Identification of Sponge-Associated Bacteria with Antibacterial Property against *Staphylococcus aureus* based on Molecular Approach

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Abstract

Staphylococcus aureus *is among human pathogens which are known to be resistant to almost clinically significant antibiotics that causes a crisis, in the treatment and management of infectious disease, and also presents a clear danger to the future of public health. Recently, invertebrate-associated microorganisms have become the target of the search for marine microbial natural products. Sponge-associated microorganisms are one of the most interesting natural product sources because they produce bioactive natural products including polyketides and nonribosomal peptides. Molecular approach based on 16S rDNA has been very successful in the identification and the search of secondary metabolite-producing microorganisms, particularly by using specific degenerated primers, for the detection of Polyketide Synthases (PKS) and Nonribosomal Peptide Synthetases (NRPS) gene fragments that are essential for the biosynthesis of bioactive products. Four bacterial isolates from sponge Dysidea sp. were found to inhibit the growth of* S. aureus*. However, only the best two isolates were selected for further identification and screening for the presence of either PKS or NRPS. The results revealed that both isolates BSP5.11 and BSP11.7 have 99% homology with* Alpha proteobacterium*. PCR analysis of PKS and NRPS gene fragments showed that they both amplified the NRPS but not the PKS gene fragments.*

Keywords: *Idenfication, sponge-associated bacteria, Polyketide Synthases*

Introduction

Marine organisms including those from coral reef ecosystems have become sources of great interest to natural product chemistry, since they provide a large proportion of bioactive metabolites with different biological activities (Faulkner 2000). In particular, marine invertebrates with high species diversity in the Indo-Pacific regions (Coll and Sammarco 1986) are often rich in secondary metabolites and are preferential targets in the search for bioactive natural products (Sammarco and Coll 1992).

Sponges (phylum Porifera) are most primitive of the multicelled animals that have existed for 700–800 million years. Of the approximately 15,000 sponge species, most occur in marine environments. Only about 1% of the species inhabits freshwater (Belarbi *et al.,* 2003).

Perhaps the most significant problem that has hampered the investigation of secondary metabolites produced by reef's invertebrates is their low concentration. In marine invertebrates many highly active compounds contribute to$<10^{-6}$ % of the body-wet weight. Providing sufficient amounts of these biologically active substances, hence, may be a difficult task (Proksch *et al.*, 2002; Radjasa *et al.,* 2007a,b,c).

The present work is aimed at screening of marine bacteria associated with sponge *Dysidea* sp. for the production of secondary metabolites against pathogenic bacterium *S. aureus* coupled with PCR-based method for the occurrence of NRPS and PKS gene fragments for estimating the genetic potential of the biologically active strains.

Materials and Methods

Colonies of sponge *Dysidea* sp. were collected from Bandengan water, Jepara, North Java Sea, Indonesia by scuba diving from a depth of approximately 5 meters. Upon collection sponge colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA) The sponges were then rinsed with sterile seawater and 1 cm^2 of sponge tissue was excised from the middle of the whole sponge and sponge surface after it was peeled off (Radjasa *et al.*, 2007c) with a sterile knife. The resultant tissues were serially diluted, spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al.*, 2000).

To screen their biological activity, a total of 98 sponge isolates were tested against pathogenic bacterium *S. aureus*. One 100 µl culture of indicator microorganism in the logarithmic phase (ca. 10^9 cells ml^{-1}) was spread on to agar medium. Several paper disks (8 mm; Advantec, Toyo Roshi, Ltd, Japan) containing 30 µl of the sponge bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 hours. Antibacterial activity was defined according to Radjasa *et al.,* (2007a) by the formation of inhibition zones greater than 9 mm around the paper disk.

To obtain genomic DNA of secondary metabolite producing-strains for PCR analysis, cell materials were taken from an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C). Amplification of peptide synthetase gene fragments was carried out with the NRPS degenerated primers A2gamF (5´-AAG GCN GGC GSB GCS TAY STG CC-3´) and A3gamR (5´-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3´) (Marahiel *et al.*, 1997) and PKS degenerated primers KSDPQQF (5´-MGN GAR GCN NWN SMN ATG GAY CCN CAR CAN MG-3´) and KSHGTGR (5´-GGR TCN CCN ARN SWN GTN CCN GTN CCR TG -3´) (Piel, 2002).

