATTC 25922, *Salmonella thypi* NCTC 786, *Vibrio cholerae* ATCC 25175, *Shigella dysenteriae ATCC 13313*, *Pseudomonas aeruginosa* ATCC 27853- and 5 Gram-positive bacteria (*Staphylococcus epidermidis* ATCC 14990, *Bacillus subtilis* ATCC 6633, *Propionibacterium acnes NCTC 737*, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175) by Agar Diffusion method (Table 1).

Morphological examination

The morphological examination of the symbiotic bacteria isolates from the three types of *Polycarpa aurata* colors, namely yellow, white, and blue, revealed that 17 symbiotic bacterial isolates exhibited distinct characteristics. The microscopic examination of the 17 isolates revealed that 8 symbiotic bacterial isolates were classified as Gram-negative bacteria, while 9 isolates were classified as Gram-positive bacteria (Table 2).

Table 1. Results of measurement of the average diameter of the *Polycarpa aurata* bacteria inhibition zone

Isolate code	Average diameter inhibition zone (mm)									
	BS	SA	SE	Pac	SM	EC	SD	PA	VC	ST
ALK10-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ALK10-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ALK10-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ALK10-4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ALP10-3	0.00	0.00	0.00	8.45	0.00	0.00	17.45	0.00	0.00	0.00
ALP10-4	0.00	0.00	0.00	8.35	0.00	0.00	9.37	0.00	0.00	0.00
ALP10-5	0.00	0.00	0.00	0.00	0.00	0.00	10.91	0.00	0.00	0.00
ALB10-2	0.00	0.00	0.00	0.00	19.56	0.00	0.00	0.00	0.00	0.00
ALB10-5	0.00	0.00	0.00	0.00	21.01	0.00	0.00	0.00	0.00	0.00
ALB10-6	0.00	0.00	0.00	0.00	13.32	0.00	0.00	0.00	0.00	0.00
ALB10-7	0.00	0.00	0.00	0.00	13.24	0.00	0.00	0.00	0.00	0.00
AQB10-1	14.27	11.06	0.00	0.00	0.00	0.00	0.00	0.00	10.62	0.00
AQB10-1	20.38	18.91	17.88	21.93	22.23	19.52	25.39	22.19	26.47	18.94
AQB10-1	18.73	20.76	21.21	20.38	0.00	18.49	20.94	20.33	24.94	22.13
AQB10-2	16.70	22.46	21.25	20.60	0.00	21.50	20.71	0.00	26.25	21.91
AQB10-3	10.69	17.06	17.55	22.69	0.00	18.38	12.52	13.09	19.08	20.70
AQB10-5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	21.55	0.00	0.00
Control (+)	30.00	18.50	22.40	28.90	26.40	23.80	21.10	19.80	21.10	26.20

Note: ALK10-1 = isolate one, yellow-coloured *Polycarpa aurata* symbiotic bacterium with seawater solvent; AQK10-1= isolate one, yellow-coloured *Polycarpa aurata* symbiotic bacterium with aquadest solvent; ALP10-1 = isolate one, white-coloured *Polycarpa aurata* symbiotic bacterium with seawater solvent; AQP10-1= isolate one, white-coloured *Polycarpa aurata* symbiotic bacterium with aquadest solvent; ALB10-1= isolate one, blue *Polycarpa aurata* symbiotic bacterium with seawater solvent; BS: *Bacillus subtilis* ATCC 6633, SA: *Staphylococcus aureus* ATCC 25923, SE: *Staphylicoccus epidermidis* ATCC 14990, PAc: *Propionibacterium acnes NCTC 737*, SM: *Streptococcus mutans* ATCC 25175, EC: *Eschericia coli* ATTC 25922, SD: *Shigella dysenteriae ATCC 13313*, PA: *Pseudomonas aeruginosa* ATCC 27853, VC: *Vibrio cholerae* ATCC 25175, ST: *Salmonella thypi* NCTC 786, Control (+) = Chloramphenicol 0.1 ppm.

Table 2. Morphological examination of *Polycarpa aurata* symbiont bacteria isolates

Isolate code	Macroscopic				Microscopic	
	Colony shape	Elevate	Edge shape	Color	Shape	Gram
ALK10-1	Round	Convex	Entire	Cream to white	Streptococcus	Negative
ALK10-2	L-form	Raised	Undulate	Cream	Coccus	Negative
ALK10-3	Mucoid	Umbonate	Undulate	Cream	Coccus	Negative
ALK10-4	L-form	Raised	Undulate	Cream	Coccus	Negative
ALP10-3	Irregular and spreading	Flat	Undulate	Translucent	Coccus	Negative
ALP10-4	L-form	Umbonate	Undulate	Cream to white	Streptococcus	Negative
ALP10-5	Irregular and spreading	Umbonate	Undulate	Cream	Streptococcus	Positive
ALB10-2	Irregular and spreading	Flat	Undulate	Translucent	Bacil	Positive
ALB10-5	Irregular and spreading	Raised	Undulate	White to translucent	Diplococcus	Positive
ALB10-6	L-form	Convex	Smooth	Cream to white	Coccus	Positive
ALB10-7	Irregular and spreading	Flat	Lobate	Translucent	Coccus	Negative
AQB10-1	Punctiform	Drop-like	Entire	Cream	Coccobacillus	Positive
AQB10-1	Round	Convex	Entire	Cream	Coccobacillus	Negative
AQB10-1	Irregular and spreading	Hilly	Lobate	Translucent	Diplococcus	Positive
AQB10-2	Round	Convex	Undulate	Cream	Baciil	Positive
AQB10-3	Irregular and spreading	Hilly	Undulate	Translucent	Diplococcus	Positive
AQB10-5	Punctiform	Convex	Entire	Cream	Coccus	Positive

