The potential of Symbiont Ba cteria in *Melo melo* Gastropod found in Pekalongan Waters as a source of MDR antibacterial active compound

ABSTRACTION

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The increasing resistance of many pathogenic microorganisms against antibiotics compounds creates an alarming issue in medical world. This concern has created research opportunities in new antibiotics compounds as alternative options. The gastropod *Melo melo* is a species whose main diet consists of other smaller gastropods. However, Melo-melo does not have any self-defense mechanism save for its thin shell. To protect itself from various pathogenic bacteria existing in its food, *Melo melo* produces secondary metabolites, which are suspected to contain bioactive compounds with antibacterial properties. This fact puts *Melo melo* as a marine biota with potential as a source of new antibacterial compounds. This research aims to discover the potency of symbiont bacteria in the gastropod *Melo melo* with capabilities in producing Multi-drug resistant (MDR) antibacterial compounds. Samples of *Melo melo* are collected from the vicinity of Pekalongan waters, Central Java, Indonesia. This research begins with the isolation of symbiont bacteria, screening of symbiont bacteria with potency in MDR antibacterial activities, antibacterial test, and isolation of MDR clinical pathogenic bacteria. These protocols are then followed by antibacterial sensitivity test, and identification of bacterial species active against MDR by biochemical test and molecular analysis. Molecular analyses are carried out sequentially by DNA extraction, DNA amplification by PCR, and DNA sequencing. Results of 16S rDNA are analyzed using Genetix program and then followed by sequence analysis of the 16S rDNA. In this research, 11 bacteria in *Melo melo* are isolated and there are 4 isolates which show antibacterial activities against MDR bacteria from *Pseudomonas* sp. and *Enterobacter* sp species. Molecular analysis of the most active isolates identifies that isolate PM 26 matches in characteristics with *Brevibacterium celere* strain KMM 3637 with 89% homology match. On the other hand, biochemical test shows that isolate PM 26 is identical with *Bacillus* sp. This research concludes that symbiont bacteria found in *Melo melo* possess antibacterial activities against bacteria of MDR strain.

Keywords: Symbiont bacteria, Gastropod, Antibacteria, *Multi Drug Resistant*

INTRODUCTION

The resistance of microorganisms towards antibiotics continuously evolves from time to time. High rate of infection cases, both endemic and epidemic, is one of the contributing factors attributed to higher microorganism resistance towards certain types of drugs.

The problem above moves researchers into discovering new sources for antibiotics both from land and sea. Indonesian water is one of the wealthiest sources of marine resources, teeming with abundant marine life of various species. Some of these marine species have been known to produce chemical compounds as their defense mechanism against predators. These chemical compounds exhibit bioactivities which show potency in its application in the field of pharmacy. Many studies prove that these compounds show capability in inhibiting bacterial and even cancer cell growth as well as many other useful bioactivities (Handayani, Noviandi, & Dachriyanus, 2008). Pringgenies *et al* (2008) has successfully isolated more than 20 species of Gastropods and identified 10 species whose symbiont bacteria appear to show promise as sources of new antibiotics, such as that found in the gastropod *Conus miles*.

Reports also show that some mollusks from gastropod family produce bioactive compounds. Activities of bioactive compounds produced by these gastropods have been studied as candidates for sources of new drugs, such as *Dolastatin* 10 found in the *Dolabella auricularia* seaslug, which shows antimitotic properties, and is currently under clinical test stage I for medicines to treat liver and breast cancer, tumor, and leukemia (Murniasih & Satari, Isolasi Substansi Bioaktif Antimikroba dari Spons Asal Pulau Pari, 1998).

Increasing resistance of pathogenic microorganisms poses a significant issue in the medical world that various studies and researches have been conducted in an effort to find the alternative antibiotic compounds to tackle this issue. Many approaches has been used in these studies and researches, which among them is discovering alternative antibiotics material from compounds found in both plants and animals (Martini, 2001).

Symbiont bacteria, particularly those found in gastropods, are viable alternative sources of bioactive compounds with activities against MDR bacteria. Osinga *et al.* (2001) in their study discovered that symbiont bacteria produce chemical compounds which serve as antibacterial, antifungal, antifouling agents as well as a self-defense mechanism against predators.

Based on the findings above, this research aims to isolate and to screen symbiont bacteria found in Melo melo which possess the capacity in producing antibacterial compounds against Multi-drug Resistant (MDR) bacteria.

METHODS

Sampling

Samples of gastropods were collected from the waters of Pekalongan, Central Java, Indonesia. Collected samples were then documented and were transported to *Laboratorium Instititut Bahan Obat Alam UNDIP Semarang* in a cool box.

Zobell 2216E Media

The creation of 1 liter of Zobell 2216E soft agar media was achieved by mixing 15 gr of bacto-agar, 2,5 gr of bacto-pepton, 0,5 gr of yeast extract powders and sea water up to 1 liter of volume, after which the mixture was put into homogenization process on a small fire. Autoclave process on 121^{o}C for 15 minutes then followed.

