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Polyketide Synthase Gene Domain Exploration of Marine Sponge Symbiont Bacteria Collected From Weh Island

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Abstract. Sponges have long been known as a source for isolating secondary metabolites. These natural compounds are biosynthetic products of symbiont bacteria from various phyla colonizing sponge tissue. Some symbiont bacteria are known to produce bioactive compounds that would have antibacterial activity, such as polyketide, due to competition in colonizing and obtaining nutrients from their hosts. In general, this study aims to explore the biosynthetic potential of seven sponge-symbiont bacteria by detecting the gene domain involved in the production of polyketide compounds. Sponge-symbiont bacteria isolation was carried out on one species of sponge collected from a depth of ±15 m in the Iboih area, Weh Island, Aceh Province, Indonesia**.** The bacteria was allowed to grow in Sea Water Complete agar medium and incubated at 28^{0}C for 10-14 days. The production of polyketide compounds involves the enzyme polyketide synthase (PKS). Polyketide synthase was detected by detecting the encoding gene domain involved in the production of polyketide compounds using PCR method. Five of the seven isolates of sponge symbiont bacteria were detected to contain the PKS gene domain. Furthermore, molecular identification confirm by 16S rRNA sequencing showed that the isolates belonged to the phylum *Firmicutes* and *Actinobacteria***.** The result indicated that the sponge symbiont bacteria collected from Weh Island had the biosynthetic potential to produce polyketide compounds**.** These compounds would have antimicrobial activities that will play a major role in the medical field. Research related to screening PKS genes in marine sponge symbionts bacteria from Weh Island has never been reported before, thus adding to the novelty of this research.

Keywords: Marine Sponge; symbion bacteria; *Actinobacteria*; 16S rRNA; PKS gene domain

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INTRODUCTION

Sponges have been widely known as a source of bioactive compounds. Several groups of bioactive compounds that have been isolated from sponges are terpenoids, alkaloids and polyketides. These compounds have antiviral, antitumor, antimicrobial, and other cytotoxic activities that play a major role in the medical field (Wang, 2006). Research related to bioactive compounds derived from sponges was first carried out by Bergmann and Burke in the 1940s (Bergmann dan Feeney, 1951). This discovery further sparked greater research interest in the isolation and identification of novel compounds from marine sponges. One of the novel bioactive compounds was nocardiopsistin which have anti-MRSA activity (Xu et al., 2018). Another example of bioactive compounds isolated from sponges is monanchocidin which has anticancer activity against leukemia cells (Makarieva et al., 2011).

Microorganisms that are symbiotic with sponges are estimated to be about 40% of the volume and 50-60% of the dry weight of the spongy tissue itself, with a population density of up to 1-5×10^6 bacteria per ml^{-1} (Wang, 2006). These symbionts also have their diversity, both found between host species and between sponge habitat locations. This occurs due to the coevolution of the host and symbionts and the transmission of symbionts from one host sponge to another (Bright dan Bulgheresi, 2010; Fisher, Henry, Cornwallis, Kiers dan West, 2017). This process automatically also affects the type of active compound produced (Brinkmann et al., 2017; Matcher et al., 2017). In fact, the type and

intensity of predators can cause the diversity of symbionts and the resulting active compounds (Marty et al., 2017).

The explanation above illustrates the potential of symbionts in marine sponges as a very large producer of bioactive compounds. Its high diversity makes it difficult to conduct conventional research (based on testing the inhibitory activity of an active compound against a broad spectrum of microorganisms). As an alternative, an approach using the polyketide pathway can be used to see the potential of these sponge symbiont bacteria as producers of active compounds. Polyketides are important intermediates in the biosynthesis of antibiotics through a catalytic process involving the enzyme polyketide synthase (PKS) (Nivina et al., 2019). Thus, the potential for these symbionts to produce active compounds can be investigated by screening the gene encoding PKS, as has been done by previous researchers (Abd El-Moneam et al., 2017).