Gram staining of symbiont bacterial isolates from *Polycarpa aurata* was carried out to identify the bacterial as Gram-positive or Gram-negative bacterial. The results of Gram staining of the 17 isolates identified, there were 9 isolates of symbiont bacterial classified as Gram-positive, namely ALP10-5, ALB10-2, ALB10-5, ALB10-6, AQK10-1, AQB10-1, AQB10-2, AQB10-3, AQB10-5 with coccus form and 8 isolates of symbiont bacterial classified as Gram-negative, namely ALK10-1, ALK10-2, ALK10-3, ALK10-4, ALP10-3, ALP10-4, ALB10-7, AQP10-1 with bacil and coccus forms. Gram-positive bacterial have the ability to bind crystal violet more strongly than safranin, so the bacterial will be purple-coloured, while Gram-negative bacterial bind safranin more strongly, so red-coloured. Symbiont bacterial isolates in Gram determination identified 2 groups of bacterial, because generally bacterial that live in the sea are in the form of bacil and coccus. The bacil form of bacterial is a bacterial that has a flagellum which is used as a means locomotion of very active (Lee et al., 2023).

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Production of secondary metabolites

The production of secondary metabolites involved utilizing several nitrogen sources in the fermentation production media, such as meat, peptone, tryptone, and yeast extract. The results from the antibacterial activity test revealed that 5 symbiotic bacteria isolates from coloured-blue *Polycarpa aurata* showed the the largest inhibition with a strong to very strong category bacteria against the pathogenic bacteria, namely ALP10-2,

ALB10-5, AQB10-1, AQB10-2, AQB103, AQB10-5, ALB10-2 and ALB10-5 isolate. The isolates AQB10-2, AQB10-3, ALB10-5 and AQB10-5 showed the largest avtivity using variations in nutrition sources from peptone, isolate AQB10-1 using variations in nutrients from tryptone and peptone, and ALB10-2 isolate using variations in nutritions from meat and yeast extract nutrient. The production of secondary metabolites by symbiont bacteria was achieved through fermentation using different nutritional variations of meat, peptone, tryptone, and yeast extract in a 100 mL fermenting media. The heaviest extract weight was obtained by the AQB10-1 isolate produced 57.2 mg of ethyl acetate extract (yeast extract), the AQB10-2 isolate resulted in 19.7 mg of ethyl acetate extract (yeast extract), the AQB10-3 isolate obtained 12.8 mg of ethyl acetate extract (meat), the AQB10-5 isolate yielded 40.9 mg of ethyl acetate extract (tryptone), and the ALB10-5 isolate, with 29.2 mg of ethyl acetate extract (meat) (Figure 3).

Growth time optimization

The optimization of the growth time of the blue and white *Polycarpa aurata* symbiont bacterial isolate by fermentation using maltose medium suggested that the symbiont bacteria isolate had a different optimization time (Figure 4).

The growth time optimization of the *Polycarpa aurata* symbiont bacterial isolate was obtained by measuring the Optical Density (OD) value using UV-Vis spectroscopy. The growth curve of the symbiotics was used to determine the rate of cell growth rate against the influence of the growth environment (growth medium). In Figure 4, the optimum time of each isolate was AQB10-1 and ALB10-5 isolates = 30 hours, AQB10-2 isolate = 22 hours, AQB10-3 isolate = 34 hours, and AQB10-5 isolate = 32 hours.

Optimization of time and nutritional variation in culture incubation conditions are important parameters to know. Optimization of culture condition is an important factor that influences the

