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Characterization of a yellow pigmented coral-associated bacterium exhibiting anti-Bacterial Activity Against Multidrug Resistant (MDR) Organism

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ABSTRACT

Multidrug resistant (MDR) infections have been a world health issue for many decades, and therefore exploration of new antibiotics to overcome this issue is urgently needed. Regarding antimicrobial properties in the coral holobiont, coral-associated bacteria are suggested as potential producer of new antibacterial agents against MDR infections. The purposes of this study include isolation and identification of pigmented coral-associated bacteria, preparation of pigmented extract and evaluation of its antibacterial property, as well as the characterization of the pigmented extract using thin layer chromatography (TLC). Nine bacteria candidates were isolated from an unidentified stony coral collected from Tanjung Gelam, Karimunjawa National Park, Jepara Region, Central Java Province, Indonesia. Bacterial pigment and other metabolites were extracted using 1-butanol, ethyl acetate and acetone. Silica gel-based TLC was applied to detect β-carotene and characterize the bacterial crude extracts. Antibacterial activity was evaluated against the extended spectrum β-lactamase (ESBL) *E. coli*, *Klebsiella pneumoniae*, and MRSA (methicillin-resistant *Staphylococcus aureus*). A yellow pigmented bacterium was isolated and identified as *Vibrio* owensii TNKJ.CR.24-7. Nonribosomal peptide-synthetase Peptide-Synthetase NRPS genes fragments were detected. All bacterial extracts from 1-butanol, ethyl acetate and acetone contained the yellow pigment that was identified as β-carotene. Only the crude extract from ethyl acetate inhibited all of MDR bacteria. TLC chromatogram showed that there were 7 bands from 1-butanol extract and 6 bands from ethyl acetate extract. However, crude extract from acetone was not well separated using silica-based TLC. Bands at R_f 0.64 and 0.81 were found both in 1-butanol and ethyl acetate extracts.

Introduction

Infection by multidrug resistant (MDR) bacteria is one of the major health issues in the world attributable to their resistance to commercial antibiotics (van Duin and Paterson, 2017; WHO, 2018). The Center for Diseases Control and Prevention (CDC) identified 18 top MDR bacteria due to their threats in the United States. Several pathogenic bacteria such as the extended spectrum β-lactamase (ESBL) Enterobacteriaceae including *Escherichia* and *Klebsiella*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* are considered as MDR bacteria which cause serious threats (CDC, 2018). To overcome this issue, exploration of new antibiotic candidates is urgently needed.

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The coral holobiont has unique and elusive competitive interaction among associated microorganisms that induces antimicrobial production involved with their competitive behavior inside of their hosts (Ahila et al., 2017; Pereira et al., 2017; van de Water et al., 2017). This bioactivity can be applicable not only to inhibit coral pathogen but also human pathogenic bacteria. Raina et al. (2016) reported that coral-associated *Pseudovibrio* sp. produced tropodithietic acid, an antimicrobial compound which inhibited coral pathogenic bacteria. Moreover, several studies applied coral-associated bacteria to inhibit human pathogenic bacteria such as *Bacillus subtilis*, *Enterobacter cloacae*, *E. coli* and *Staphylococcus epidermidis* (El-Ahwany et al., 2013; Pham et al., 2015; Ahila et al., 2017). Studies of antimicrobial activity from marine bacteria have led to an interesting assumption that most of the pigmented bacteria are able to produce antimicrobial compounds to inhibit human pathogenic bacteria (Choi et al., 2015; Offret et al., 2016; Kalinovskaya et al., 2017).