These media were intended as seeding media for seeding of test bacteria and test pathogenioc bacteria, which would be utilized later during the anti-pathogenic bacteria activity test. The creation of 1 liter of Zobell 2216E soft agar media was achieved by mixing 15 gr of bacto-agar, 2,5 gr of bacto-pepton, 0,5 gr of yeast extract powders and sea water up to 1 liter of volume, after which the mixture was put into homogenization process on a small fire. The media were then poured into 50 ml Pyrex vials, all of which are put into autoclave process on 121^{o}C for 15 minutes.

Isolation of symbiont bacteria found in the gastropod Melo melo

Isolation of symbiont bacteria of Melo melo was carried out according to the processes mentioned in Brock dan Madigan (1991) in Radjasa *et. al.,* (2003). Pieces of Melo melo samples were pulvirized in a blender. 1 gram of pulverized sample was mixed with setriilized sea water, and the mixture was then put into a homogenization process. 1 gram of homogenized sample was again mixed with 9 ml of sterilized sea water, and then was put into another homogenization process. This process was repeated until five dilution (10^{-5}) was achieved. 0.1 ml sample of each dilution process was removed to be put into prepared petri dish with Zobell 2216E media. All petri dishes were innoculated in room temperature for 2 x 24 hours.

Screening and purification of bacteria isolates

Screening and purification of the bacteria was carried out using scratch method. Different bacterial colonies are scratched from each petri dish from every dillutied sample (Radjasa, Sabdono, Subagyo, A. S., Trianto, & Djunaedi, 2003). Bacteria isolates were screened based on the difference in color, texture, and physiology.

Taslihan *et al* (2001) explains that bacteria isolate purification can be performed repeatedly until the required result is obtained. Purified isolate must then be kept in a tilted agar media.

Bacteria culture maintenance

Purified culture of bacteria isolated from the gastropod Melo melo as well as of test bacteria is developed in tilted Zobell 2216E agar media in a reaction tube using scratch method (Radjasa, Sabdono, Subagyo, A. S., Trianto, & Djunaedi, 2003). Displacement and maintenance of the cultures in this research was carried out by transferring the culture to newly prepared tilted agar media. The displacement was performed as soon as any of the used agar media exhibited signs of degradation.

Bacterial culture in liquid media

Bacterial culture using liquid media in this research was achieved by putting one oz of cultured bacteria from tilted agar media into a reaction tube filled with 5ml of liquid Zobell 2216E media. The tubes were then put into shaker and then were incubated in room temperature for 1 x 24 hours. The development of cultured bacteria in the media was observed from the change in turbidity of the liquid media.

Antibacterial susceptibility test (Qualitative test)

The qualitative antibacterial test in this research was performed using the overlay method (McKillip, 2001). According to this method, symbiont bacteria isolates are firstly inoculated using dot method onto the surface of Zobell 2216E media in one petri dish, after which it will be incubated in room temperature for 4 x 24 hours. Test bacteria are also culture in liquid Zobell 2216E media, which also undergo a 24 hour incubation period in room temperature. The following process involves removing 1% (from the total volume of soft agar) of each cultured bacteria and then mixed with soft agar, after which the soft agar will be put into media with symbiont bacteria isolate which have gone through an incubation period of 2 x 24 hours. Antibacterial activities will then be determined by observation of the formation of inhibition zone on the periphery of the isolate.

Anti MDR bacteria test (Quantitative test)

The quantitative antibacterial test in this research was performed using Kirby-Bauer diffusion method (Volk & Wheller, 1999). The diffusion method begins by streaking 100 µl of 24-hour old, liquid cultured test bacteria onto Zobell 2216E media in L-shaped spreaders. The mixture is left for several minutes for media impregnation process. Sterile paper discs are placed on the surface on the media, each gently pressed to retain its position. The paper displacement

process is perfomed asceptically by using sterilized pincers. 30 µl 5 x 24 hours incubated microbial broth of the symbiont bacteria is dripped onto the paper discs, after which they will be incubated in room temperature for 2 x 24 hours. The inhibition zones formed are measured with calipers.

Morphological and biochemical tests

Morphological and biochemical (characterization) test are performed to identify physiological and morphological properties of bacteria. Djatmiko *et al.* (2007) listed the test process as the usage of carbon, nitrogen, and macromolecules, salt reaction test, oxidase test, catalase test, pigment formation, lysine decarbosilase, incubation temperature variation growth test, medium pH growth test, salt (NaCl) tolerance, enzyme activity, motility and anaerobic growth.