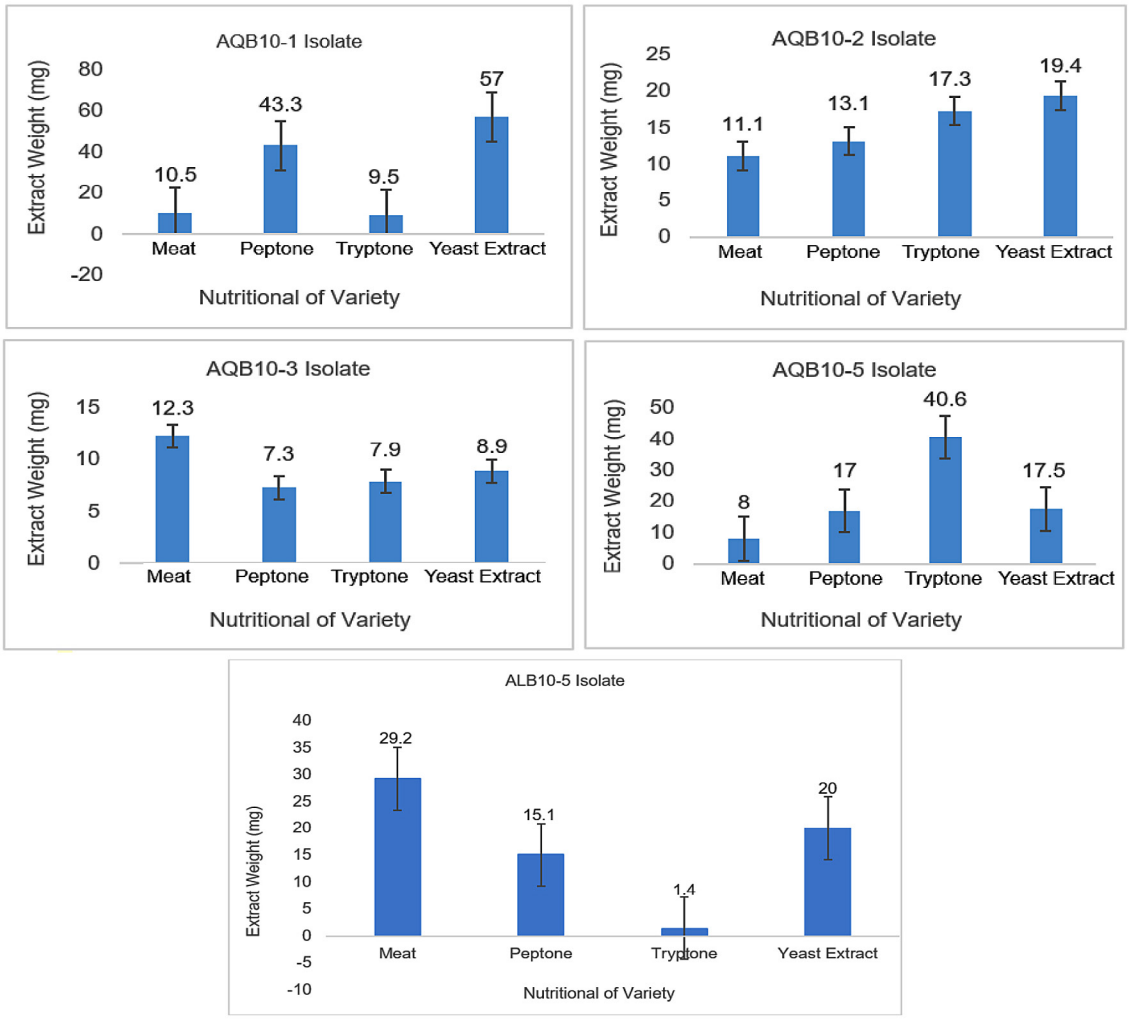

Figure 3. Diagram of ethyl acetate extract weight of symbiont bacterial isolate based on nutrition variation

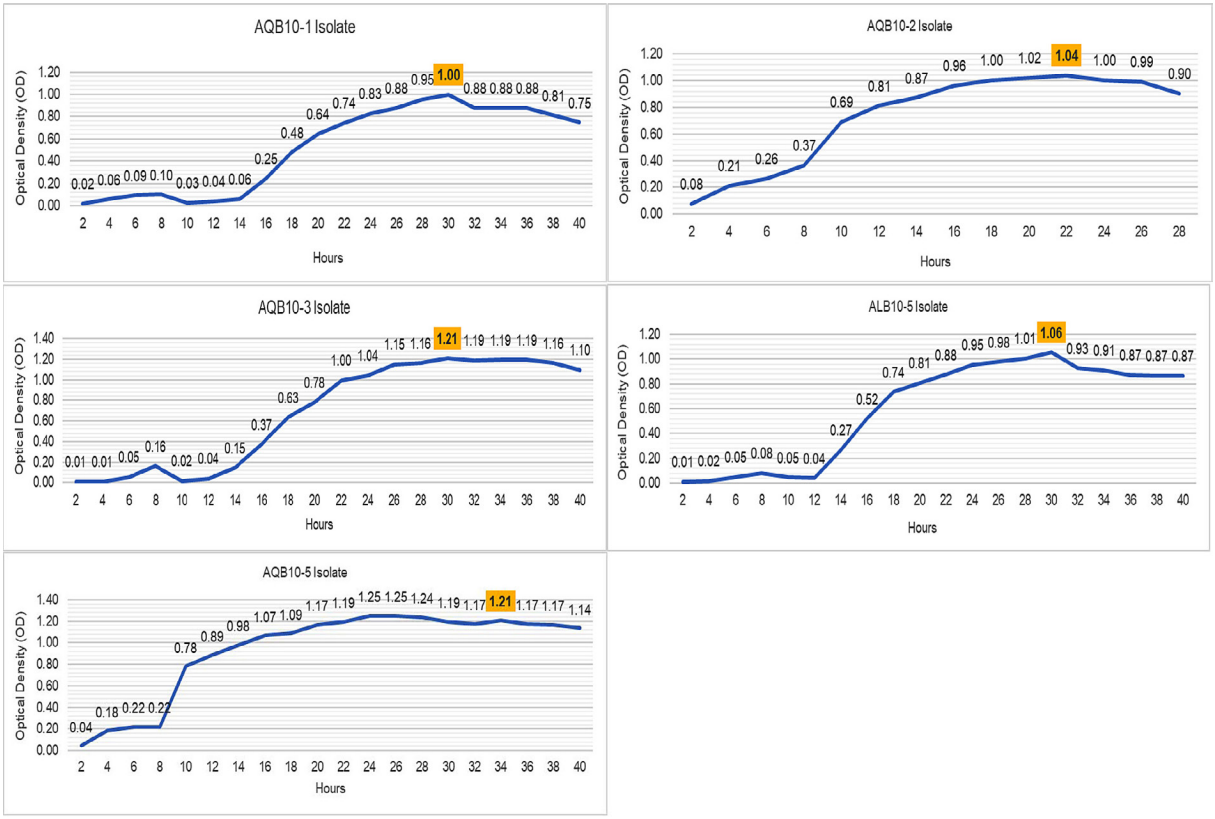

Figure 4. Growth time optimization of *Polycarpa aurata* symbiont bacterial isolate