Ayuningrum et al. (2017) isolated orange-pigmented *Pseudoalteromonas flavipulchra* from an Indonesian stony coral. This bacterium inhibited several MDR bacteria such as *Acinetobacter baumannii*, *Enterobacter aerogenes*, *E. coli*, MRSA and *Staphylococcus hemolyticus*. In addition, several compounds such as marinoazepinone B; marinoquinoline I; marinopyrazinone B; marinoquinolines A, C and D; diisobutyl-, dibutyl- and bis(2-ethylhexyl) phthalates were reported as potential antimicrobial candidates produced by marine pigmented bacteria (Choi et al., 2015; Romanenko et al., 2015; Kalinovskaya et al., 2017). Due to their antibacterial properties, pigmented coral-associated bacteria are suggested as potential candidates to overcome multidrug resistant (MDR) infections. This study was designed to isolate and identify pigmented coral-associated bacteria, to obtain their pigment extracts and evaluate their antibacterial property, and to characterize the pigmented extracts using thin layer chromatography (TLC).

Methodology

Sample collection

An unidentified coral was collected from Tanjung Gelam, Karimunjawa National Park, Jepara Region, Central Java Province, Indonesia (Fig. 1) with permission number 1096/T.34/TU/SIMAKSI/7/2017 by SCUBA diving at 15 m in depth. Approximately 2 cm fragment of the coral was collected using pliers and then kept in a sterilized zip lock for bacterial isolation.

Bacterial isolation and identification

The coral sample and 10 mL of sterilized natural seawater were homogenized with a mortar under aseptic condition, and then diluted until 10^{-6} in sterilized marine water. The dilution of 10^{-4}, 10^{-5} and 10^{-6} were spread onto marine agar (MA) consisting of marine broth (MB) from Difco™ and agar, and then the agar plates were incubated at room temperature (27 °C) for 5×24 h. Everyday the isolation media were observed to obtain the pigmented strain. Then, the pigmented strain was purified for the further investigations. One selected isolate was identified by Gram staining and molecular identification which was carried out by Ayuningrum et al. (2017). Primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3) and 1492 R (5′- GGT TAC CTT GTT ACG ACT T-3′) were applied for bacterial 16S rDNA amplification and sequenced by 1st Base Laboratories Sdn Bhd, Malaysia. Basic Local Alignment Search Tool (BLAST) was carried out to compare the sample sequence to GenBank to obtain bacterial homologues. Phylogenetic tree was reconstructed using MEGA 7 software package with neighbor-joining method and 1000 number of bootstrap replication (Tamura et al., 2011).

NRPS and PKS-I genes detection

Non-ribosomal peptide synthetase (NRPS) was detected using the primers A2gam (5′-AAG GCN GGC GSB GCS TAY STG CC-3′) and A3gam (5′-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3′), while type I polyketide synthase (PKS-I) was detected using the primers K1 (5′-TSA AGT CSA ACA TCG GBCA-3′) and M6R (5′-CGC AGG TTS CSG TAC CAG TA-3) (Ayuso-Sacido and Genilloud, 2005; Radjasa et al., 2007). The presence of these genes were confirmed using electrophoresis.

Bacterial cultivation and pigment extraction

Bacteria were cultivated in marine broth (MB) from Difco™. Seed culture was prepared by adding 1 cm^2 of agar culture of candidate isolates into 50 mL of MB and then cultured at room temperature (27 °C) for 3 days on a shaker (120 r.p.m.). Production cultures were prepared by adding 1% of the seed culture to 100 mL of MB in Erlenmeyer flasks and then cultured for 6 days at room temperature (27 °C) on a shaker (120 r.p.m.).

Three different organic solvents (1-butanol, ethyl acetate and acetone) were added into broth culture with single extraction method in a ratio of 1:2 (broth: solvent v/v). The mixture was agitated using a shaker (120 r.p.m.) for 1 h. The organic layer was separated from the broth using a separatory funnel and was evaporated to obtain the pigment extract. The crude pigment extracts were dried using rotary vacuum centrifugation for the next experiments.