Molecular analysis of bacteria isolates

Bacteria isolate was dissolved in 1 ml of sterile water in 1.5 ml micro centrifuge tube. The mixture was put into centrifuge with 10,000 – 12,000 rpm rate. The supernatant resulted from this process was then removed. 200 µl of instagene matrix (bacterial residue) is added, and then the mixture was spun in the vortex. Prepared sample was then incubated in 56 ^{o}C of temperature inside a heatblock for 30 minutes, after which it was re-spun using the vortex for another 10 minutes. The tube was put back into incubation in 100 ^{o}C of temperature inside the heatblcok for 8 minutes, after which it was re-spun using the vortex in high speed for the first 10 seconds and in 12,000 rpm for the next 3 minutes. 20 µl of resulting supernatant (DNA genome compound) is used in every 50 µl of PCR reaction process.

***Polymerase Chain Reaction* (PCR)**

The primer used in the DNA amplification process PCR 16S rDNA in this research was Forward (5'-AGAGTTTGATCMTGGCTCAG-3') position 8-27 and 1492 Reverse (5'-TACGGYTACCTTGTTACGACTT-3'). Temperature treaments of the primer were as follows: denaturation at 94 ^{o}C for 2 minutes followed by 30 cycles (*annealing* at 50 ^{o}C for 40 seconds with extension at 72 ^{o}C for 1 miunte, re-denaturation at 94 ^{o}C at 40 seconds), at 42 ^{o}C for 1 munte, 72 ^{o}C for 5 minutes at lastly at 4 ^{o}C ~.

The mixture consisted of $MgCl_2$ 25mM (5 µl), dNTP 2.5 mM (4 µl), 10x buffer (5 µl), LA Taq (0.25 µl), ddH_2O (26.75 µl), template (5 µl), primer 8F (2 µl), primer 1492R (2 µl) up to a total volume of 50 µl, after which all the materials were mixed and poured into the PCR tubes.

The PCR products were put into electrophoresis using 0.8% agarose gel. The agarose gel used in the PCR process were made by mixing 0.32 gr of agarose with 40 ml of TAE 1x, heated until perfectly homogenous, and then simmered down. 1/1000 gel volume of *CyBr Safe DNA* gel stain was added, after which the mixture was poured into gel mold and left to solidify. The resulting gel was then put into an electrophoresis device until it is soaked in TAE 1x compounds, after which loading dye-added PCR products were sorted into the well along with ladder in the first well. The electrophoresis process was carried out for approximately 30 minutes with 100 V of current. Visualization process ensued to conduct observation of DNA bands. DNA bands obtained were then separated and were stored in -20 ^{o}C of temperature.

Purification of PCR 16s rDNA Products

DNA bands obtained from the electrophoresis and PCR phases were purified with using 400 µl of DF *Buffer,* added into the tubes. The tubes were then diluted by heating using a heatblock at 60 ^{o}C for approximately 15 – 20 minutes. Following the dilution process, the products were placed inside DF columns and were spun in a centrifuge for 1 minute at 13,000 rpm. At the end of this process, the supernatant formed was removed and 750 µl of wash buffer was added, after which the products were again spun in a centrifuge for 1 minute at 13,000 rpm. The subsequently formed supernatant was removed, and the DF columns were returned to their initial positions, and were spun under empty condition for 2 minutes at 13,000 rpm. Afterwards, the DF columns were placed in new tubes, followed by the addition of 20 µl of elution buffer into all of the DF columns, before the columns were incubated in 60 ^{o}C for 3 minutes. The incubated columns were then put in centrifuge for 2 minutes at 13,000 rpm. The resulting supernatant from this process was the DNA used in this research, which was then stored in -20 ^{o}C of temperature.

DNA Sequencing

PCR cycle sequence process was carried out prior to DNA sequencing. In the PCR cycle sequence, primers 765R and 1114R were put into purified DNA obtained from the purification process. The resulting product of the PCR cycle sequence was then purified by adding ddH2O equal volume (20 µl), EDTA 125 mM (4 µl), NaOAc 3M (4 µl), absolute ethanol 100 %. After thorough dilution was achieved, the container was wrapped in aluminum foil and was incubated for 15 minutes. The incubated product was then put into centrifuge for 30 minutes at 3000 G. The resulting supernatant formed from this process was removed, and 70% alcohol was added into the remaining product. This initial purified product was put into centrifuge at 3000 G for 15 minutes, after which the supernatant resulting from this process was removed, was flashed, and

its container was cleaned using tissues. The resulting product was then put in a desicator for 7 minutes, and then was diluted by adding 15 µl nucleus free water before it was flashed. The resulting product was then heated inside a heatblock under a temperature of 52 ^{o}C for 8 minutes, and was re-flashed for 20 minutes at 6000 G. The purified product of the PCR Cycle Sequence was put inside a sequencer machine (Automatic Sequencer ABI 3130 XL Genetic Analyzer Applied Biosystem) for automatic analysis.

Analysis of DNA Sequence

BLAST is one of the most commonly used methods in tracing sequence database. Genetic codes obtained from sequencing using ABI 3130XL (DNA sequencing application) are inserted into GENE BANK via the internet using Basic Local Alignment Search Tool (BLAST) database tracing program at the National Center for Biotechnology Information (NCBI), National Institute for Health, USA (Atschul, et al., 1997).