In Indonesia, there are several studies that have reported the exploration of the PKS gene for symbiont bacteria. A study reported in 2017 screened bacteria of the genus *Candidatus entotheonella* isolated from the sponge *Rhabdastrella sp*. in the Kapoposang Island area, South Sulawesi (Kurnia et al., 2017). Another study succeeded in obtaining and analyzing the PKS gene domain from 174 bacterial isolates which were the result of exploration on sponge *Aaptos sp*. and *Hyrtios sp*. in the area of Pramuka Island, Jakarta (Rini et al., 2017). The small number of studies reported related to screening PKS genes in marine sponge symbionts in Indonesia indicates that this study has high novelty. In addition, research related to marine sponges from Weh Island has never been reported before, thus adding to the novelty of this research (Bahri et al., 2015; Barber and Bellwood, 2005; Fadli et al., 2019; Fastawa et al., 2016).

In this study, bacterial symbionts were identified by using 16S rRNA gene analysis. The biosynthetic potential of sponge symbiont bacteria was analyzed by screening the PKS gene's presence in the bacterial genome. The results of the study are expected to provide information regarding species of marine sponge symbiont bacteria from Weh Island, which have the potential to produce polyketide compounds.

METHODS

Sample collection

A sponge sampling was carried out in the Iboih area, Sabang, using the SCUBA diving method at a depth of ±15 m. The sponge obtained was placed in a sterile box and stored in a coolbox during its journey to the Integrated Research Laboratory, Faculty of Veterinary Medicine, Syiah Kuala University.

Symbiont bacteria isolation

The symbiont bacteria were isolated by crushing 1 gram of the mesohyl sponge that had been washed with sterile water. The mesohyl part that has been destroyed is then dissolved in 0.9% NaCl with a ratio of 1:1 (Kim et al., 2006). This suspension is then diluted through serial dilutions 10^{-1} to 10^{-7}. Every 1 ml of dilution levels 10^{-5}, 10^{-6} and 10^{-7} was spread on the Sea Water Complete (SWC) media surface and then incubated at 28 ^{0}C for 10-14 days. Bacterial colonies growing in the media were purified using the same medium to obtain a single bacterial colony.

Gram staining

Gram staining is done by smearing the bacterial culture on an object glass and fixing it. The fixed object was stained with crystal violet and allowed to stand for 1 minute, followed by washing with H_2O. The clean preparations were stained again using Iodine solution for 1 minute, washed, then 96% alcohol was added and rinsed with running water. The final stage of Gram staining is immersing the object with safranin for 2 minutes, rinsing and drying and then observing the cells' shape and color.

Genomic DNA extraction

Genomic DNA of marine sponge symbiont bacteria from Sabang waters was carried out using a commercial Genomic DNA Purification kit regarding the kit protocol without any modifications.

Amplification of 16S rRNA gene and molecular analysis

The amplification of the 16S rRNA gene was carried out using the Polymerase Chain Reaction (PCR) method using 27F and 1492R primers. This primer will initiate the elongation reaction of the target DNA chain fragment at about 1400 bp. The composition of the PCR reaction mixture was 25 uL consisting of 2 uL 27F primer, 2 uL 1492R primer, 3 uL DNA template, 5.5 uL sterile distilled water and 12.5 uL PCR Master Mix kit (Promega, USA) according to the instructions manual protocol. All solutions were mixed in a 0.2 mL

PCR tube. Reaction conditions used in the PCR were carried out under the Promega protocol. PCR amplicon was analyzed by 2% (w/v) agarose gel electrophoresis in 1x TAE pH 7.8 ultra Pure Grade against a molecular size marker 100 bp (Promega).