Antibacterial assay

ESBL *E. coli*, *Klebsiella pneumoniae*, and MRSA strain MDR are from the clinical pathogenic collection in General Hospital Dr. Kariadi, Semarang, Central Java. Gram-negative ESBL *E. coli* and *K. pneumoniae* were refreshed onto MacConkey Agar (HiMedia) while MRSA onto Nutrient Agar (NA, HiMedia) for 24 h at 32 °C before bioassay. Pigment extracts from three different solvents were diluted in dimethyl sulfoxide (DMSO) at the concentrations of 1000, 500, 250 and 125 μg/mL while amoxicillin 10 μg/disc was used as a positive control and DMSO without extract as a negative control. The presence of clear zone indicated the antibacterial activity of the pigment extract. The antibacterial assay was carried out according to CLSI (2016).

Pigment extract characterization

As the bacterial pigment had bright yellow to dark orange color, β-carotene (Wako, Japan) was used as a pigment standard. Crude extracts and β-carotene were loaded onto a TLC silica gel 60 F_{254} plate (Merck) as the stationary phase. For mobile phase, combination of acetone and hexane in a ratio of 6:4 v/v was employed. The same color and R_f of standard sample and bacterial pigment extract indicated the positive result in this analysis.

In addition, other compounds contained in the pigment extracts were fractionated using TLC method. Fractionation was performed using dichloromethane, ethyl acetate, and methanol as mobile phase in a ratio of 5:3:2. TLC spots were visualized by illumination of 356 nm UV lamp.

