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ANTIBACTERIAL ACTIVITY OF SPONGE ASSOCIATED-BACTERIA ISOLATED FROM NORTH JAVA SEA

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ABSTRACT

A total of 90 bacterial isolates were obtained from association with sponges collected from Bandengan water, Jepara and Karimun island of Karimunjawa islands in the north java sea. Antibacterial screening by using a pathogenic Escherichia coli indicated that two isolates BSP.12 and MKSP.5 inhihited the growth of tested strain. Molecular identification based on 16S rDNA approach revealed that isolate BSP.12 was closely related to Vibrio harveyi with a homology of 100% and isolate MKSP.5 showed highest similarity to Brachybacterium rhamnosum (99%), respectively.

A PCR-based approach by using specific primers targeting the occurrence of gene fragments necessary for the biosynthesis of major natural products, namely Non-ribosomal peptide synthetases (NRPS) and Polyketide synthases (PKS) was applied to estimate the genetic potential of these active strains. Both isolates were capable of amplifying the NRPS gene fragments but not the PKS gene fragments.

Key words: antibacterial, sponge associated-bacteria, PKS, NRPS, North Java Sea

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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

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Original Paper

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 for the production of secondary metabolites against pathogenic *Escherichia coli* 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

Sampling and isolation of sponge-associated bacteria

Colonies of sponges were collected from Bandengan water, Jepara, and Karimun island, Karimunjawa islands, 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 cubic centimeter of sponge tissue was excised from the middle of the whole sponge and sponge surface after it is 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).

Screening of sponge bacteria with biological activity

Screening and antibacterial tests as well as molecular based-works were carried out at Marine Microbiology Laboratory, Diponegoro University, Semarang, Indonesia. To screen their biological activity, a total of 90 sponge isolates **(Table 1)** were tested against isolate pathogenic bacterium *Escherichia coli*. 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 coral 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.

Table 1. Number of isolate obtained from sponges

Location	Number of Sponge	Media Zobell	Media Actinomycetes	Number of isolate
• Bandengan	6	42	15	60
• Karimun	4	25	5	30
Total	10	67	20	90

PCR-based analysis of NRPS and PKS producing bacterial strains

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

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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). All primers were manufactured by MWG-Biotech (Ebersberg, Germany).

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. *Pseudomonas* 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.

PCR amplification and sequencing of 16S rRNA gene fragments.

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 rRNA gene 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

Inhibitory interactions among sponge-associated bacteria that occur within the sponge surface are of great interest to search for secondary metabolite-producing bacteria. Isolation and screening for secondary metabolite-producing bacteria in coral reef ecosystems have been strongly neglected until now in comparison with the invertebrate parts.

The present study indicated that 2 among 90 marine bacteria associated with sponges showed growth inhibition against indicator microorganism **(Table 1).** This offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria such as *Escherichia coli*.

Table 2. Inhibitory interaction of sponge associated-bacteria agains *E.coli*

No	Isolate	Zone of inhibition (mm)
1	BSP.12	8.9 ± 0,004
2	MKSP.5	10.3 ± 0,02

Molecular identification based on 16S rDNA approach showed that the two active isolates are the member of genera *Vibrio* and *Brachybacterium* **(Table 2)**.

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Table 3. Molecular identification of active isolates

No	Isolate	Closest relative	Homology(%)	Acc. number
1.	MKSP.5	*Vibrio harveyi* strain LB5	100	DQ146936
2.	BSP.12	*Brachybacterium rhamnosum*	99	AJ415376

As shown in the Table 2, isolate MKSP.5 has a high homology to *Vibrio harveyi* with (100%), on the other hand, isolate BSP.12 is closely related to *Brachybacterium rhamnosum* (99%). The detailed alignments of DNA sequences between isolate MKSP.5 and *Vibrio harveyi* LB5 (accession number DQ146936) and isolate BSP.5 and *Brachybacterium rhamnosum* (AJ41537) are shown in the **fig. 1 and 2.**

The members of Alteromonadales and Vibrionales of the proteobacteria, such as *Pseudoalteromonas* and *Vibrio* have been known as the dominant antibiotics producers (Long and Azam, 2001; Grossart et al, 2004). Furthermore, a bacterium BSP1.12 isolated from sponge *Haliclona* sp. that closely related to *Vibrio parahaemolyticus* and inhibited only the growth of the tested bacterium *Aeromonas hydrophila* known as the causative agent of the disease *Motile Aeromonas Septicemia* (MAS) in many freshwater fishes (Radjasa et al, 2007c).

One of the active isolate BSP.12 showed close relativeness to the member of actinomycetes. The actinomycetes, although not all the Actinobacteria, are easy to isolate from the marine environment. However, their ecological role in the marine ecosystem is largely neglected and various assumptions meant there was little incentive to isolate strains for search and discovery of new drugs. However, the marine environment has become a prime resource in search and discovery for novel natural products and biological diversity, and marine actinomycetes turn out to be important contributors (Ward and Bora, 2006).

Among these five, the Class Actinobacteria, and more specifically, bacteria belonging to the Order Actinomycetales (commonly called actinomycetes) account for approximately 7000 of the compounds reported in the Dictionary of Natural Products. Looking individually at the more than 140 currently described actinomycete genera, it becomes clear that even within this Order it is a few well-known soil genera that account for the vast majority of microbial natural products discovered. In fact, the genus Streptomyces alone accounts for a remarkable 80% of the actinomycete natural products reported to date, a biosynthetic capacity that remains without rival in the microbial world (Jensen et al, 2005).