growth and biomass of bacterial cells in producing secondary metabolites (Sari et al., 2021; Al Tanto et al., 2016). Exploration of secondary metabolites in isolates of the symbiont bacterial *Polycarpa aurata* with nutritional variations using a variety of nitrogen sources (meat, peptone, tryptone and yeast extract). The concentration and source of nitrogen will affect the pH value of the medium, the growth and activity of microorganisms in the fermentation (Zárate-Chaves, 2013). Some excess can cause inhibition by the substrate. Increasing the substrate concentration will increase the specific growth rate, but to a certain extent the substrate concentration has no real effect on the specific growth rate. Constant cell growth rate can influence the inhibition of secondary metabolite production (Oh et., al., 2007). The growth time optimization of the *Polycarpa aurata* symbiont bacterial isolate was obtained by measuring the Optical Density (OD) value using UV-Vis spectroscopy. The growth curve of the symbiotics was used to determine the rate of cell growth rate against the influence of the growth environment (growth medium). In Figure 4, the optimum time of each isolate was AQB10-1 and ALB10-5 isolates = 30 hours, AQB10-2 isolate = 22 hours, AQB10-3 isolate = 34 hours, and AQB10-5 isolate = 32 hours.

Antibacterial activity

Based on nitrogen sources variations

The antibacterial activity of isolates AQB10-1, AQB10-2, AQB10-3, AQB10-5, and ALB10-5 were categorized as having a strong to very strong inhibition zone with a diameter ranging from 10 to 20 mm, and as very strong with a diameter >20 mm. Isolates ALB10-2 and ALP10-3 were classed as having a strong inhibition zone with a diameter ranging from 10 to 20 mm (Figure 5).

Based on optimization time variations

The activity of the symbiont bacterial isolates (AQB10-1, AQB10-2, AQB10-3, AQB10-5, and ALB10-5) was tested on the basis of growth optimization time using three-time variations of each isolate. The AQB10-1 isolate (blue-coloured *Polycarpa aurata* isolate) exhibited the largest barrier zone diameter against *Vibrio cholerae* ATCC 25175 bacteria at the 28th hours, measuring 23.80 mm (Table 3).

The antibacterial activity of the six isolates of the *Polycarpa aurata* symbiont bacteria (AQB10-1, AQB10-2, AQB10-3, AQB10-5, and ALB10-5) provided antibacterial activity against 10

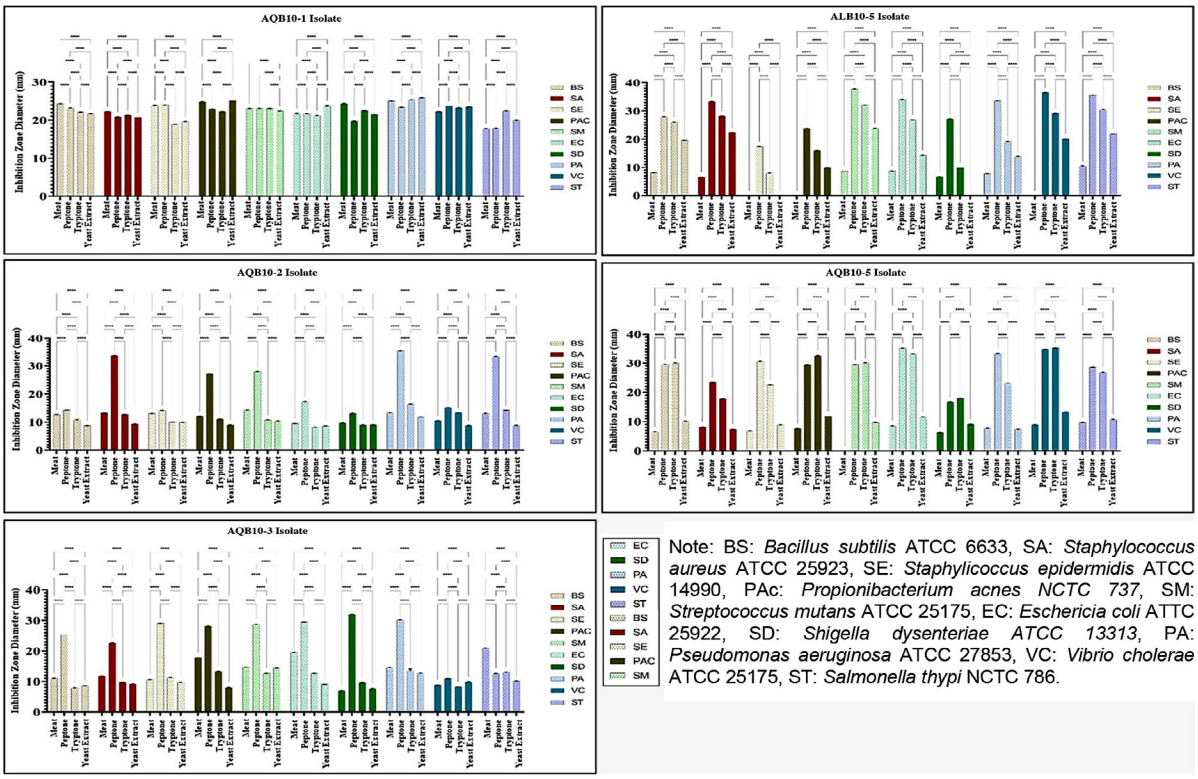

Figure 5. Antibacterial activity diagram based on nutritional variation (meat, peptone, tryptone and yeast extract)

Table 3. Barrier zone diameter results of symbiont bacterial isolate based on time variation