PCR was performed with an ProgeneThermal cycler (Techne, Burkhardtsorf, Germany) as follows: 1 µl template DNA, 1 µl of each of the appropriate primers, and 23 µl DNA free water (Fluka, Sigma-Aldrich Chemie GmbH, Germany) were added to puReTaq Ready-To-Go PCR beads (Amersham Biosciences Europe GmbH, Germany). The NRPS-PCR run comprised 40 cycles with denaturing conditions for 1 min at 95°C, annealing for 1 min at 70°C and extension for 2 min at 72°C, respectively. P*seudomonas* sp. DSM 50117 was used as positive control. The amplification of PKS gene fragments included an initial denaturating step at 94°C for 2 min, followed by 45 cycles at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min. *Bacillus subtilis* 168 was utilized for positive control.

Amplification was conducted according to method of Radjasa *et al* (2007a). Genomic DNA of secondary metabolite producing-strains for PCR analysis were obtained from cell materials taken from an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C). PCR amplification of partial 16S rDNA of sponge bacteria, purification of PCR products and subsequent sequencing analysis were performed according to the method of Radjasa *et al* (2007b). The determined DNA sequences of strains were then compared for homology to the BLAST database.

Results and Discussion

The present study indicated that among 98 marine bacteria associated with sponges, 4 isolates showed growth inhibition against *S. aureus* (Table 1). This offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria such as *S. aureus*.

Table 1. Inhibitory interaction of sponge associated-bacteria against *S. aureus*

Isolate	Diameter of inhibition zone (mm)			Average
	1	2	3	
BSP5.11*	12.2	12.3	12.0	12.2 ± 0.01
BSP11.3	9.5	11.0	8.9	9.8 ± 0.06
BSP11.7*	11.7	10.6	11.2	11.2 ± 0.03
BSP11.9	10.3	10.8	10.0	10.4 ± 0.02

* Isolates selected for further identification

Molecular identification based on 16S rDNA approach was carried out only on two isolates showing the best growth inhibition (Tabel 1 and Fig 1) and the results showed that the two active isolates are the member of *Alpha proteobacterium* (Table 2).

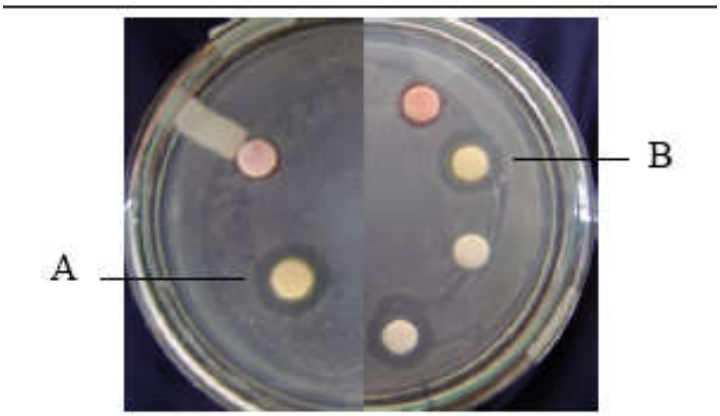


Fig 1. Zone inhibition on *S. aureus* lawn by sponge-associated bacteria. (A). BSP5.11, (B) BSP11.7