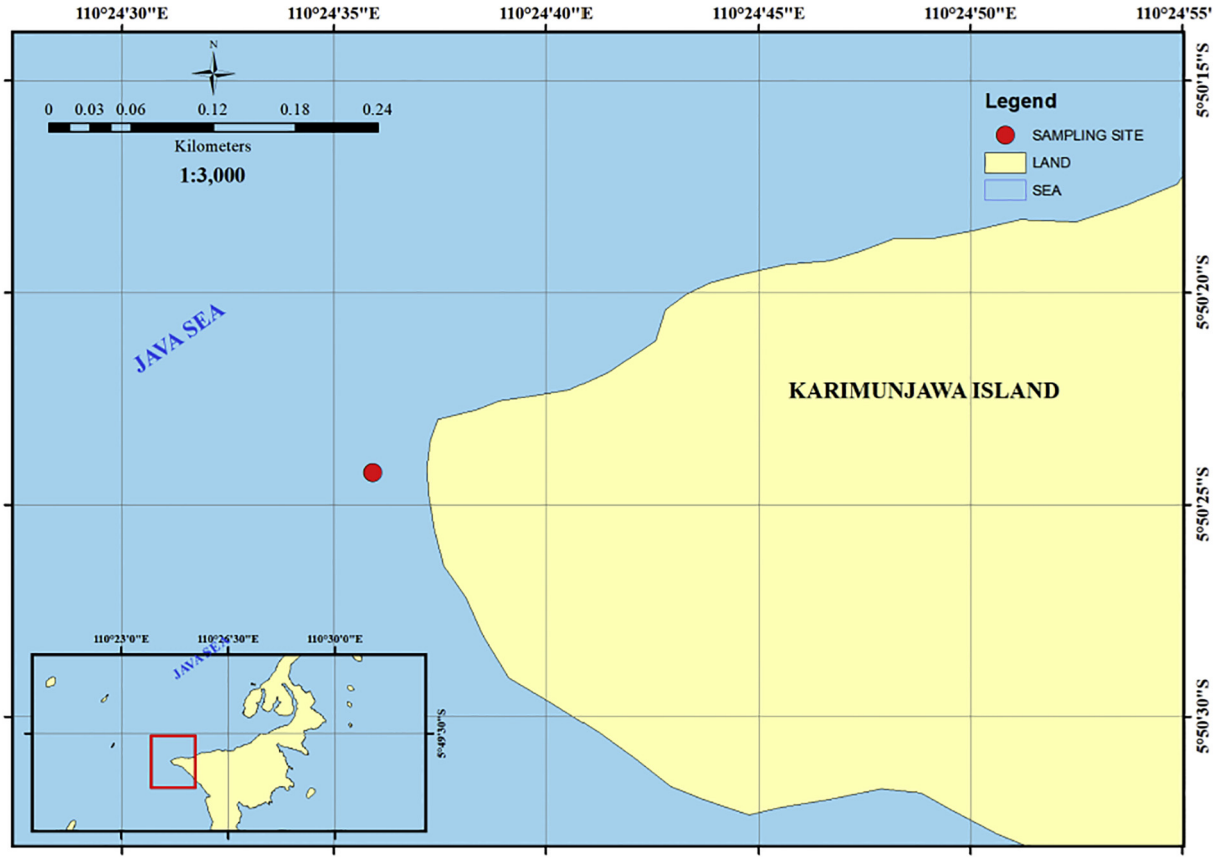

Fig. 1. Sampling site in Tajung Gelam, Karimunjawa National Park, Central Java, Indonesia.

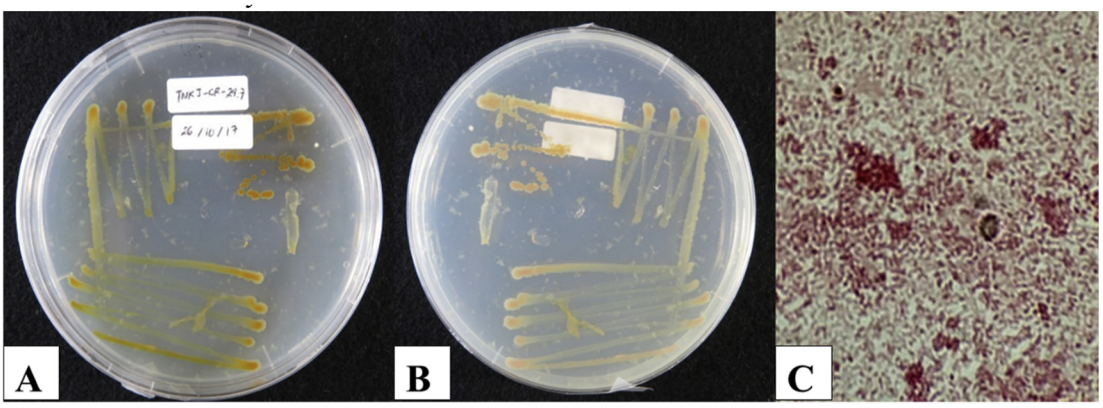

Fig. 2. (A and B) Isolate TNKJ.CR.24-7 on marine agar (MA) after 26 h of incubation at room temperature (27 °C). (A and B: bacterial colony, C: Gram staining).

Result

Isolation of coral-associated bacteria gave 9 bacterial isolates from dilution 10^{-4} to 10^{-6}, however only one isolate showed color production. An isolate TNKJ.CR.24-7 only existed at dilution 10^{-4} on marine agar. Fig. 2 shows that this isolate produces yellow pigment in the colony after 26 h of incubation on agar. Coral-associated TNKJ.CR.24-7 was selected as a prospective candidate for further analysis.

Bacterial characterization was done using Gram staining, molecular identification, and NRPS and PKS-I genes detection. The colony was nonluminecent, nonswarming, smooth and round. Based on the Gram staining test, this isolate was assigned to Gram-negative bacteria. Advance identification was carried out through molecular approach, by which the isolate TNKJ-CR.24-7 was identified as *Vibrio owensii* with 99% similarity to *V. owensii* MG896198.1. This isolate has been registered in Gen-Bank with accession number MH488980.1. The phylogenetic tree of this isolate is shown in Fig. 3. Bacterium *V. owensii* TNKJ-CR.24-7 was confirmed to possess NRPS gene cluster, while PKS-I gene was not detected by using K1 and M6R primers (Fig. 4).

V. owensii TNKJ.CR.24-7 produced yellow pigment after 5 days of cultivation in MB medium, which was readily recognized by the color change to yellow. The yield of culture extracts is presented in Table 1.