Isolate code	Time	Average barrier zone diameter (mm)									
		BS	SA	SE	Pac	SM	EC	SD	PA	VC	ST
AQB10-1	28’	18.47	17.72	19.51	16.10	15.49	18.36	14.20	16.51	23.80	14.71
	30’	18.31	18.05	18.52	15.99	15.09	17.63	15.95	14.98	23.48	16.46
	32’	18.26	18.26	17.86	15.81	16.88	19.56	17.87	17.00	23.50	17.72
AQB10-2	24’	17.08	17.72	16.32	10.28	11.85	14.10	10.74	14.58	19.18	14.50
	26’	17.35	17.18	16.52	10.66	12.32	14.50	12.55	17.67	19.24	15.30
	28’	15.49	16.87	16.78	11.33	13.13	16.75	12.82	15.19	19.42	14.39
AQB10-3	34’	13.33	10.96	12.94	18.05	16.56	21.20	17.52	12.54	18.20	18.45
	36’	14.04	13.77	14.93	18.93	13.69	21.33	20.16	16.55	17.87	18.53
	38’	14.20	13.10	11.32	18.54	16.44	19.50	19.18	13.68	18.43	18.84
AQB10-5	32’	8.30	8.55	7.38	7.89	8.02	7.95	6.35	7.95	8.37	8.37
	34’	8.32	8.75	7.65	8.03	8.03	8.12	6.87	8.02	9.93	8.78
	36’	8.61	9.65	7.61	8.38	8.21	8.56	7.18	8.27	10.92	9.13
ALB10-5	28’	7.05	8.78	10.06	8.74	8.37	10.84	6.65	7.98	10.32	8.45
	30’	7.09	8.86	7.20	7.69	8.32	10.95	6.75	8.05	10.33	8.43
	32’	8.20	9.55	9.40	8.02	8.39	10.96	6.92	8.19	10.56	8.76

pathogenic bacteria (5 Gram-negative bacteria and 5 Gram-positive bacteria). The diameter of the barrier zone of the symbiont bacterial isolate was classified in the average, strong and very strong barrier area diameter (Table 4). Based on the classification of the activity groups, the diameter zone belonged to the medium category between 5–10 mm, the strong category was 10-20 mm and the very strong category was >20 mm (Kohram et al., 2021).

Molecular identification

Molecular identification of symbiont bacterial isolates (isolates QB10-1, AQB10-2,

Table 4. Results of DNA isolation gene extraction of symbiont bacteria

Sample name	Isolate code	Conc. (ng/µl)	$A_{260/280}$	$A_{260/230}$	Volume (µl)
Coloured-blue *Polycarpa aurata*	AQB10-1	4.8	1.90	0.49	50
	AQB10-2	5.0	1.75	1.04	50
	AQB10-3	2.2	1.39	0.19	50
	AQB10-5	5.5	1.73	0.46	50
	ALB10-5	10.4	1.93	1.93	50

AQB10-3, AQB10-5, and ALB10-5) was performed with DNA gene extraction using the Quick-DNATM Magbead Plus kit (Zymo Research, D4082) (Table 4).

The PCR products were electrophoresed with a 1% TBE agarose gel, a 1 kb DNA sequence (loaded in 2.5 µL), and the nucleotide sequence was sequenced. PCR product with barcoding bacterial species (~1400 bp) using primary 16S (forward: 5-GAGAGTTTGATCCTGG-3; reverse: 5-TACCGCGGCTGCTGGCAC-3) (Figure 6).

The results of phylogenetic analysis of blue-coloured *Polycarpa aurata* symbiosis bacterial isolates revealed code AQB10-1 isolate (bacterial strains of *Bacillus licheniformis*), AQB10-2 isolate (bacterial strains of *Bacillus licheniformis*), AQB10-3 isolate (bacterial strains of *Bacillus licheniformis*), and AQB10-5 isolate (bacterial strains of *Paenibacillus alvei*), and ALB10-5 isolate (bacterial strain *Pseudomonas aeruginosa*) (Figure 7).

In the discovery of 16S rRNA, another method that can be used is the DECIPHER method, which could be used for a search-based approach to 16S RRNA. This method is based on the detection of short fragments that are uncommon in phylogenetic groups where query rows are classified but often found in other phylogenetic groups 47. The 16S RNA is an important gene for determining Gram-negative and Gram-positive bacterial strain based on genetic characteristics of 16S DNA ribosomes (Rocchetti et al., 2020).

The symbiont bacterial isolates of *Polycarpa aurata* obtained bacterial strains of *Bacillus licheniformis* and *Paenibacillus alvei* belong to the *Bacillus sp*. bacteria, namely a group of Gram-positive bacteria that have spore and vegetative forms and serve as probiotics. This bacterial has high stability against atmospheric conditions such as heat, stomach conditions and humidity. This strain of bacteria has antimicrobial, anticancer, antioxidant and vitamin-producing properties (Ariani, et al., 2019). The strain of bacteria *Pseudomonas aeruginosa* is a Gram-negative pathogenic bacterium that can be isolated in small quantities from a variety of hosts, both in soil and in water, but is easily found in almost any environment occupied by animals and humans (Wright et al., 2012). *Pseudomonas aeruginosa* bacterial are Gram-negative bacterial opportunistic pathogenic bacterial and can be isolated in small numbers from various hosts, both in soil, and waters, but these bacterial are easily found in almost all environments inhabited by animals and humans. *Pseudomonas aeruginosa* bacterial are pathogenic and cause death in humans with chronic conditions and are difficult to treat, because the bacterial have antibiotic resistance mechanisms and tend to form multicellular biofilm