Phylogenetic Analysis

ClustalX computer software was employed in the phylogenetic analysis phase of this research for its capability in matching DNA tree sequences based on DNA codes obtained from earlier phases. Obtained bacteria DNA were cross-referenced with several closely matched bacteria DNA codes in the GENE BANK through BLAST screening process.

The phylogenetic analysis can be made by comparing the 16S rDNA of bacteria PM 26 with 30 of the closely matched bacteria DNA sequence code in the GENE BANK database during the BLAST analysis. The data from the resulting 30 closely matched sequences are processed using ClustalX software to obtain phylogenetic tree and percentage of DNA sequence similarity.

RESULTS

Isolation of symbiont bacteria of Melo melo

11 bacteria isolates were obtained from bacteria isolation process performed on Melo melo (Image 1). These bacteria isolates were distinguished by the difference in color, shape, and texture of each colony.

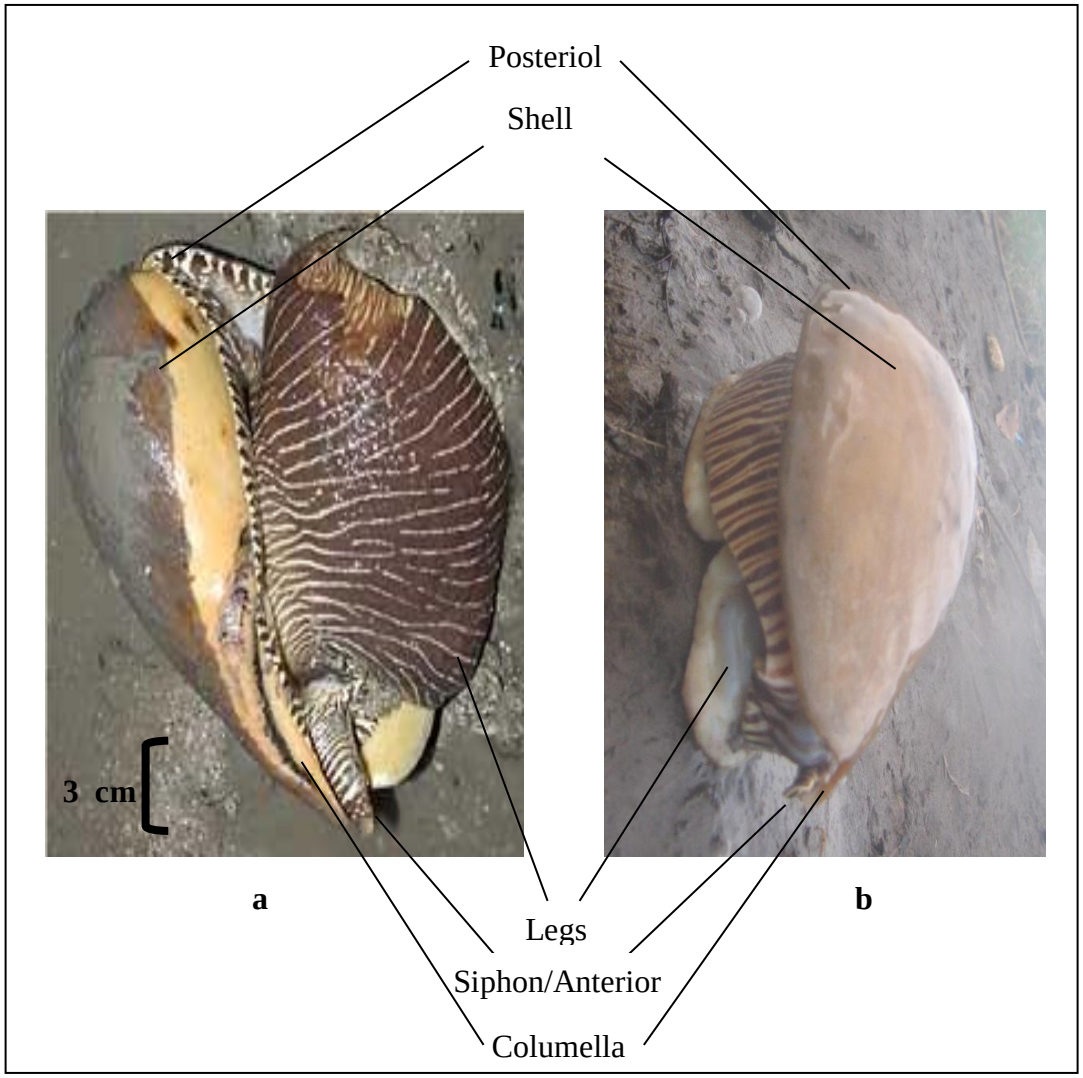

Image 1. *Melo* sp.(http://wildsingapore.com) (a) ; *Melo melo* (research documentation) (b)

Antibacterial susceptibility test (Qualitative test)

From 11 isolates tested against MDR bacterial strain *Klebsiella* sp., *E. coli*, *Coagulase Negatif Staphylococcus* (CNS), *Enterobacter* 5, *Enterobacter* 10 and *Pseudomonas* sp., 4 symbiont bacteria of Melo melo exhibited antibacterial activities capable of hindering the growth of MDR bacteria. The specifics on antibacterial activities of each species is listed in Table 1.