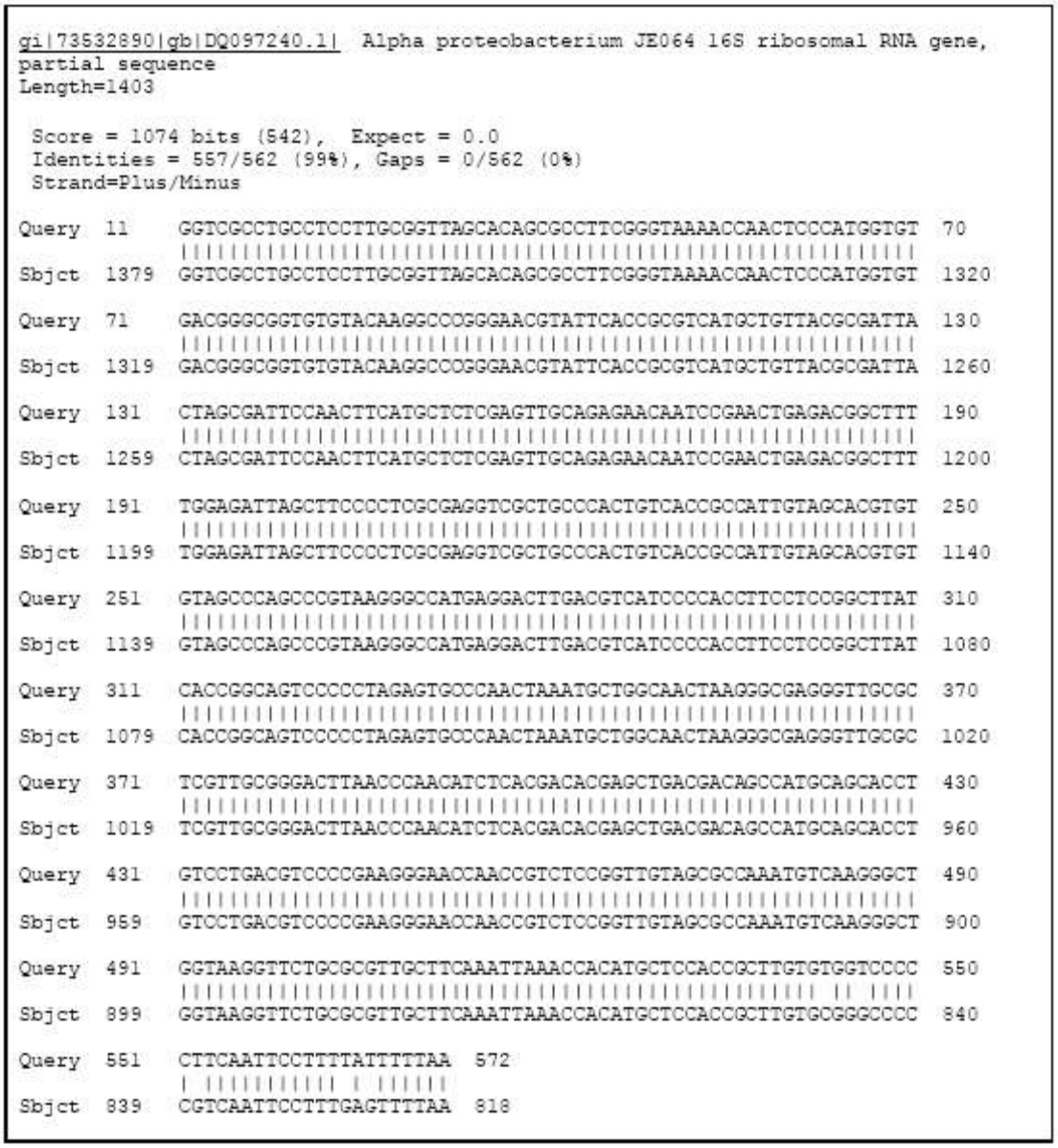
Table 2. Molecular identification of the best two active isolates

No	Isolate	Acc. Number	Closest relative	Homology (%)
1	BSP5.11	DQ097240	*Alpha proteobacterium* JE064	99
2	BSP11.7	AY762960	*Alpha proteobacterium* Z143-1	99

As shown in the Table 2, both isolates BSP5.11 and BSP11.7 had high homologies to *Alpha proteobacteria* (99%). The detailed alignments of DNA sequences both isolates are shown in the Figure 2 and 3.

One of the active isolate BSP11.7 showed a high homology to *Alpha proteobacterium* Z143-1(99%), a marine bacterium isolated from Philippine tunicate that produce anti-*Staphylococcus aureus* metabolite heptylprodigiosin (de Guzman, unpublished). Interestingly our isolate BSP.11 also inhibited the growth of tested strain *S. aureus*. On the other hand, isolate BSP5.11 had closest relation with *Alpha proteobacterium* JE064 (Acc. No. DQ097240), a culturable Alphaproteobacterial symbiont of many marine sponges collected from geographically different regions , namely *Axinella corrugata, Mycale laxissima, Monanchora unguifera*, and *Niphates digitalis* from Key Largo, Florida; *Didiscus oxeata* and *Monanchora unguifera* from Discovery Bay, Jamaica; an *Acanthostronglyophora* sp. from Manado, Indonesia; and *Microciona prolifera* from the Cheasapeake Bay in Maryland (Enticknap *et al*, 2006).