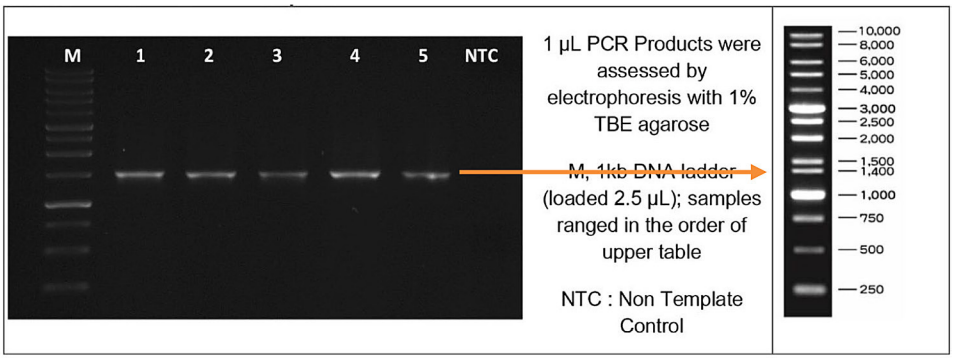

Figure 6. DNA electrophoresis of symbiont bacteria isolate (Note: M=DNA ladder 100 bp, 1=AQB10-1 Isolate, 2= AQB10-2 Isolate, 3= AQB10-3 Isolate, 4= AQB10-5 Isolate, 5= ALB10-5 Isolate)

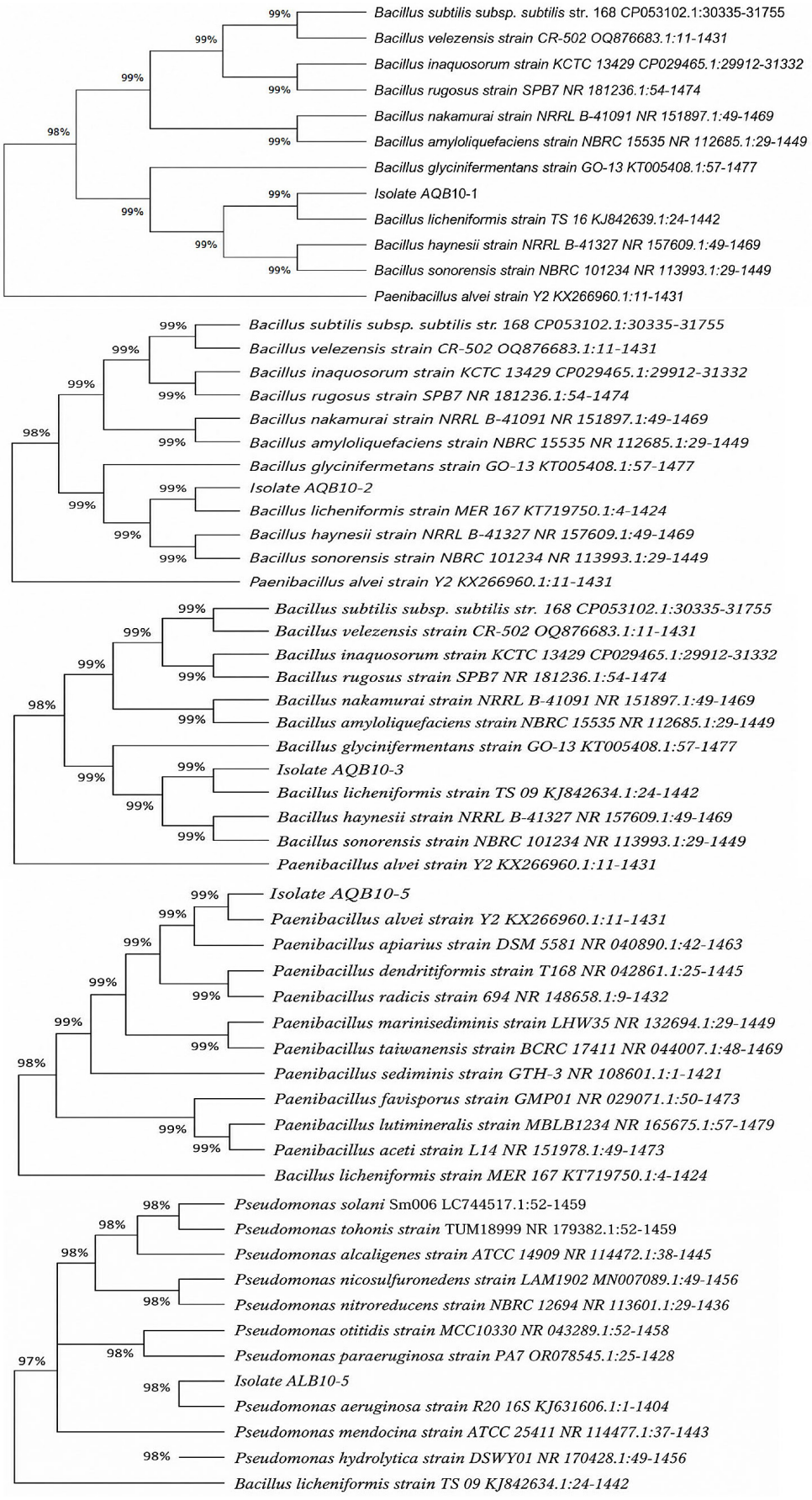

Figure 7. Phylogenetic tree of symbiont bacteria isolate AQB10-1, AQB10-2, AQB10-3, AQB10-5 and ALB10-5

Table 5. Hasil analisis spektroskopi GC-MS ekstrak etil asetat isolate IB10-2

Peak	Real time	Compounds name	Area %	Molecular formula	Molecular weight
2	5.627	1,4-dimethyl-	1.53	C_8H_{10}	106
3	6.017	Butanoic acid	5.43	$C_5H_{10}O_2$	102
11	11.508	Azulene	1.99	$C_{10}H_8$	128
22	19.094	3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	2.59	$C_8H_{12}N_2O_2$	168
24	20.018	Cyclo(L-prolyl-L-valine)	5.03	$C_{10}H_{16}N_2O_2$	196
29	20.988	Hexadecanoic acid	2.92	$C_{24}H_{38}O_4$	390
41	24.843	cis-13-Octadecenoic acid	3.33	$C_{19}H_{36}O_2$	296
60	33.627	1,2-Benzenedicarboxylic acid	20.06	$C_{24}H_{38}O_4$	390
68	38.079	Lycopersen	2.29	$C_{40}H_{66}$	546
72	46.410	Dodec-11-enylbenzene	1.44	$C_{18}H_{28}$	244

(Lee et al., 2019). The main cuse of *Pseudomonas aeruginosa* bacterial infection is an infection of wouds, eyes, and the respiratory system which can spread systematically through the bloodstream (Diggle et al., 2020).