Table 1. Antibacterial susceptibility test results of symbiont bacteria of Melo melo

Isolate Code	Test MDR bacteria					
	Klebsiella	*Pseudomonas*	*E.coli*	CNS	*Enterobacter* 5	*Enterobacter* 10
PM 16	-	-	-	-	-	-
PM 17	-	-	-	-	-	-
PM 18	-	-	-	-	-	-
PM 19	-	-	-	-	-	-
PM 20	-	-	-	-	-	-
PM 21	-	-	-	-	-	-
PM 22	-	-	-	-	-	-
PM 23	-	-	-	-	-	-
PM 24	-	+	-	-	-	-
PM 25	-	+	-	-	-	+
PM 26	-	+	-	-	-	+
PM 27	-	+	-	-	-	-
Number	0	4	0	0	0	2

Legend : + = inhibitive

\- = non-inhibitive

Anti MDR bacteria test (Quantitative)

Kirby-Bauer diffusion test method was used in performing anti MDR bacteria test in this research. The results of this test is displayed in table 2 below.

Tabel 2. Quantitaive test results of antibacterial activities of symbiont bacteria of Melo melo against MDR test pathogenic bacteria

Isolate Code	Diameter if inhibition zone formed (mm)					
	Klebsiella	Pseudomonas	E.Coli	CNS	Enterobacter 5	Enterobacter 10
PM 24	-	9.76 $\pm$ 0.75	-	-	-	-
PM 25	-	8.69 $\pm$ 0.38	-	-	-	9.98 $\pm$ 0.10
PM 26	-	9.15 $\pm$ 0.58	-	-	-	10.26 $\pm$ 0.20
PM 27	-	10.16 $\pm$ 1.13	-	-	-	-

Based on the formation rate and size of inhibition zone, isolate PM 26 showed the best results, and were therefore proceeded to the next test.

Molecular Analysis of Bacteria Isolates

DNA Amplification

The results of DNA amplification process of isolate PM 26 can be seen in Image 2. The image shows that isolate PM 26 displayed a single band of 1500 bp (base pair), according to the DNA marker.

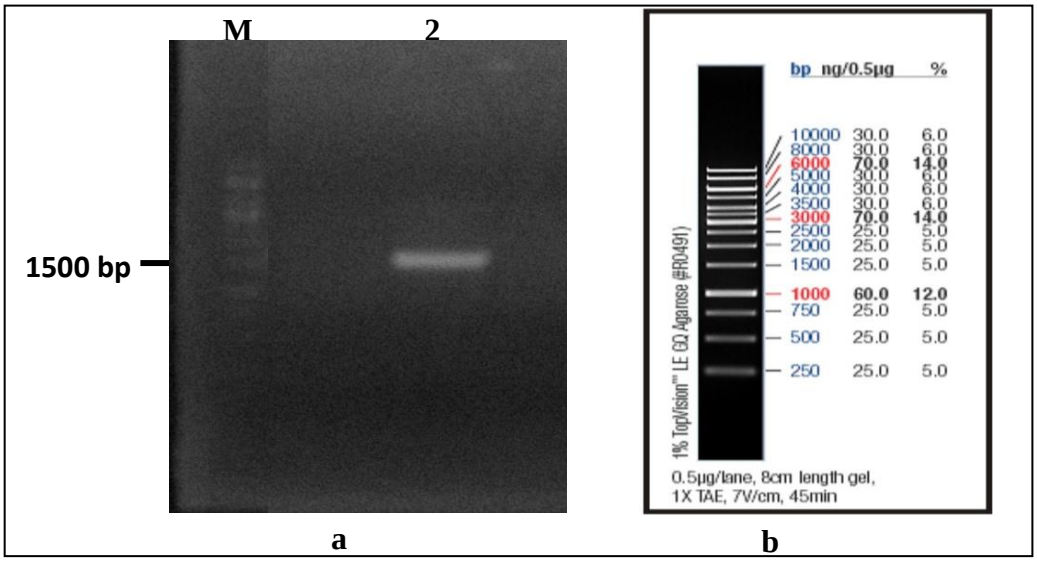

Image 2. Results from genetic amplification 16s rDNA of isolate PM 26 (M: DNA Marker, 2 : Isolate PM 26) (a); DNA Marker (b)

Molecular phylogenetic analysis

Sequencing results of 16S rDNA gene of screened bacteria isolate can be viewed in Table 4, which displays 308 base pairs.