The 16S rRNA gene sequence obtained from the sequencing results was analyzed with the MEGA program and compared with other bacterial 16S rRNA gene data at the National Center for Biotechnology Information (NCBI) Gene Bank database on the website www.ncbi.nml.nih.gov by the method Basic Alignment Search Tool (BLAST). The output of the BLAST program is used as input in the construction phase of the phylogenetic tree using the MEGA program (http://www.megasoftware.net/).

Screening of biosynthetic domain gene (PKS)

Screening of the PKS gene domain with a target length of about 700 bp was carried out using BioRad C1000 thermal cycler. The specific primers used for this domain are degKS2F (5′-GCSATGGAYCCSCARCARCGSVT-3) and degKSR5 (5′-GTSCCSGTSCCRTGSSCYTCSAC-3’) (Schirmer et al., 2005). The composition of the PCR reaction mixture was 25 uL consisting of 2 uL degKS2F primer, 2 uL degKSR5 primer, 3 uL DNA template, 5.5 uL sterile distilled water and 12.5 uL PCR Master Mix kit (Promega, USA). PCR was carried out under the following condition: initial denaturation at 94^{0}C for 1 minute, denaturation at 94^{0}C for 40 seconds, annealing at 50^{0}C for 40 seconds, elongation at 72^{0}C for 75 seconds and final elongation at 72^{0}C for 5 minutes (Schirmer et al., 2005). PCR amplicon was analyzed by 2% (w/v) agarose gel electrophoresis in 1x TAE pH 7.8 ultra Pure Grade against a molecular size marker 100 bp (Promega).

RESULTS AND DISCUSSION

Sponge Symbiont Bacteria Isolation

Bacterial symbiont isolation was carried out on one species of sponge collected from a depth of ±15 m in the Iboih area, Weh Island, Aceh Province, Indonesia (Figure 1). A total of 7 bacterial isolates were isolated from the mesohyl of the sponge, where extracellular heterotrophic bacteria are commonly found (Figure 2). The morphological evaluation results showed that the sponge symbiont bacteria isolates had similar shapes, edges, elevations and colony colors (Table 1).

The bacterial isolates of marine sponge symbionts from Sabang waters grew aerobically at 28^{0}C with an incubation period of 10-14 days. The isolate has a tolerance to NaCl with a concentration of 0-15% (w/v) and a pH of 6-8. The Gram staining results showed that the isolates were Gram-positive bacteria which was indicated by the formation of a purple color at the end of the staining. In addition, six isolates were known to be bacteria with coccus-shaped cells, while one other isolate (ID: PSB-2) was bacteria with rod-shaped cells.

Symbiotic bacteria can be defined as bacteria that colonize the internal parts of the sponge tissue (host), both intra and extracellular, without giving negative effects to the host. Many sponges have been colonized by bacterial symbionts, and only a few have been thoroughly studied for their bacterial symbiont content. Until 2014, there were at least 32 bacterial phyla known to exist in sponges, of which the bacteria commonly found and successfully cultivated came from the phyla Proteobacteria (alpha, beta and gamma), Bacteroldetes, Plantomycetes, Nitrospira, Cyanobacteria, Chloroflexi, Acidobacteria, Spirochaetes, Gemmatimonadetes, Firmicutes and Actinobacteria. (Abdelmohsen et al., 2014).

Sponges, together with symbiotic bacteria, are known to produce various secondary metabolites, which have many variations both biologically and chemically, which can protect sponges from pathogenic bacteria, viruses, parasites and fungi. Some sponge symbiont bacteria are even known to produce more antibacterial compounds due to the high competition in colonizing tissues and obtaining nutrients from their hosts. One method which is considered the most effective that can be used in the search for these compounds is the cultivation of symbiotic bacteria. Several groups of compounds commonly found in sponge symbiont bacteria come from the terpenoids, alkaloids, fatty acids, peptides and polyketides (Bibi et al., 2016; Paul et al., 2021).