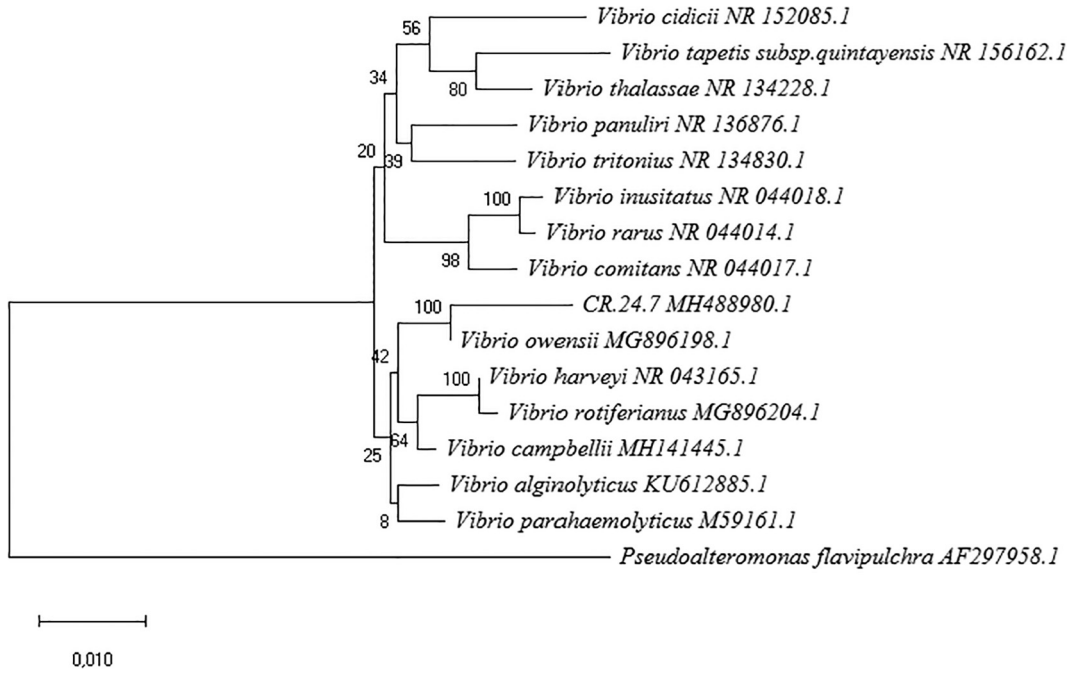

Fig. 3. Phylogenetic tree of TNKJ.CR.24-7 according to 16S rRNA region.

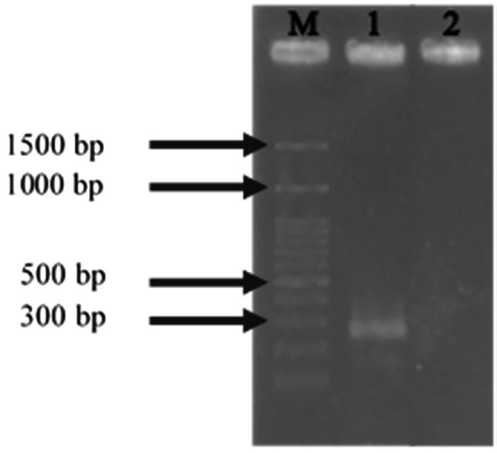

Fig. 4. Detection of secondary metabolite biosynthetic genes (M: marker, 1: NRPS, 2: PKS-I).

Table 1
Bacterial pigment crude extract fom different organic solvents.

Organic solvent	Yield (% w/v)
1-butanol	1.14 ± 0.01
Ethyl acetate	0.13 ± 0.05
Acetone	0.22 ± 0.08

(Data were average ± standard deviation).

Extraction with 1-butanol gave the highest yield of crude extract with the amount of 1.14 ± 0.01%, then followed by acetone (0.22 ± 0.08%) and ethyl acetate (0.13 ± 0.05%). Furthermore, all of the bacterial crude extracts contained carotenoid pigments displaying the same characteristics with the β-carotene standard such as yellow to orange color and R_f value of 0.94 (Fig. 5).