Homology search results of the 16S rDNA of isolate PM 26 with the sequences in the DNA database GENE BANK using the BLAST system can be seen in Image 3. Homology of isolate bacteria matched with bacteria species form the DNA database GENE BANK is displayed in Table 4.

Table 3. Sequence of the Melo melo symbiont bacteria isolate

Isolate	Sequence (308 bp)
PM 26	GACTGTTATGACAGAGTCGCGCCTTCGCCCCGGTGTTCCTCCTGATATCTGC GCATTTCACCGCTCCACCCGGGAATTCCAGAATCCCCTACTGCACTCTAGTC AGCCCCGTACCCACTGCACGCGCAACGTTAAGCGGTTGCGTTTTCCCCAGCA GACGTGACAACCAACCCACAAGCTTCTTTTACGCCCAATAATTTCCAGAGAA CGCGTCGGTACCCCTACGTATTACCGCGGCTGCTGGGGCGTAGTTAGCCGGG TACTTCTTCTGCAGGTACGGACTTTCGCTTCTCCCTGCGAGAAGCGGTTACA CCAGGAGTCATCACCAACTGGTCCTGCAAGGATCTCTCATGGGAAAGCCACT GCGCTACTAGAGTTGACCGTATCATCA

gb|AY228463.1| Brevibacterium celere strain KMM 3637 16S ribosomal RNA gene, partial sequence
Length=1523

Score = 374 bits (202), Expect = 2e-100
Identities = 276/308 (89%), Gaps = 20/308 (6%)
Strand=Plus/Minus

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Query  12   CAGAGTCGCGCCTTCGCC-CCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTCCACC  70
            ||||||| |||||||||| |||||||||||||||||||||||||||||||||||| ||||
Sbjct  715  CAGAGTCCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACC  656

Query  71   CGGGAATTCCAGAATCCCCTACTGCACTCTAGTCAGCCCCGTACCCACTGCACGCGCAAC  130
             || ||||||||| |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  655  AGG-AATTCCAGACTCCCCTACTGCACTCTAGTCAGCCC-GTACCCACTGCACGCGCAAC  598

Query  131  GTTAAGCGGTTGCGTTTTCCCCAGCAGACGTGAC-AACCAACCCACAAGCTTCTTTTACG  189
            |||||||| |||||||| || ||||||||||||| ||||| || || |||| |||| |||
Sbjct  597  GTTAAGCG-TTGCGTTT-CCACAGCAGACGTGACCAACCA-CCTACGAGCT-CTTT-ACG  543

Query  190  CCCAATAATTTCCAGAGAACGCGTCGGTACCCCTACGTATTACCGCGGCTGCTGGGGCGT  249
            |||||||||| || || ||||| ||| ||||| ||||||||||||||||||||||  |||
Sbjct  542  CCCAATAATT-CCGGACAACGC-TCG-TACCC-TACGTATTACCGCGGCTGCTGGCACGT  487

Query  250  AGTTAGCCGGGTACTTCTTCTGCAGGTACGG--ACTTTCGCTTCT-CCCTGC-GAGAAGC  305
            |||||||||| |||||||||||||||||| |  |||||||||||| |||||| || ||||
Sbjct  486  AGTTAGCCGG-TACTTCTTCTGCAGGTACCGTCACTTTCGCTTCTTCCCTGCTGA-AAGC  429

Query  306  GGTT-ACA  312
            |||| |||
Sbjct  428  GGTTTACA  421
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Image 3. Search results of the sequence homology of 16S rDNA of Isolate PM 26 with sequences in DNA Database GENE BANK using the BLAST system.

Tabel 4. BLAST Homology of symbiont bacteria isolate of Melo melo

Isolate	Relative similarity	Homology (%)	Access No
PM 26	*Brevibacterium celere* strain KMM 3637	89	AY228463.1

