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Antimycobacterial Activities from Seagrass *Enhalus* sp. Associated Bacteria Against Multi Drug Resistance Tuberculosis (MDR TB) Bacteria

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ABSTRACT

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and the most important public health problem in the world. In 2012, there were an estimated 8.6 million incident cases of TB globally, and estimeated 67,000 deaths among TB cases in 2012. Indonesia ranks 4th on the list of TB high burden countries in the world with 450,000 cases, estimated cases of MDR TB were 5700 and 65.000 deaths among TB cases in Indonesia, 2011.This research was conducted to isolate and characterize of seagrass *Enhalus* sp. -associated bacteria that having antimycobacterial activity against multi drug resistant tuberculosis bacteria. There were 9 isolates collected from *Enhalus* sp. One isolate from *Enhalus* sp.-associated bacteria were successfully screened for antimycobacterial against tuberculosis bacteria. EKJP9 was found to inhibit the growth of tuberculosis bacteria (MDR TB strain HE , SR, HR). Based on DNA Data Bank of Japan the accession number of EKJP9 is AB851799. It is *Bacillus* sp.

Key words: *Mycobacterium tuberculosis*, *Enhalus* sp, MDR-TB, antimycobacterial activities, associated bacteria

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1. Introduction

Tuberculosis (TB) remains a major global health problem. The disease is spread in the air. In general,

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people infected with *Mycobacterium tuberculosis* will develop TB disease which increase the numbers of infected people moving into the community and thus expose the general population to the risk of contracting a resistant strain of infection. In 2012, there were an estimated 8.6 million incident cases of TB globally,equivalent to 185 cases per 100,000 population, And estimated 67,000 deaths among TB cases. Indonesia ranks 4th on the list of TB high burden countries in the world. It is estimated that total cases of TB were 460,000, and cases deaths were 67,000 [1]. Today some strains of bacterial infections are treatable with only a single drug; some no longer have effective treatments. Infections caused by resistant microbes may fail to respond to treatment, resulting in prolonged illness and greater risk of death. The past 20 years have seen the worldwide appearance of multidrug-resistant (MDR) TB. MDR TB is caused by *Mycobacterium tuberculosis* that is resistant at least to isoniazid and rifampicin, the most effective anti-TB drugs. These drugs are considered first-line drugs and are used to treat all persons with TB disease [2]. Globally in 2012, there were an estimated 450,000 cases of MDR TB in the world and estimated cases MDR TB in Indonesia were 5,700[1].

The oceans, with their unique and wide range of biodiversity, producing unusual metabolites, emerges as a good candidate for new antimycobacterial agents. The development of marine organisms-derived compounds into drugs has been held back by supply limitations. Several marine organisms have adapted themselves through symbiotic association among themselves that help them survive under harsh environments. Symbioses between microorganisms and marine organisms are abundant and widespread in the sea. Most marine invertebrates and algae harbor diverse microbial symbionts including prokaryotic bacteria, archaea, cyanobacteria, and fungi. Increasing evidence implicates microbial symbionts as the true source of many marine organism-derived compounds, which makes marine microbial symbionts a hotspot in the field of marine microbiology and marine natural products because of their potential for solving the bottleneck problem of marine natural product supply[2] Although marine microorganisms are not well defined taxonomically, preliminary studies in this field unequivocally indicate that the wealth of microbial diversity in the world's oceans, coupled with its biochemical uniqueness, make this a promising frontier for the discovery of new medicines[3]. Appear of resistant TB drugs need a new antibiotic alternative to richness the kind of TB drugs. On the other side Indonesia has biodiversity in ocean that needs to know it's big potentially, so we were very interest to know the antimycobacterial activity from sea grass *Enhalus* sp. associated bacteria against Multi Drug Resistant Tuberculosis bacteria. The research purpose was to isolate and characterize of seagrass *Enhalus* sp. -associated bacteria that having antimycobacterial activity against multi drug resistant tuberculosis bacteria.