Polyketides, derived from symbiotic bacteria, are the product of the biosynthesis of the multidomain polyketide synthase (PKS) enzyme. Screening for the biosynthetic potential of secondary metabolites in symbiont bacteria can be carried out using a molecular approach utilizing this pathway. In this study, the biosynthetic potential of isolates of marine sponge symbiotic bacteria from Sabang waters was focused on the detection of the PKS gene domain that acts as an enzyme for the biosynthesis of polyketide.

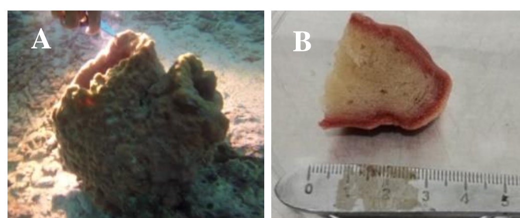

Figure 1. Sponge sample. (A) Sponge at a depth of ±15 m . (B) Mesohyl sponge

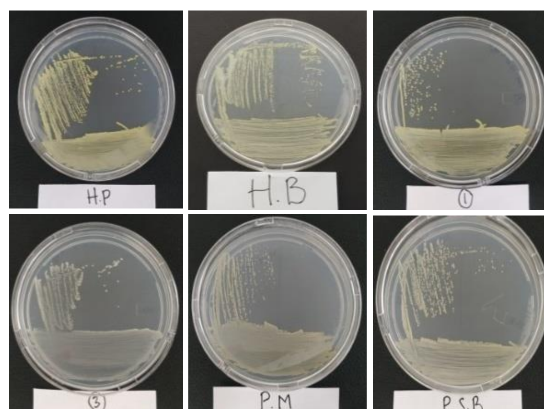

Figure 2. Pure culture of marine sponge symbiont bacteria

Table 1. Colony morphology of marine sponge symbiont bacteria.

ID	Color	Shape	Edge	Elevation	Gram	Cell Shape
HP	Yellow	Round	Irregular	Convex	+	Coccus
HB	Yellow	Round	Irregular	Convex	+	Coccus
1	Yellow	Round	Irregular	Convex	+	Coccus
3	Pale Yellow	Round	Entire	Convex	+	Coccus
PM	Pale Yellow	Round	Entire	Raised	+	Coccus
PSB	Pale Yellow	Round	Irregular	Convex	+	Coccus
PSB-2	Cream	Round	Entire	Raised	+	Rod

Amplification of 16S rRNA gene and molecular analysis

The 16S rRNA gene of sponge symbiont bacteria was amplified using primers 27F and 1492R (Figure 3). Amplification products (1400bp) were purified and analyzed using the Sanger sequencing technique. The 16S rRNA gene sequence showed that the isolates were closely related to *Kocuria* (2 isolates), *Micrococcus* (2 isolates), *Oceanobacillus* (1 isolate) and *Uncultured bacterium* (2 isolates). Two isolates of the genus *Kocuria* have similarities with *K. Rhizophilla* and *K. palustris*. Two isolates of the genus Micrococcus have similarities with *M. Luteus* and *M. yunnanensis*, respectively. The isolates of *Oceanobacillus* were similar to *O. Kimchii*. While the other two isolates were closely related to *Uncultured bacterium clone butcher F36* (Table 2).

Percent similarity shows the similarity of the 16S rRNA gene sequence between the sponge symbiont bacteria and the bacterial gene sequences in the GeneBank database, both at the genus and the species level. Bacterial isolates with percent similarity above 99.8% were considered to have similar sequences to the species level. So it can be concluded that the isolates of sponge bacteria that have a value above 99.8% similarity with the reference gene of NCBI bacteria are two bacteria that come from the same species (Zhu et al., 2020).