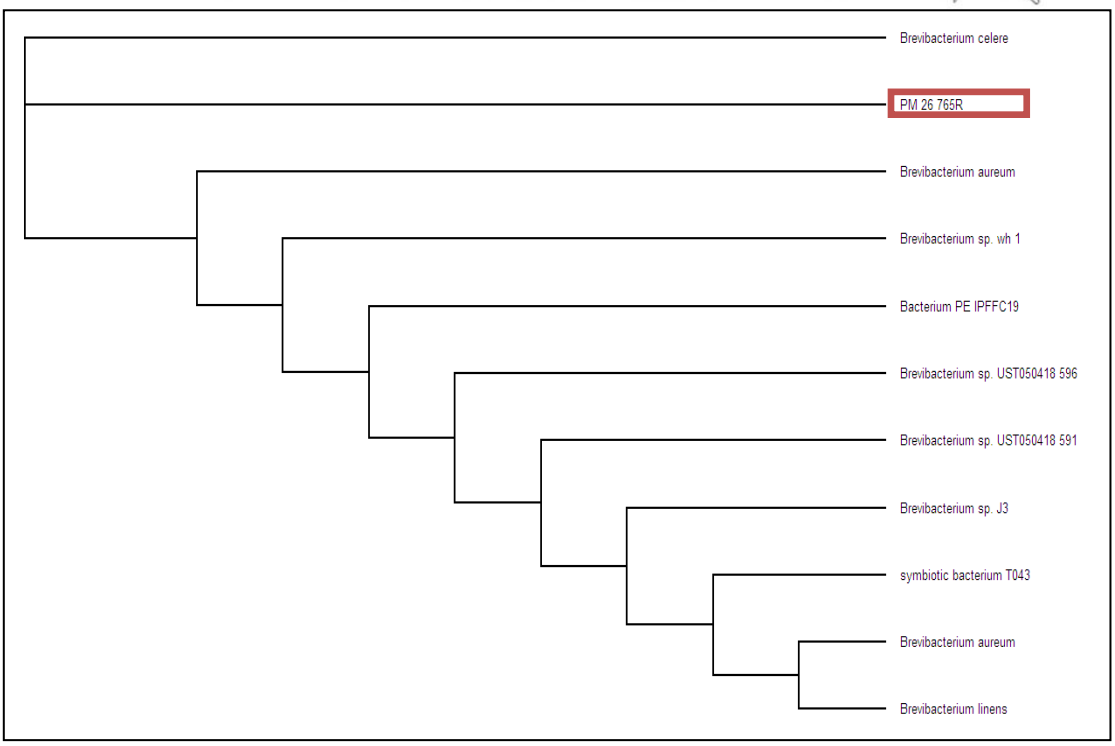

Image 4. Dendogram bacteria isolate PM 26of

Morphological and Biochemical Tests

Bacteria isolate PM 26 was put through morphological and biological tests to identify characteristics, genus and species of the bacteria present in the isolate.

Table 5. Characterization tets of bacteria isolate PM 26

Uji Biokimia	PM 26
Gram	+
Bentuk	Rod
Acid Fast	-
Spore	+
Position and form of spore	TYX
Cell length > 3 µm	-
Motility	+
Aerobic	+
Anaerobic	+[F]
	+

Catalase	
Oxidase	-
Glucose acid	+
Carbohydrate (OF)	F
Growth with 10% NaCl	+
Nitrate reduce	+
Indol	-
ONPG	-
VP	-
Hydrolysis of:	
- Starch	-
- Urea	-
- Casein	+
Acid from AAS medium :	
- Glucose	+
- Celibiose	-
- Galactose	-
- Rafinose	-
- Salicin	-
- Xylose	-
Growth on 50^0	+
Utilization of Citrate	+

Legends:

- TYX : Terminal – Round – Oval (ellipoidal)
- $+^F$: Anaerobic facultative
- F : Fermentative

From the results of charaterization tests above, the symbiont bacteria found in the gastropod Melo melo is classified into the genus Bacillus sp (Cowan, S.T and Steel, K.J., 1993).

Discussion

Out of 11 symbiont bacteria of Melo melo going through screening process, four species exhibit antibacterial activities against the test bacteria, as seen from observation of inhibition zone formed which hinder the growth of test MDR bacteria (Table 4). The growth of MDR bacteria is prevented by the existence of secondary metabolites in symbiont bacteria isolates. According to Murniasih (2005), secondary metabolites are produced by organisms as a reponse towards its environmental influence. Murniasih (2005) added that, other than as a self defense mechanism, production of secondary metabolites serves the function of interaction media with other organisms, as a preventive measure against infection from other microorganism, and as media in reproduction process. Secondary metabolites produced by bacteria associated with marine ecosystems possess various antibacterial substances with roles specific to each of their respective host. Antibiotics substances produced by these symbiont bacteria play a vital role in ecological competition.

When two species compete in an ecosystem, then there will also be a competition between the two for space and nutrients, which leads to variation of growth strategies. Manitto (1981) explains that the aforementioned phenomenon can be attributed to varying self defense mechanisms found in bacteria. Chemical substances often prove to be important for a species in its effort to preserve itself, and in competition against other microrganisms. Chemical substances produced by microoganisms has also been used to eradicate competitors, harming their growth and development. The variation in chemical interaction between organisms affects the production and secretion of antimicrobial seconday metabolites.

Results of quantitaive test shows that isolates PM 25 amd PM 26 display antibacterial activities againts two MDR species, namely *Pseudomonas* sp. and *Enterobacter* sp. On the other hand, isolates PM 24 and PM 27 are found to be potent only in hampering the growth of MDR bacteria *Pseudomonas* sp (table 5). The difference in the antibacterial activities exhibited in different isolates indicates the difference in the chemical contents in each of the repective isolates. Brocks and Madigan (1991) elaborate that the size of inhibition zone formed in the periphery of the paper disc depends highly on the properties of the antibacterial compounds produced by the respective species. Hence, the molecule diffusion rate of antibacetrial compounds in agar media is affected by the existing molecule in and its action against the agar media. The more size and the molecoles of a compound have compared to that of the agar's, the greater their diffusion rate will be. However, the difussion rate several antibacterial compound molecules have been known to be stalled by its agar media.