2. Material and Method

The study was conducted at Karimunjawa-Jepara,North Java Sea Indonesia. Sea grass *Enhalus* sp. were put into sterile plastic bags (Whirl-Pak, Nasco USA). The tissue were rinsed with sterile seawater and homogenized with blender. The homogenized tissue were serially diluted, spread on half strength ZoBell 2216E marine agar medium and incubated at room temperature for 2x24 hours. On the basis of morphological features colonies were randomly picked and purified by making streak plates[4].

2.1. Antibacterial test

Antibacterial test of *Enhalus* sp. associated bacteria against MDR TB bacteria was performed by using an overlay and dilution method. MDR TB bacteria (MDR TB strain HE resistant to Isoniazid and Ethambutol,strain SR resistant to Streptomycin and Rifampicin, and strain HR resistant to Isoniazid and Rifampicin) used in this study were obtained from Health Laboratory Of Center of Java Provence-Semarang. Culture of each bacterium in the logarithmic phase was mixed with Middle brook 7H9+OADC soft agar medium (1% v/v), which were poured on to the respective agar surface previously inoculated with *Enhalus* sp. associated bacteria that had incubated for 4 days at room temperature. Then the plates were incubated at room temperature 2x24 hours. Antibacterial activity was defined by the formation of inhibition zones around the MDR TB bacterial colonies and the minimum inhibitory concentration of extract EKJP9 at 25%, 50%,75% and 100%.

2.2.PCR amplification and DNA sequencing

PCR amplification was carried out according to the method of Radjasa et al (2007). Universal primers described by Weisburg et al (1991) was used for PCR amplification. Genomic DNA of 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 or partial 16S rRNA gene of *Enhalus* sp. associated bacteria and subsequent sequencing analysis were performed according to method of Radjasa et al (2007)[5]. The determined DNA sequences of strains were compared for homology to the BLAST database.

3. Results and Discussion

There were nine isolates association bacteria from sea grass *Enhalus* sp. The morphology identification shown in Table 1.

Tabel 1. Morphology Identification of *Enhalus* sp. Assosiated Bacteria

Sample No.	Code of bacteria	Type of biota	Shape	Colour	Textur
47	EKJP1	Enhalus	Round	Brown	Convex
48	EKJP2	Enhalus	Round	Yellow	Convex
49	EKJP3	Enhalus	Round	White	Rough
50	EKJP4	Enhalus	Oval	White	Smooth
51	EKJP5	Enhalus	Oval	Yellow	Convex
52	EKJP6	Enhalus	Round	White	Rough
53	EKJP7	Enhalus	Round	Brown	Convex
54	EKJP8	Enhalus	Round	Transparant	Rough
55	EKJP9	Enhalus	Little Round	Yellow	Rough

Only one isolate association bacteria were found to inhibit the growth of MDR TB bacteria (MDR TB strain HE, strain SR, and strain HR) as shown in Table 2.

Table 2. Inhibit zone from EKJP9 against MDR TB

No.	Isolate	Inhibit zone (cm)
1	No 55 EKJP9 to MDR TB HE	0.390
2	No 55 EKJP9 to MDR TB SR	0.240
3	No 55 EKJP9 to MDR TB HR	0.330

Multi drugs resistant tuberculosis (MDR TB) that used for this research were MDR TB strain HE (*Mycobacterium tuberculosis* resistant Isoniazid and Etambutol), MDR TB strain SR (*Mycobacterium tuberculosis* resistant Streptomycin and Rifampicin), MDR TB strain HR (*Mycobacterium tuberculosis* resistant Isoniazid and Rifampicin). Isoniazid, Rifampicin, Etambutol and Streptomycin are the first line drugs for tuberculosis. The Sea grass, *Enhalus* sp. associated bacteria EKJP9 had potential antibacterial activities. Their potential antibacterial were from secondary metabolites microorganism that produced when bacteria cell finished the logarithmic phase and going into stationer phase. This phase is called "idiophase". Secondary metabolite microorganism can come from the conversion of primary metabolites[6].