GC-MS analysis

The results of the Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis of isolate AQB10-2 from the symbiont bacteria *Polycarpa aurata* obtained 73 peaks as chemical compounds and 10 peaks were the highest peaks (Table 5, Figure 8).

The symbiont bacteria isolate *Polycarpa aurata* (AQB10-2 isolate code) is the strain *Bacillus licheniformis* bacteria and based on GC-MS spectroscopy analysis of the ethyl acetate extract of symbiont bacteria isolate AQB10-2 contains 10 chemical compounds as higest peack, such as 1,4-dimethyl-, butanoic acid, azulena, 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, Cyclo(L-prolyl-L-valine), Hexadecanoic acid, cis-13-Octadecenoic acid, Lycopersen, Dodec-11-enylbenzene, and 1,2-Benzenedicarboxylic acid (Figure 9).

Gas chromatography–mass spectrometry (GC-MS) is employed to analyze chemical

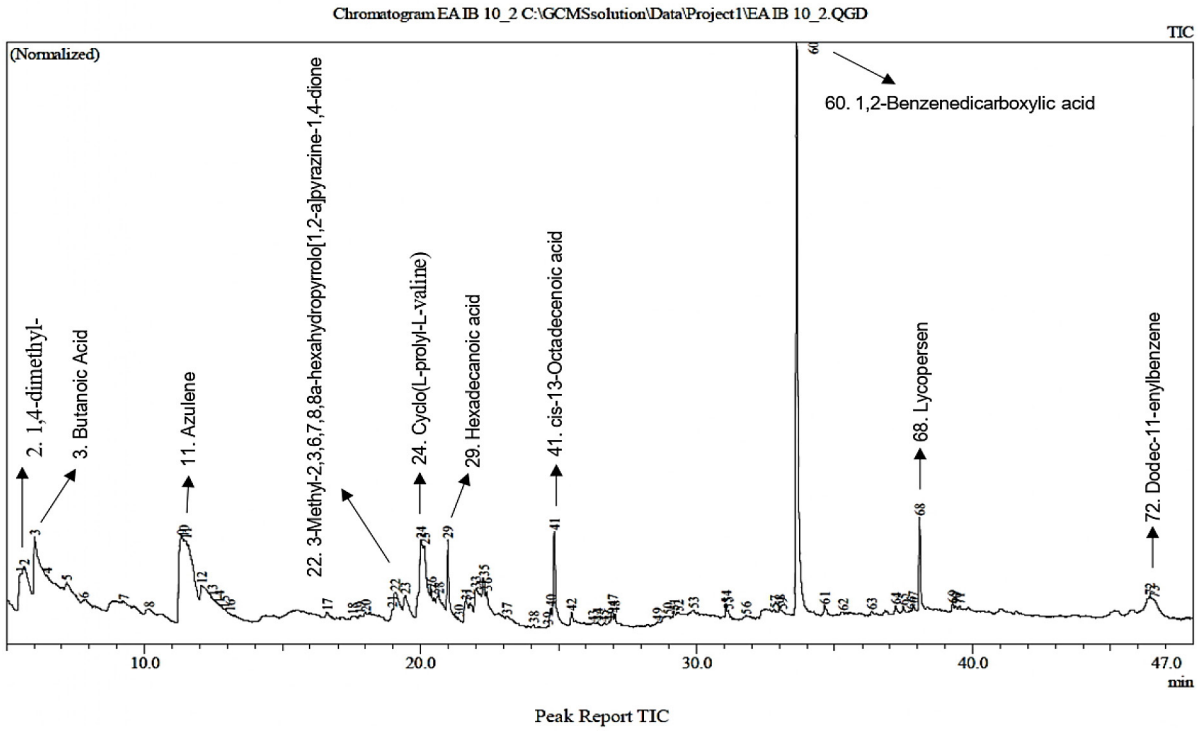

Figure 8. GC-MS spectrum of ethyl acetate extract isolate AQB10-2 symbion bacteria *Polycarpa aurata*