To confirm the phylogenetic relationship and strain types of the isolates, the construction of a phylogenetic tree based on the alignments of the 16S rRNA gene was also carried out. Phylogenetic analysis based on the Neighbor-Joining method using matrix pairwise comparisons showed that the seven isolates of sponge symbiont bacteria belong to *Kocuria*, *Micrococcus*, *Oceanobacillus* and *Uncultured bacterium.* The isolates of *Kocuria* genus were distributed into two different clusters. In this cluster, two isolates (HB

and PSB-1) are known to have very close relationships with *Kocuria sp. strain MMun 160* and *Kocuria Palustris strain Rb-107*. Isolates 1 and HP-1 were phylogenetically identical, and both isolates were related to *Unculture bacterium clone butcher F36*. Two isolates of the genus *Micrococcus* (PM and 3) were divided into two different clusters where the isolates have successive relationships with *M. Yunnanensis strain DSM 1677* and *M. Luteus strain* 10240. The other isolate, which belongs to the genus *Oceanobacillus*, has a very close phylogenetic similarity with *O. Kimchii strain FJAT-45433* (Figure 4).

Analysis of the 16S rRNA gene sequence showed that 4 of the seven isolates of sponge symbionts belong to the phylum *Actinomycetes*. *Actinomycetes* or *Actinobacteria*

is a bacterial phylum consisting of Gram-positive bacteria, considered an intermediate group between bacteria and fungi. *Actinobacteria* are spread in almost all environments and habitats, acting as pathogenic bacteria and symbionts. Various marine invertebrate organisms are also a source of cultivation of this bacteria, where sponges are generally the main source of cultivation. *Actinobacteria* is even known as the most abundant secondary metabolites producer, followed by *Proteobacteria*, *Cyanobacteria*, *Firmicutes* and *Bacteroldetes*. The recoverability of *Actinobacteria* in sponge samples varied depending on the species of sponge and the environment in which the sponge was obtained (Abdelmohsen et al., 2014). For example, Abdelmohsen et al only succeeded in isolating three *Actinobacteria* from *Hyrtios erectu* and four *Actinobacteria* from *Amphimedon sp.* that were collected from the Red Sea (Abdelmohsen et al., 2010, 2014). Meanwhile, zero *Actinobacteria* have been isolated from *Amphimedon complanata* collected from Puerto Rico (Vicente et al., 2013). In addition, another thing that also needs to be considered to increase the efficiency of isolation is the use of bacterial growth media, where the more variety of media used, the greater the variety of actinobacteria genera that can be found.

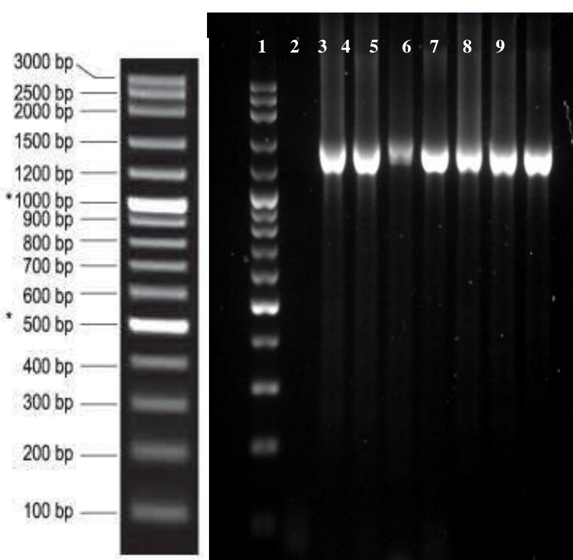

Figure 3. 16S rRNA amplification product (±1400 bp). Lane 1: Marker DNA 100 bp, Lane 2: Negative control, Lane 3: HP, Lane 4: HB, Lane 5: sample 1, Lane 6: sample 3, Lane 7: PM, Lane 8: PSB, Lane 9: PSB-2.

Table 2. Similarity data on the 16S rRNA gene of sponge symbiont isolates with the NCBI bacterial gene collection.