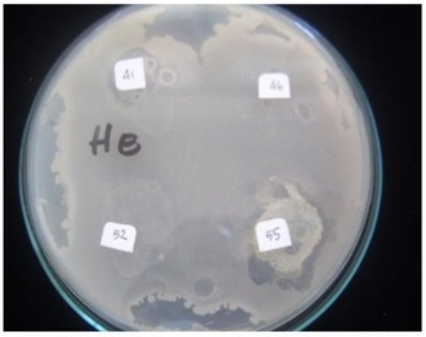

Fig 1. Inhibit the growth of MDR TB HE

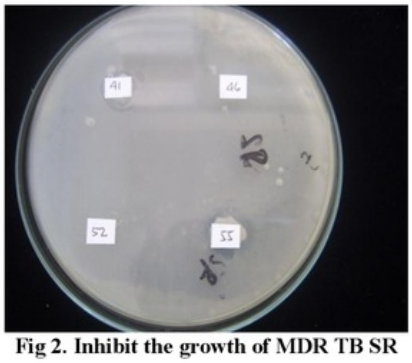

Fig 2. Inhibit the growth of MDR TB SR

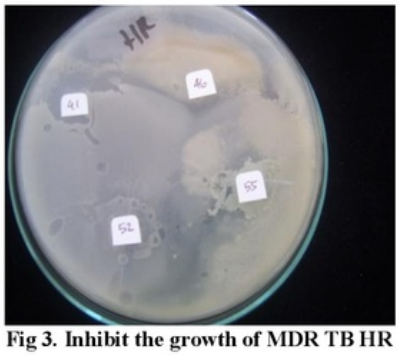

Fig 3. Inhibit the growth of MDR TB HR

The previous study showed that associated bacteria from soft coral *Sinularia* sp. exhibited secondary metabolite producing marine bacteria with antibacterial activity that potential against tuberculosis bacteria: *Mycobacterium tuberculosis H37Rv* and the resistant strains: MDR TB strain HE and MDR TB strain SR[7].

Table 3. Turbidity level at variation contration of extract EKJP9 to MDR TB HE

Incubation (hours)	Concentration of extract EKJP9(%)					
	100	75	50	25	0	Control
0	1744	1.600	1.254	0.592	0.018	0.027
24	2.080	2.024	1.761	1.295	0.920	1.011
48	1.862	1.716	1.570	1.991	1.237	1.690

Minimum turbidity from table 3 was 0.018 at 0% concentration of extract EKJP9 and the maximum was 2.080 at 100% concentration of extract EKJP9. There were turbidity decrease from 50%, 75% until 100% concentration of extract EKJP9.

Table 4. Turbidity level at variation contration of extract EKJP9 to MDR TB HR

Incubation (hours)	Concentration of extract EKJP9(%)					
	100	75	50	25	0	control
0	1.910	1.649	1.428	0.873	0.076	0.139
24	1.922	1.714	1.578	1.219	0.606	0.853
48	1.748	1.237	1.270	1.156	0.842	1.210

Minimum turbidity from table 4 was 0.076 at 0% concentration of extract EKJP9 and the maximum was 1.922 at 75% concentration of extract EKJP9. There were turbidity decrease from 25%, 50%, 75% until 100% concentration of extract EKJP9.

Table 5. Turbidity level at variation contration of extract EKJP9 to MDR TB SR

Incubation (hours)	Concentration of extract EKJP9(%)					
	100	75	50	25	0	control
0	1.748	1.539	1.320	0.784	0.030	0.024
24	1.874	1.792	1.648	1.372	0.718	0.460
48	1.730	1.433	1.384	1.551	1.280	0.764

Minimum turbidity from table 5 was 0.024 at control concentration of extract EKJP9 and the maximum was 1.874 at 100% concentration of extract EKJP9. There were turbidity decrease from 50%, 75% until 100% concentration of extract EKJP9.