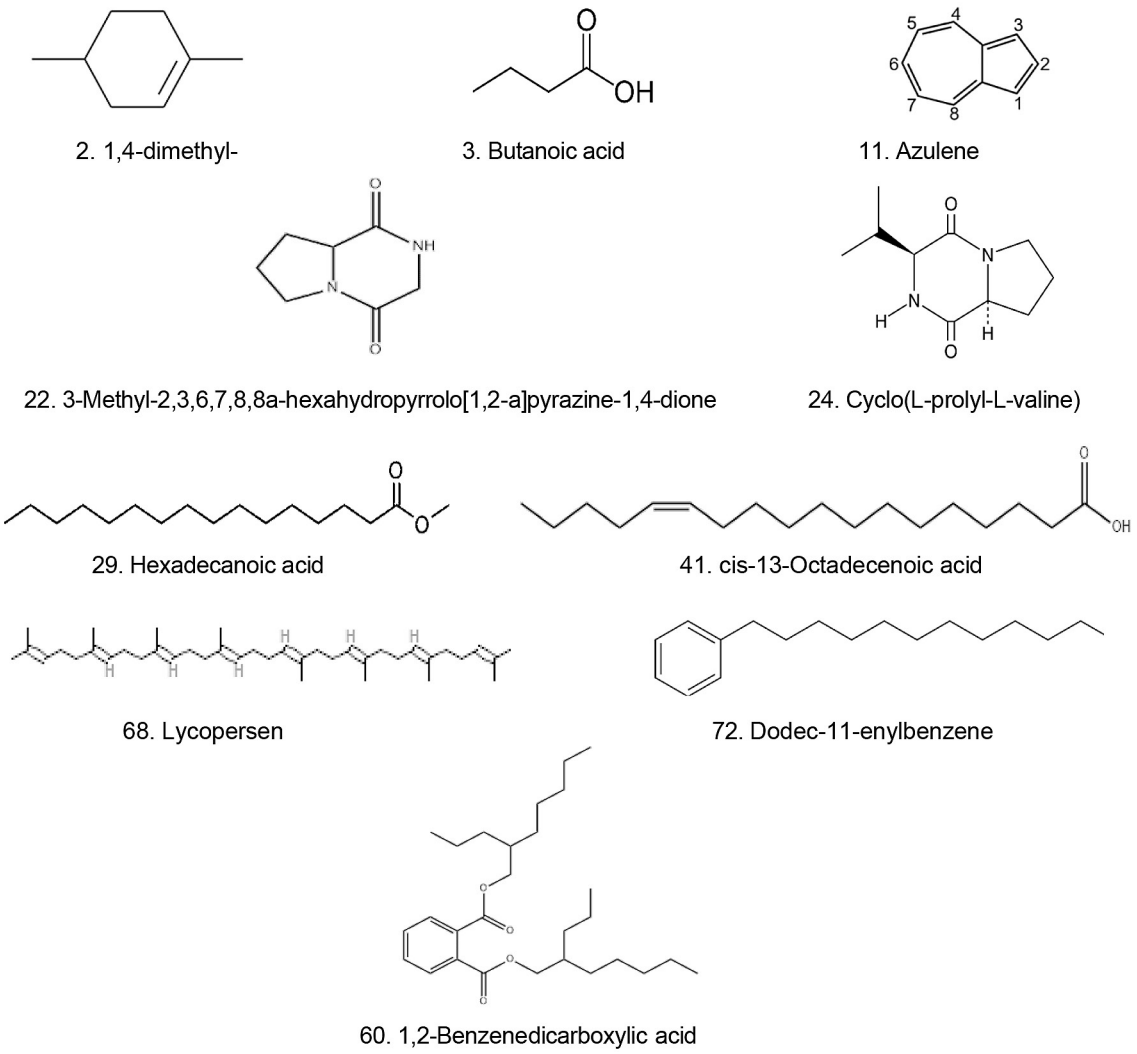

Figure 9. Chemical structure of isolate IB10-2 symbiont bacteria of *Polycarpa aurata*

constituents in medicinal plants, including alkaloids, steroids, and flavonoids. The compound structure of ethyl acetate extract of isolate AQB10-2 in figure 9, such as chemical compound 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a] pyrazine-1,4-dione ($C_8H_{12}N_2O_2$; 168) which provides activity as antimicrobial againts *Saccharomyces cerevisiae ATCC 9763, Candida albicans ATCC 10231, Staphylococcus aureus BAA-2313, Escherichia coli ATCC 25922, Bacillus subtilis ATCC 6633*. Chemical compound of 1,2-Benzenedicarboxylic acid which provides activity as anticancer based on tested in the bioassay NCl yeast anticancer drugs screen (PubChem, 2025). Chemical compound of Cyclo(L-prolyl-L-valine) ($C_{10}H_{16}N_2O_2$;196) which could give great contributions to milky, roasting, fruity, sweetness, and nutty aromas. This is chemical compound of Cyclo(L-prolyl-L-valine) obtained to from endophytic bacteria *Bacillus velezensis* as potential flavor precursors (Li et al., 2025). Chemical compound of lycopersen ($C_{40}H_{66}$; 546) potential as anti-fouling (Ravi et al., 2023).

CONCLUSIONS

The growth optimization revealed that the optimal growth times symbiont bacteria isolates of coloured-blue *Polycarpa aurata* for the production of secondary metabolites, namely AQB10-1 and ALB10-5 isolates = 30 hours, AQB10-2 isolate = 22 hours, AQB10-3 isolate = 34 hours and AQB10-5 isolate = 32 hours with the nutritions used in AQB10-1 and AQB10-2 isolate is yeast extract, AQB10-3 and ALB10-5 isolate is meat, and AQB10-5 isolate is triptone. Molecular analysis using 16S rRNA sequencing identified the most active isolates as strains of *Bacillus licheniformis* (AQB10-1, AQB10-2, AQB10-3

isolate), (AQB10-5 isolate), and *Pseudomonas aeruginosa* strain (ALB10-5 isolate). These strains of *Bacillus licheniformis* from AQB10-2 isolate containing chemical compounds 1,4-dimethyl-, butanoic acid, azulena, 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, cyclo(L-prolyl-L-valine), hexadecanoic acid, cis-13-Octadecenoic acid, lycopersen, dodec-11-enylbenzene, and 1,2-Benzenedicarboxylic acid. The antibacterial activity of ethyl acetate extract of isolate AQB10-2 which provides activity as antibacterial with the largest inhibition zone diameter in against *Vibrio cholerae* ATCC 25175.

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