No.	ID	Species	*% Similarity*	*Accession number*
1	HP	Uncultured bacterium F36	98.69	FJ873670.1
2	HB	*Kocuria* sp. strain MMun 160	98.90	MG980081.1
3	1	Uncultured bacterium F36	98.27	FJ873670.1
4	3	*Micrococcus luteus 10240*	98.05	CP041689.1
5	PM	*Micrococcus yunnanensis DSM 1677*	98.22	MN175946.1
6	PSB-1	*Kocuria palustris RB-107*	96.98	JQ085396.1
7	PSB-2	*Oceanobacillus kimchii FJAT-45433*	99.93	KY849479.1

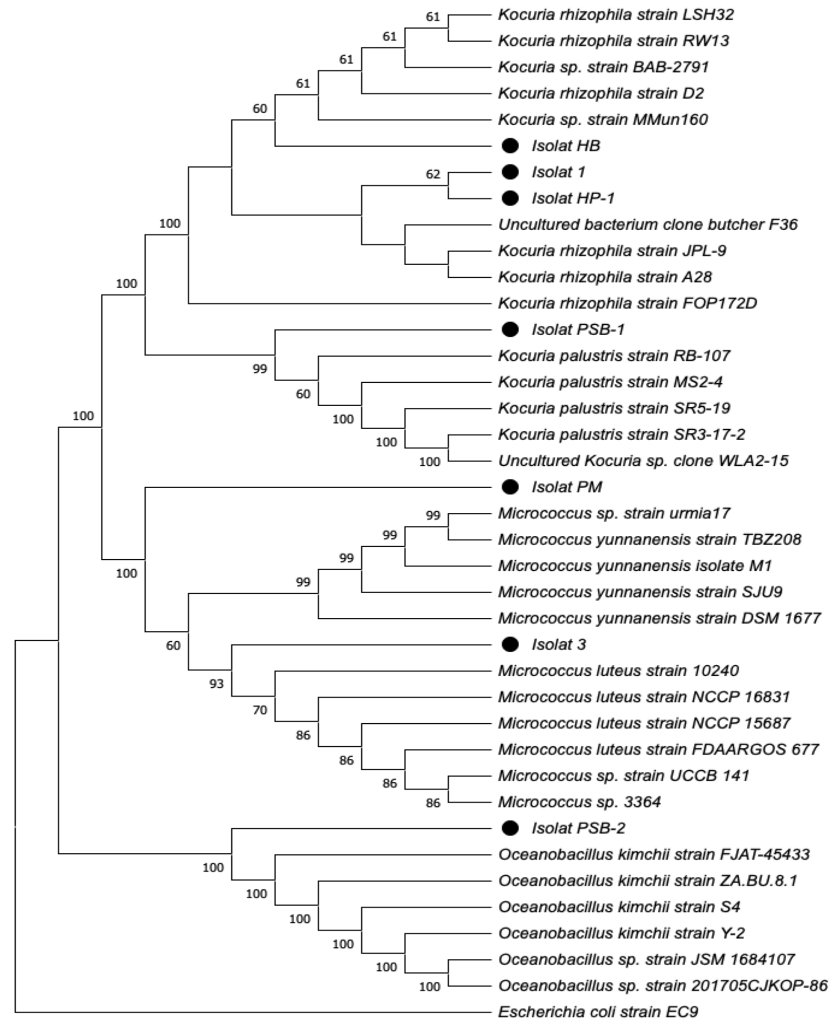

Figure 4. Phylogenetic tree of marine sponge symbiont bacteria collected from Weh Island.

Screening of PKS Gene Domain

In this study, the PKS gene domain was determined by the presence of a DNA band at 700 bp (Figure 5). The results indicated that isolate HP, isolate 1, isolate HB, isolate PSB-1 and isolate 3 (5 out of 7 symbiont bacteria) had PKS gene domains which play a role in the metabolic pathways of bioactive compounds. Those bacteria are closely related to *the Uncultured bacterium F36*, *Kocuria sp. MMun strain 160*, *Kocuria palustris strain RB-107* and *Micrococcus luteus strain 10240*. Sponge-associated genera *Kocuria* and *Micrococcus* have been known to possess the BGC gene and have the capacity to produce bioactive compounds. Majority of the BGC present in *Actinobacteria* are polyketide synthase (PKS) or Non-Ribosomal Peptide Synthase (NRPS) or both PKS and NRPS (Dhakal et al., 2019).