The minimum inhibitory concentration of EKJP9 extracts that can inhibit the growth of MDR TB bacteria was the concentration of 75%, because there were no difference between the minimum inhibitory concentration of EKJP9 extract concentration 75% to 100% concentration. Extraction was done in this study still produces many compounds, it was not pure compounds. The compounds such as alkaloids, saponins and triterpenoids. The mechanisms involved in inhibiting the growth of MDR TB bacteria can not be known, whether the mechanism of the synergism of the various chemical constituents from many compounds, or only one of the activities of the chemical constituents.

Isolate EKJP 9 Complete sequence (1272 nukleotida)

aacctgcctg taagactggg ataactccgg gaaaccggag ctaataccgg atagttcctt gaaccgcatg gttcaaggat gaaagacggt ttcggctgtc acttacagat ggacccgcgg cgcattagct agttggtgag gtaacggctc accaaggcga cgatgcgtag ccgacctgag agggtgatcg gccacactgg gactgagaca cggcccagac tcctacggga ggcagcagta gggaatcttc cgcaatggac gaaagtctga cggagcaacg ccgcgtgagt gatgaaggtt ttcggatcgt aaagctctgt tgttagggaa gaacaagtgc aagagtaact gcttgcacct tgacggtacc taaccagaaa gccacggcta actacgtgcc agcagccgcg gtaatacgta ggtggcaagc gttgtccgga attattgggc gtaaagggct cgcaggcggt ttcttaagtc tgatgtgaaa gcccccggct caaccgggga gggtcattgg aaactgggaa acttgagtgc agaagaggag agtggaattc cacgtgtagc ggtgaaatgc gtagagatgt ggaggaacac cagtggcgaa ggcgactctc tggtctgtaa ctgacgctga ggagcgaaag cgtggggagc gaacaggatt agataccctg gtagtccacg ccgtaaacga tgagtgctaa gtgttagggg gtttccgccc cttagtgctg cagctaacgc attaagcact ccgcctgggg agtacggtcg caagactgaa actcaaagga attgacgggg gcccgcacaa gcggtggagc atgtggttta attcgaagca acgcgaagaa ccttaccagg tcttgacatc ctctgacaac cctagagata gggctttccc ttcggggaca gagtgacagg tggtgcatgg ttgtcgtcag ctcgtgtcgt gagatgttgg gttaagtccc gcaacgagcg caacccttga tcttagttgc cagcattcag ttgggcactc taaggtgact gccggtgaca aaccggagga aggtggggat gacgtcaaat catcatgccc cttatgacct gggctacaca cgtgctacaa tggacagaac aaagggctgc gagaccgcaa ggtttagcca atcccacaaa tctgttctca gttcggatcg cagtctgcaa ctcgactgcg tgaagctgga atcgctagta atcgcggatc agcatgccgc ggtgaatacg ttcccgggcc tt

Sequencing of the 16S rRNA gene is presently the most commonly used molecular approach in bacterial phylogeny (Woese et al., 1990). Using this methodology, The EKJP9 isolate was *Bacillus* sp., as the accession number AB851799 from the DNA Data Bank of Japan. The confrontation test showed that the isolate produced active substances, the active substances could be antibiotics since most natural antimicrobial agents of bacterial origin are mainly synthesized by *Bacillus* and actinomycetes[8]. The accesion number of EKJP9 is AB851799 from the DNA Data Bank of Japan (DDBJ).