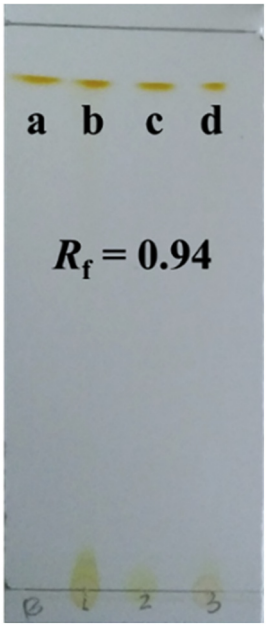

Fig. 5. TLC chromatogram of carotenoid detection in bacterial pigment extract a) β-carotene standard, b) 1-butanol extract, c) ethyl acetate extract, d) acetone extract.

The characteristics of other compounds from the bacterial pigment extracts are shown in Fig. 6 and Table 2. The compounds in bacterial extracts from 1-butanol and ethyl acetate were well fractionated in silica gel-based TLC, whereas the bacterial extract from acetone solvent was not well separated displaying the smeared bands (Fig. 6). Table 2 and Fig. 6 show that two bands with the same color and R_f values (0.64 and 0.81) were detected in bacterial extracts from 1-butanol and ethyl acetate.

In the antibacterial testing using paper-disc method, bacterial pigment extracts from 1-butanol and acetone did not inhibit any MDR bacteria, while the extract from ethyl acetate showed inhibi-

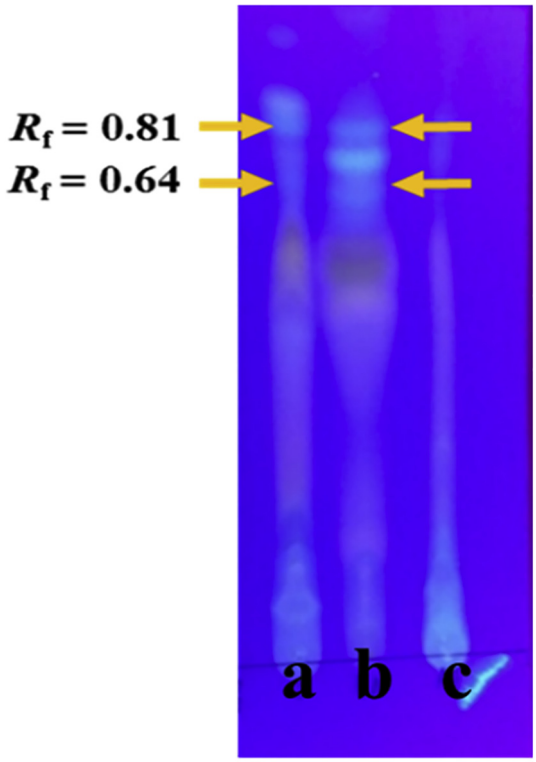

Fig. 6. TLC chromatogram of bacterial crude extracts from different solvents a) 1-butanol, b) ethyl acetate, c) acetone under UV illumination at 356 nm.

Table 2
Retention factors of bacterial crude extracts from different solvents.

Extract	Retention factor (R_f)
From 1-butanol	0.10; 0.25; 0.64*; 0.66; 0.81†; 0.84; 0.93
From ethyl acetate	0.20; 0.58; 0.64*; 0.64; 0.77; 0.81†
From acetone	Not well separated

(Same notation indicates the same R_f values).

Table 3
Antibacterial activity of pigment extract from ethyl acetate solvent against MDR bacteria.