Dananjoyo (2009) explains that antibacterial compounds produced by microorganisms selectively impede the growth of other bacteria, making the antibacterial activities of the compounds mutually exclusive for specific target species without affecting other bacteria in the same environment. According to Levinson (2004), antibacterial compounds can be classified into

two spectrums based on the number of species with which the compounds react; the broad spectrum antibacterial compounds which are potent against a large number of species of pathogenic bacteria, and the narrow spectrum compounds which specifically target a limited number of specific bacteria species.

The results of quantitative tests conducted in this research, isolates displays the most significant antibacterial properties and formation of inhibition zones against their respective test bacteria were selected for further testing. Based on the screening results, isolate PM 26 was chosen for its high levels of antibacterial activities against 2 of the test bacetria namely *Pseudomonas* sp., and *Enterobacteria* sp. strain 10 with diameter of inhibition zones formed 9.15 mm dan 10.26 mm respectively (Table 5).

DNA amplification with PCR 16s rDNA of isolate PM 26 shows that the isolate produces a single band of 1500 bp, as displayed by the DNA marker as comparison (Image 10). The size of this band is expected from genes of 16s rDNA bacteria with 1500 base pair lengths. Sabdono (2001) elaborates that DNA amplification of single-banded often requires the usage of specific primer to amplify the gene into 16S rDNA. The amplification of 16S rDNA has become a standard in stidying phylogenetics and diversity of marine organisms (Radjasa *et al.*, 2004).

After the sequencing of isolate PM 26 had yielded results, DNA database search was performed with GENE BANK using BLAST method through National Center for Biotechnology Information, National Institute for Health, USA website (http://www.ncbi.nlm.nih.gov). Search results identifies that the bacteria in isolate PM 26 homologically has 89% similarity with the bacteria *Brevibacterium celere* strain KMM 3637. Categorizes similarity levels of taxonomical homology from top to bottom with 97-100% being accurate identification at the species level, 93-96 % being accurate identification at the genus level, and 86%-92% being accurate identification at related organisms level.

The bacterium *Brevibacterium celere* is taxonomically classified into the phylum of Actinobacteria, the class of Actinobacteria, the sub-class of Actinobacteridae, the order of Actinomycetales, the sub-order of Micrococcineae, the family of Brevibacteriaceae, the genus of Brevibacterium and the species of *Brevibacterium celere*.

Among the characteristics of *Brevibacterium celere* are gram positive, non-motile, sporeless, light yellowish in color, salt tolerant (thrives in 0-15% NaCl), naturally grow in 12-24 °C with optimal growth rate in 8.5 – 9 pH value, alkali tolerant, negative catalase class, positive oxidase, and aerobic. Gelatin, laminarin, and alginate are hydrolisized, whereas casein and starch are not. Nitrate is not reduced to nitric, and negative value in urease and pyrazinamidase test (Ivanova, et al., 2004).

The bacterium *Brevibacterium celere* can be isolated by degradation of the thallus of the brown algae *Fucus evanescens*. This species of bacteria has been identified with 97% similarity to the bacterium *Fucus casei*.

Biochemical test of the characteristics of symbiont bacteria found in Melo melo shows that the bacteria take a rod (bacillus) shape, gram positive, positive catalase, positive motility, facultative anaerobic and aerobic, negative oxidase, glucose acid positive, carbon (OF) fermentative, grows in 10% of salt concentration, indol negative and glucose positive (Table 6). According to Cowan, S.T and Steel, K.J. (1993) bacteria with characteristics mentioned above fall into the category of the genus *Bacillus* sp. Gram positive bacteria with rod shape and positive catalase can be identified with the genus *Bacillus* sp and *Brevibacterium* sp. The two genuses can be distinguished based on the presence of spores. *Bacillus* sp has been known to show thick spore whereas *Brevibacterium* sp displays the opposite characteristic.

The seemingly multiple interpretations of identification based on the results of both biochemical and biomolecular tests results are expected, since the isolate show low levels of homology in 89%. One of the factors which may have affected the accuracy of the results is the ambiguity in DNA sequence from automated sequencer results, making the produced DNA base sequence difficult and too short to read.

CONCLUSIONS

1. 11 species of bacteria are identified as the symbiont bacteria in the gastropod Melo melo.
2. 2. Only 4 isolates show antibacterial activities against MDR test bacteria, identified as *Pseudomonas* sp. dan *Enterobacter* sp.
3. Based on the biomolecular test results, the isolate containing symbiont bacteria of Melo melo is identified with the bacterium species *Brevibacterium celere*.

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