Furthermore, Hentscel *et. al* (2002), reported that Alpha proteobacterium was also found in association with *Theonella swinhoei*, *Aplysina aerophaba*, *Rhopaloides odorabile* dan *Halicondria panicea*. The finding that A*lpha proteobacterium* dominated the sponge *Rhopaloides* odorabile in geographically different area, has confirmed that this associated species is sponge-specific bacterium (Lee *et al*, 2001).



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gi|73532890|gb|DQ097240.1|  Alpha proteobacterium JE064 16S ribosomal RNA gene,
partial sequence
Length=1403

 Score = 1074 bits (542),  Expect = 0.0
 Identities = 557/562 (99%), Gaps = 0/562 (0%)
 Strand=Plus/Minus

Query  11    GGTCGCCTGCCTCCTTGCGGTTAGCACAGCGCCTTCGGGTAAAACCAACTCCCATGGTGT  70
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Sbjct  1379  GGTCGCCTGCCTCCTTGCGGTTAGCACAGCGCCTTCGGGTAAAACCAACTCCCATGGTGT  1320

Query  71    GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGTCATGCTGTTACGCGATTA  130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1319  GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGTCATGCTGTTACGCGATTA  1260

Query  131   CTAGCGATTCCAACTTCATGCTCTCGAGTTGCAGAGAACAATCCGAACTGAGACGGCTTT  190
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1259  CTAGCGATTCCAACTTCATGCTCTCGAGTTGCAGAGAACAATCCGAACTGAGACGGCTTT  1200

Query  191   TGGAGATTAGCTTCCCCTCGCGAGGTCGCTGCCCACTGTCACCGCCATTGTAGCACGTGT  250
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1199  TGGAGATTAGCTTCCCCTCGCGAGGTCGCTGCCCACTGTCACCGCCATTGTAGCACGTGT  1140

Query  251   GTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGCTTAT  310
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1139  GTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGCTTAT  1080

Query  311   CACCGGCAGTCCCCCTAGAGTGCCCAACTAAATGCTGGCAACTAAGGGCGAGGGTTGCGC  370
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1079  CACCGGCAGTCCCCCTAGAGTGCCCAACTAAATGCTGGCAACTAAGGGCGAGGGTTGCGC  1020

Query  371   TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCT  430
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Sbjct  1019  TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCT  960

Query  431   GTCCTGACGTCCCCGAAGGGAACCAACCGTCTCCGGTTGTAGCGCCAAATGTCAAGGGCT  490
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Sbjct  959   GTCCTGACGTCCCCGAAGGGAACCAACCGTCTCCGGTTGTAGCGCCAAATGTCAAGGGCT  900

Query  491   GGTAAGGTTCTGCGCGTTGCTTCAAATTAAACCACATGCTCCACCGCTTGTGTGGTCCCC  550
             ||||||||||||||||||||||||||||||||||||||||||||||||| || ||||
Sbjct  899   GGTAAGGTTCTGCGCGTTGCTTCAAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC  840

Query  551   CTTCAATTCCTTTTATTTTTAA  572
             | |||||||||||| | ||||||
Sbjct  839   CGTCAATTCCTTTGAGTTTTAA  818
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Figure 2. The DNA alignment showing the homology of isolate BSP5.11 and *Alpha proteobacterium* JE064 based on BLAST search.

Polyketides and non-ribosomal peptides are two of the largest groups of multifunctional proteins that create a multitude of secondary metabolites (Hutchinson, 2003), many of them are used as therapeutic agents (Piel *et al.*, 2003). Products of the microbial non-ribosomal peptide synthesis include the immunosuppressant cyclosporine and other antibiotics such as gramicin S, tyrocin A and surfactins (Kleinkauf and von Doehren, 1996). Among clinically important polyketides are the antibiotics aunorubicin, erythromycin, lovastatin and rapamycin (Due *et al.*, 2001).

With advanced techniques of molecular biology such as polymerase chain reaction (PCR), it is now become possible to carry out a screening on the presence of polyketides and non ribosomal peptides by using specific primers of polyketide synthases (PKS) (Piel, 2002) and non ribosomal polypeptide synthetases (NRPS) (Marahiel *et al.*, 1997).