Bacteria	Concentrations (μg/mL)	Inhibition zone (mm)
ESBL *E. coli*	DMSO (Negative control)	0.00 ± 0.00[a]
	125	1.25 ± 1.76[b]
	250	2.05 ± 0.21[b]
	500	4.30 ± 1.31[d]
	1000	5.11 ± 0.02[d]
	Amoxicillin 10 μg/disc	3.75 ± 0.21[c]
K. pneumoniae	DMSO (Negative control)	0.00 ± 0.00[a]
	125	0.00 ± 0.00[a]
	250	2.15 ± 0.15[b]
	500	4.05 ± 0.07[c]
	1000	4.25 ± 0.07[d]
	Amoxicillin 10 μg/disc	6.30 ± 1.27[e]
MRSA	DMSO (Negative control)	0.00 ± 0.00[a]
	125	1.40 ± 0.42[b]
	250	2.35 ± 1.35[b]
	500	4.35 ± 1.06[c]
	1000	5.45 ± 1.65[c]
	Amoxicillin 10 μg/disc	4.10 ± 0.84[c]

(Data were average ± standard deviation. Different notations indicate significant difference at $P < 0.05$).

tion against ESBL *E. coli*, *K. pneumoniae*, and MRSA. The inhibition zones of bacterial pigment extract from ethyl acetate solvent are summarized in Table 3.

Pigment extract from ethyl acetate displayed weak activity against ESBL *E. coli* and MRSA at concentration 125 μg/mL with values 1.25 ± 1.76 mm and 1.40 ± 0.42 mm, respectively, while *K. pneumoniae* was weakly inhibited at concentration 250 μg/mL with values 2.15 ± 0.1 mm. Moreover, bacterial pigment extract showed stronger antibacterial activity than amoxicillin 10 μg/disc against ESBL *E. coli* and MRSA at concentration 1000 μg/mL with values 5.11 ± 0.02 mm and 5.45 ± 1.65 mm, respectively.

Discussion

V. owensii is known as a marine bacterium commonly isolated from crustaceans and corals. This species was firstly isolated from crustaceans in Australia with characteristics such as bright yellow, nonluminiscent, nonswarming and round colonies (2–3 mm) (Cano-Gómez et al., 2009). Our strain TNKJ.CR.24-7 also displayed all these characteristcs. *V. owensii* is also known as a pathogen of shrimp and corals (Wilson et al., 2012; Liu et al., 2018). Wilson et al. (2012) revealed that *V. owensii* was a prevalent species among *Vibrio* isolates associated with *Acropora* white syndrome (AWS). Moreover, this bacterial species was also reported as an inducer of the tissue loss disease of *Montipora* white syndrome (MWS) in Hawaii (Ushijima et al., 2012). According to Fig. 3, strain TNKJ.CR-24.7 belongs to *Harveyi* clade with *V. harveyi*, *V. campbellii, V. rotiferianus*, *V. alginolyticus* and *V. parahaemolyticus*. This result was consistent with the work of Cano-Gómez et al. (2009) reporting that *V. owensii* belongs to Harveyi clade with 16S rRNA gene sequence close to *V. harveyi*, *V. campbellii* and *V. rotiferianus*.

Since 1-butanol is able to extract not only bacterial metabolites but also water-soluble compounds, the yield of bacterial crude extract from this solvent was higher than the extracts from ethyl acetate and acetone. *V. owensii* TNKJ.CR.24-7 produces carotenoid pigments that give yellow color to its colonies. Carotenoid is a fat-soluble terpenoid with yellow, orange or reddish color produced by most of the living organisms (Avalos and Limón, 2015). Kusmita et al. (2017) reported that several symbiotic bacteria such as *Pseudoalteromonas shioyasakiensis*, *P. rubra*, *P. spongiae* and *Virgibacillus salaries* from an Indonesian soft coral *Sarcophyton* sp. produced yellow carotenoid pigments. Based on the chemical structure and oxygen functionality carotenoids are classified into two types, there are carotenes and carotenoid hydrocarbons such as lycopene and β-carotene that consist of only carbon and hydrogen; whereas xanthopylls and oxygenated carotenoids such as lutein, canthaxanthin, zeaxanthin, violaxanthin, capsorubin and astaxanthin are oxygenated and may contain epoxy, carbonyl, hydroxyl, methoxy or carboxylic acid functional group (Rivera and Canela-Garayoa, 2012; Fassett and Coombes, 2012). It should be noted that the yellow pigment produced by *V. owensii* TNKJ.CR.24-7 is β-carotene according to the result of TLC analysis (Fig. 5). In microorganisms, carotenoid has important roles for UV protection, cell coloration, and resistance to oxidative stress (Avalos and Limón, 2015; Rodrigo-Baños et al., 2015).