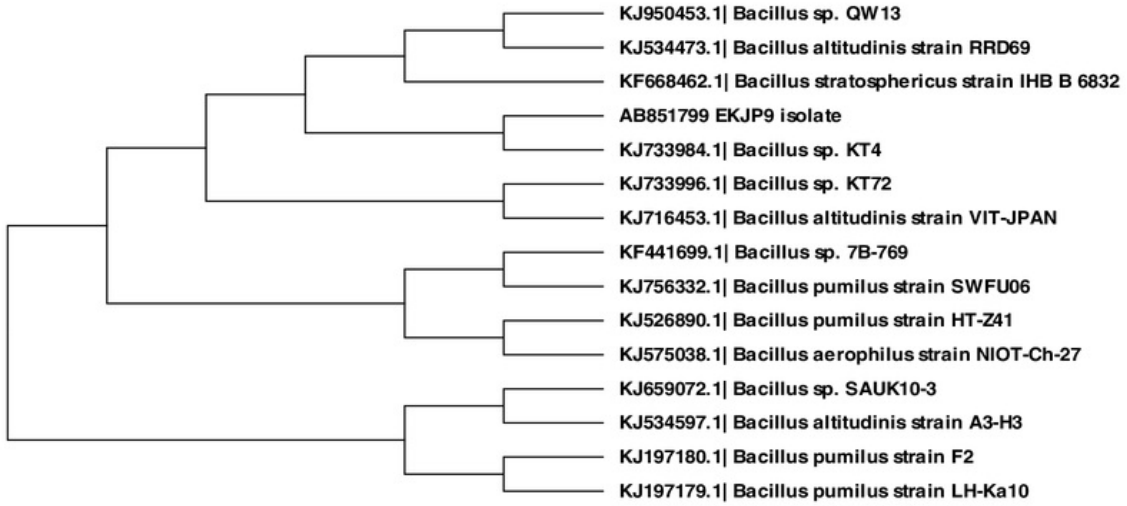

Fig 4. Molecular Phylogenetic analysis by Maximum Likelihood method

Recently, the systematic of the *Bacillus* group has been widely modified. Few publications are devoted to the study of the *Bacillus* species isolated from the marine environment. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats. Species of *B. marinus, B. badius B. subtilis, B. cereus, B. licheniformis, B. firmus,* and *B. lentus* were often isolated from marine habitats. The marine bacilli showed that strains of *B. marinus, B. subtilis, B. pumilus, B. licheniformis, B.cereus,* and *B. mycoides* are common inhabitants of the Pacific Ocean habitat. A group of marine *Bacillus* strains from the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bioorganic Chemistry (Vladivostok, Russia) has been taxonomically studied in view of their ability to produce biologically active compounds. One isolate, KMM 1717, associated with a sponge from the Coral Sea was identified as *B. pumilus.* Two strains, *Bacillus* KMM 1916 and KMM 1918, showed antibiotic sensitivity profiles similar to *B. licheniformis,* but they had a distinct fatty acid composition and peculiar phenotypic traits. A few bacilli of marine origin have been reported to produce unusual metabolites different from those isolated from terrestrial bacteria. These metabolites include an antibiotic, 3-amino-3-deoxy-D-glucose, a new glucanase, and cyclic acylpeptides [9].

Bacillus is a gram_positive bacteria with rod shaped and a member of the division Firmicutes. *Bacillus* species can be obligate aerob or facultative anaerob, and test positive for the catalase enzyme. Ubiquitous in nature, *Bacillus* includes both free-living and pathogenic species[4].

The number of antibiotics produced by genus Bacillus was 167. These antibiotics are mainly polypeptides. 66 different peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis*. Polymyxin and the closely related colistin, bacitracin, the tyrothricin complex (linear gramicidin plus tyrocidine), and gramicidin S have been used, to some extent, for antibacterial therapy. Most of the peptide antibiotics produced by bacilli are active against gram-positive bacteria; however, compounds such as polymyxin, colistin, and circulin exhibit activity almost exclusively upon gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts[10].

The *Bacillus* sp. showed inhibitory effect on mycobacterial growth of *Mycobacterium smegmatis* and *Mycobacterium aurum* (Non Tuberculosis Mycobacteria)[8]. The strain of *B. pumilus* capable of inhibit mycobacterial growth (*M. smegmatis*, *M. aurum* A+ and *M. bovis* BCG) through the action of a protein metabolite[11].

4. Conclusion

The EKJP9 had antimycobacterial activity that can inhibit the growth of MDR TB bacteria with inhibitory growth zone and with extract of EKJP9 75%. Isolate EKJP9 identified as *Bacillus* sp. based on accesion number AB851799 by DNA Data Bank of Japan.

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