Sponge-associated bacteria from the Red sea water were detected to have PKS genes involved in their metabolic processes. (Palomo et al., 2013). However, some studies have reported the absence of the PKS gene domain in sponge-associated genera *Micrococcus*. For example, a comprehensive study on *Halichondria panicea-associated Actinobacteria* from the Baltic Sea has reported that *Micrococcus luteus* did not contain PKS and NRPS gene domains (Schneemann et al., 2010).

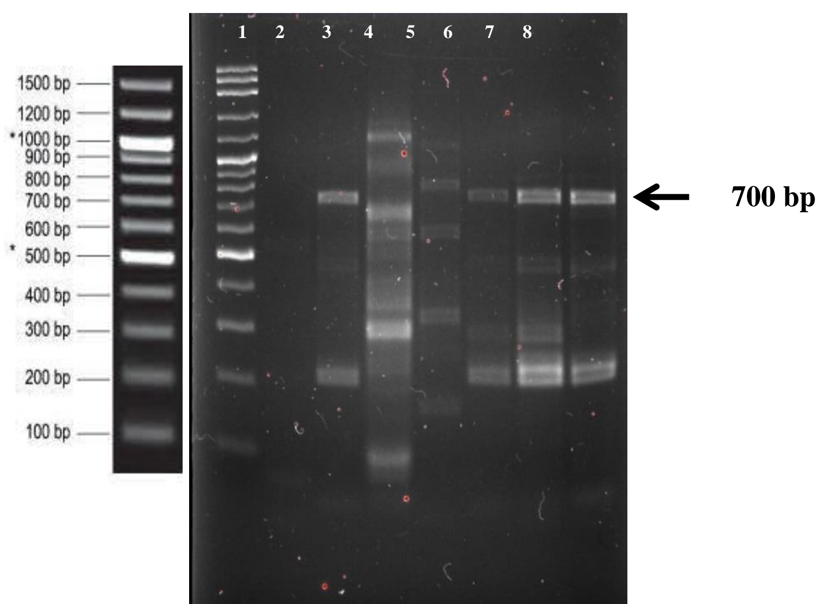

Figure 5. Amplification of PKS gene domain. Lane 1: Marker DNA 100 bp, Lane 2: Negative control, Lane 3: HP, Lane 4: PSB-2, Lane 5: (1), Lane 6: HB, Lane 7: PSB-1, Lane 8: (3).

It should be noted that the absence of the PKS gene domain does not indicate the inability of sponge-associated bacteria to produce bioactive compounds. This can be possible if the metabolic pathway is synthesized by another BGC (NRPS) or at least the presence of the BGC gene, in this case, the PKS gene domain, or it can also be caused by the slight similarity of the primer sequence used with the PKS gene domain conserved area in the target bacteria. Furthermore, the positive results of BGC amplification, in this case, the PKS gene domain, in bacteria also do not provide a guarantee that the bioactive compound products will be expressed properly because it is possible that the presence of the detected BGC gene is actually the gene involved in the production of pigments or compounds that serves as a constituent of the structure of the bacterial cell itself (Palomo et al., 2013).

CONCLUSION

Sponge-symbiont bacteria collected from Weh Island are bacteria in the phylum Firmicutes and Actinobacteria. The results indicated that 5 out of 7 symbiont bacteria had PKS gene domains. Those bacteria are closely related to the Uncultured bacterium F36*,* *Kocuria* sp. MMun strain 160*,* *Kocuria palustris* strain RB-107 *and Micrococcus luteus* strain 10240.

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