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gi|54300652|gb|AY762960.1|  Alpha proteobacterium Z143-1 16S ribosomal RNA gene,
complete
sequence
Length=1442

 Score = 1322 bits (667),  Expect = 0.0
 Identities = 673/675 (99%), Gaps = 0/675 (0%)
 Strand=Plus/Minus

Query  4     GGTCGCCTGCCTCCTTGCGGTTAGCACAGCGCCTTCGGGTAAAACCAACTCCCATGGTGT  63
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Query  64    GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGTCATGCTGTTACGCGATTA  123
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Sbjct  1340  GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGTCATGCTGTTACGCGATTA  1281

Query  124   CTAGCGATTCCAACTTCATGCTCTCGAGTTGCAGAGAACAATCCGAACTGAGACGGCTTT  183
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Sbjct  1280  CTAGCGATTCCAACTTCATGCTCTCGAGTTGCAGAGAACAATCCGAACTGAGACGGCTTT  1221

Query  184   TGGAGATTAGCTTCCCCTCGCGAGGTCGCTGCCCACTGTCACCGCCATTGTAGCACGTGT  243
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Sbjct  1220  TGGAGATTAGCTTCCCCTCGCGAGGTCGCTGCCCACTGTCACCGCCATTGTAGCACGTGT  1161

Query  244   GTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGCTTAT  303
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Sbjct  1160  GTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGCTTAT  1101

Query  304   CACCGGCAGTCCCCCTAGAGTGCCCAACTAAATGCTGGCAACTAAGGGCGAGGGTTGCGC  363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1100  CACCGGCAGTCCCCCTAGAGTGCCCAACTAAATGCTGGCAACTAAGGGCGAGGGTTGCGC  1041

Query  364   TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCT  423
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Sbjct  1040  TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCT  981

Query  424   GTCCTGACGTCCCCGAAGGGAACCAACCGTCTCCGGTTGTAGCGCCAAATGTCAAGGGCT  483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  980   GTCCTGACGTCCCCGAAGGGAACCAACCGTCTCCGGTTGTAGCGCCAAATGTCAAGGGCT  921

Query  484   GGTAAGGTTCTGCGCGTTGCTTCAAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC  543
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  920   GGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC  861

Query  544   CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATGCTTAATGCG  603
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Sbjct  860   CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATGCTTAATGCG  801

Query  604   TTAGCTGCGTCACCAAATAGCAAGCTACCTGACAACTAGCATTCATCGTTTACGGCGTGG  663
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Sbjct  800   TTAGCTGCGTCACCAAATAGCAAGCTACCTGACAACTAGCATTCATCGTTTACGGCGTGG  741

Query  664   ACTACCAGGGAATCT  678
             |||||||||| ||||
Sbjct  740   ACTACCAGGGTATCT  726
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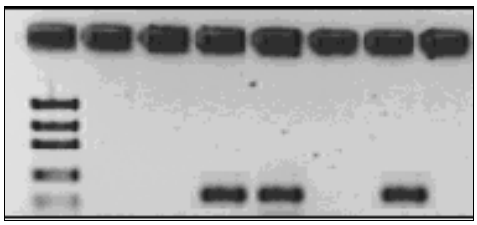
Figure 3. The DNA alignment showing the homology of isolate BSP.11.7 and *Alpha proteobacterium* Z143-1 based on BLAST system.

The present study revealed that both active isolates were able to amplify the NRPS gene fragments that are essential in the biosynthesis of peptide bioactive products (Fig 4), but not the PKS gene fragments.



M BSP5.11 BSP11.7 +

Figure 4. PCR amplification of NRPS gene fragments; + control *Pseudomonas* sp DSM 50117

The discovery of the potential for the synthesis of peptides among sponge-associated bacteria in particular the member of Alpha proteobacteria, should be of interest because of promising applications in the development of pharmacological peptides. Furthermore, considering the fact that research on sponge-bacteria association has been fairly limited , the future study on the search for secondary metabolite producers among coral colonizers should be given prominence.

Conclusion

In conclusion, sponge *Dysidea* sp. exhibited secondary metabolite producing-marine bacteria with antibacterial potential against *S. aureus*. The present study highlighted the PCR-based method by using specific degenerated primers NRPS and PKS as a powerful tool in estimating the genetic potential of sponge associated-bacteria that is essential in the biosynthesis of secondary metabolites.

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