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)

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Original Paper

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 Score = 1604 bits (809),  Expect = 0.0
Identities = 809/809 (100%), Gaps = 0/809 (0%)
Strand=Plus/Minus

Query  4     GCGTCCTCCCGAAGGTTAAACTACCTACTTCTTTTGCAGCCCACTCCCATGGTGTGACGG  63
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  GCGTCCTCCCGAAGGTTAAACTACCTACTTCTTTTGCAGCCCACTCCCATGGTGTGACGG  1376

Query  64    GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGC  123
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGC  1316

Query  124   GATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTTTGG  183
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1315  GATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTTTGG  1256

Query  184   GATTCGCTCACTATCGCTAGCTTGCTGCCCTCTGTATGCGCCATTGTAGCACGTGTGTAG  243
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1255  GATTCGCTCACTATCGCTAGCTTGCTGCCCTCTGTATGCGCCATTGTAGCACGTGTGTAG  1196

Query  244   CCCTACTCGTAAGGGCCATGATGACTTGACGTCGTCCCCACCTTCCTCCGGTTTATCACC  303
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1195  CCCTACTCGTAAGGGCCATGATGACTTGACGTCGTCCCCACCTTCCTCCGGTTTATCACC  1136

Query  304   GGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGGCAAACAAGGATAAGGGTTGCGCTC  363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1135  GGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGGCAAACAAGGATAAGGGTTGCGCTC  1076

Query  364   GTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGT  423
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075  GTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGT  1016

Query  424   CTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTAGG  483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1015  CTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTAGG  956

Query  484   TAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCG  543
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  955   TAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCG  896

Query  544   TCAATTCATTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTAACGCGTT  603
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  895   TCAATTCATTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTAACGCGTT  836

Query  604   AGCTCCGAAAGCCACGGCTCAAGGCCACAACCTCCAAGTAGACATCGTTTACGGCGTGGA  663
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835   AGCTCCGAAAGCCACGGCTCAAGGCCACAACCTCCAAGTAGACATCGTTTACGGCGTGGA  776

Query  664   CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCATCTGAGTGTCAGTATCTG  723
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Sbjct  775   CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCATCTGAGTGTCAGTATCTG  716

Query  724   TCCAGGGGGCCGCCTTCGCCACCGGTATTCCTTCAGATCTCTACGCATTTCACCGCTACA  783
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715   TCCAGGGGGCCGCCTTCGCCACCGGTATTCCTTCAGATCTCTACGCATTTCACCGCTACA  656

Query  784   CCTGAAATTCTACCCCCCTCTACAGTACT  812
             |||||||||||||||||||||||||||||
Sbjct  655   CCTGAAATTCTACCCCCCTCTACAGTACT  627
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Fig1. The DNA alignment showing the homology of isolate MKSP.5 and *Vibrio harveyi* based on BLAST system.

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 Score = 1092 bits (551),  Expect = 0.0
 Identities = 554/555 (99%), Gaps = 0/555 (0%)
 Strand=Plus/Minus


Query  1     GTTACCGACTTTCGTGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC  60
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Sbjct  1378  GTTACCGACTTTCGTGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC  1319

Query  61    AGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCC  120
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Sbjct  1318  AGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCC  1259

Query  121   AATCCGAACTGAGACCGGCTTTTTGGGATTCGCTCCACCTCACAGTTTCGCAACCCATTG  180
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  1258  AATCCGAACTGAGACCGGCTTTTTGGGATTCGCTCCACCTCACAGTTTCGCAACCCTTTG  1199

Query  181   TACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1198  TACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCG  1139

Query  241   TCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATGAGTCCCCGCCATTACGCGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1138  TCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATGAGTCCCCGCCATTACGCGCT  1079

Query  301   GGCAACATGGAACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1078  GGCAACATGGAACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA  1019

Query  361   GCTGACGACAACCATGCACCACCTGTGCACCAGTCCGAAGAAAACCCCATCTCTGGAGTC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1018  GCTGACGACAACCATGCACCACCTGTGCACCAGTCCGAAGAAAACCCCATCTCTGGAGTC  959

Query  421   GTCCGGTGCATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  958   GTCCGGTGCATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGC  899

Query  481   TCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  898   TCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCC  839

Query  541   CCAGGCGGGGCACTT  555
             |||||||||||||||
Sbjct  838   CCAGGCGGGGCACTT  824
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Fig 2. The DNA alignment showing the homology of isolate BSP.12 and *Brachybacterium rhamnosum* based on BLAST system.

and non ribosomal polypeptide synthetases (NRPS) (Marahiel *et al.*, 1997).

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. Vibrio is among the dominant producers of peptide bioactive products. Liu et al (2005) reported that *Vibrio anguillaum* produced substance as virulent factor that was synthesized by a non-ribosomal peptide synthetases (NRPS). Two member of Vibrio, namely *V. coralliitycus* MJ.5 and *V. parahaemolyticus* MJ.11 were obtained from coral *Porites lutea* that have antibacterial activity against *Bacillus subtilis* and *Staphylococcus lentus.* Interestingly, isolate *V. coralliitycus* MJ.5 was also amplified NRPS gene fragments (Radjasa *et al*, unpublished).

Ayuso-Sacido and Genilloud (2004) reported that the members of Actinomycetes have been reported to produce various antibacterial compounds which are also synthesized by non-ribosomal peptide synthetases (NRPS). In this study, an

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occurrence of NRPS gene fragment within *Brachybacterium rhamnosum* is reported for the first time. It is indicated that there are more genera within the Actinomycetes that offer possibility as the source of antibacterial compounds.

CONCLUSION

In conclusion, sponges from North Java Sea exhibited secondary metabolite producing-marine bacteria with antibacterial potential against *E. coli*. 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 search for secondary metabolite-producers among sponge colonizers.

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