Antibacterial activity of bacteria is related to the existence of NRPS and PKS-I genes (Wu et al., 2015; Falanga et al., 2016; Chakraborty et al., 2017; Schinke et al., 2017). However, Fig. 4 shows that only NRPS gene fragments were detected from *V. owensii* TNKJ.CR.24-7 at 300 bp. This result is consistent with the previous studies in which the set primers A2gamF/A3gamR amplify NRPS fragments of 200–300 bp (Sibero et al., 2018). The diameter of inhibition zone is related to the antibacterial components present in the crude extracts. Interestingly, only the crude extract from ethyl acetate showed antibacterial activity against all of

MDR bacteria. Moreover, the crude extract had wider inhibition zones against ESBL *E. coli* and MRSA rather than amoxicillin. Due to the existence of NRPS fragments, the production of antimicrobial peptides (AMPs) is expected as the reason of antibacterial properties in *V. owensii* TNKJ.CR.24-7. AMPs from marine bacteria include fijimycins A–C, peptidolipins B–F and champacyclin (Agrawal et al., 2017; Schinke et al., 2017). In fact, there are no reports of AMPs from marine *Vibrio owensii* (DNP, 2018).

The result of TLC analysis showed that the crude extract from ethyl acetate had 6 different bands under UV illumination at 356 nm (Table 2). Owing to its antibacterial acitivity, activity-guided isolation method is applicable to isolate lead compounds from this extract. In addition, two bands with the same color and R_f were found in the bacterial extracts from 1-butanol and ethyl acetate (Fig. 6), while β-carotene (Fig. 5) was found in all of the crude extracts. It was suggested that antibacterial activity of the crude extract from ethyl acetate was not related to the existence of the β-carotene. Furthermore, the two bands in 1-butanol which also existed in ethyl acetate were also suggested to be irrelevant to the antibacterial activity. Therefore, we proposed that the presence of active compounds and the ratio between the active compounds and the junk compounds in a crude extract may influence the antibacterial performance.

Conclusion

One pigmented bacterium from an unidentified coral was isolated and identified as *Vibrio owensii* TNKJ.CR.24-7 with accession number MH488980.1. NRPS fragments were detected in this isolate. This isolate produced yellow pigment which was identified as β-carotene, a member of carotenoids. According to the result of bacterial metabolite extraction, β-carotene was found in all of the crude culture extract obtained by using 1-butanol, ethyl acetate and acetone. Interestingly, only the crude extract from ethyl acetate inhibited ESBL *E. coli*, *Klebsiella pneumoniae*, and MRSA strain MDR. TLC chromatogram showed that there were 7 bands from 1-butanol extract and 6 bands from ethyl acetate extract. However, crude extract from acetone was not well separated using silica gel-based TLC. Bands at R_f 0.64 and 0.81 were found in both of 1-butanol and ethyl acetate extracts.

Conflict of interest

None.

Authors' contributions

The present research was designed by Mada Triandala Sibero (MTS), Yasuhiro Igarashi (YI), Ocky Karna Radjasa (OKR), Agus Sabdono (AS), Agus Trianto (AS). The sample collection was done by MTS, Enjuro Harunari (EH) and Amit Raj Sharma (ARS). The bacterial isolations and molecular characterization were done by EH, ARS, Tiara Ulfa Bachtiarini (TUB), Adindalifa Hayu Lupita (AHL) and Defi Puspita Sari (DPS). The chemical characterization was done by MTS, TUB and YI. The manuscript was written and corrected by MTS, TUB and YI. All authors have read and approved the final manuscript.

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