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Phylogenetic Analysis and Screening of Hydrolytic Bacteria with Hydrolase Enzyme Activity from Hospital Wastewater of Semarang, Central Java, Indonesia

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ABSTRACT

Hydrolytic bacteria have the ability to convert organic material which is very useful in the bioremediation of biomedical waste. The objective of the study was to investigate the hydrolytic bacterial diversity that has the hydrolase enzyme activity. The medical waste sampling was carried out at Roemani Hospital and KRMT Wongsonegoro Hospital, Semarang Central Java in mid-August 2016. Microbiological and molecular studies, covering isolation and purification, hydrolase productivity tests, and bacterial determination, were conducted at the Tropical Marine Biotechnology Laboratory, Faculty of Fisheries and Science, UNDIP. The 16S rDNA gene sequence analysis was carried out by using the BLAST GeneBank NCBI homology test, while the phylogenetic tree construction was carried out by means of Clustal X software and Phylegenetic analysis using parsimony (PAUP vers 4.0). The test results showed that 26 hydrolytic bacterial isolates were able to produce four types of extracellular hydrolase enzymes. The results of the enzyme production test showed that there were only 2 bacterial isolates capable of producing the four types of enzymes. Microbiological and molecular polyphasic identifications showed that the 2 isolates, R1-17, and R2-6 isolates are closely related to *Virgibacillus salarius* strain B-11 and *Bacillus subtilis* strain VITVB1, respectively. Even though there are only 2 isolates capable of producing the 4 types of extracellular enzymes, it seems that hospital biomedical waste is a potential source for obtaining hydrolytic bacteria.

Keywords: hydrolytic bacteria, 16S rDNA gene, medical waste, *Virgibacillus salarius*, *Bacillus subtilis*

INTRODUCTION

Liquid biomedical waste mainly contains organic matter, while the role of hydrolytic bacteria in the degradation of organic waste is widely known. However, the microbiology of hospital wastewater remains poorly explored [Shiferaw et al. 2012; Babanyara 2013]. Investigating the diversity of indigenous hydrolytic bacteria in hospital waste reservoirs is among the crucial steps to develop a bacterial bioremediator for hospital wastewater [Colin et al. 2012]. The research aimed to investigate the culturable hydrolytic bacterial diversity with various hydrolytic enzymes from liquid biomedical wastes. The amount of liquid biomedical wastes is expected to increase due to the higher number of hospitals in Indonesia,

particularly in the Central Java Province [Ethica and Sabdono 2017]. Liquid biomedical wastes of hospitals mainly consist of organic matter coming from clinical activities [Radha et al. 2009]. Fortunately, the ability of hydrolytic bacteria in improving the water pollution parameter values of organic wastes is widely known [Abd El-Salam 2010]. Hence, the use of hydrolytic bacteria as bioremediation agents to solve the liquid biomedical waste problem is quite promising [Ethica and Sabdono 2017; Ethica et al. 2017; 2018]

In Indonesia, the studies on the diversity of the bacterial community related to bioremediation on the aquatic ecosystem are mainly associated with corals [Agus Sabdono et al. 2005; Sarjito et al. 2008; Sabdono and Radjasa 2008]. The research on the biodiversity of hydrolytic bacteria from

hospital waste was reported from several countries. However, the results of the studies achieved were not as intensive as the microorganisms isolated from other wastes. Chitnis et al. [2004] reported on the dynamics of bacterial populations at several waste treatment facilities in India. Likewise, the research conducted by [Anitha and Jayraaj 2012] reported that several types of bacteria isolated from hospital wastewater had the potential to degrade liquid medical wastes. Li and Yu [2015] demonstrated that the bacteria isolated from hypersaline environments are capable of not only producing extra polymer hydrolase enzymes but also exhibit antimicrobial activity.

The genetic diversity of culturable bacteria inhabiting biomedical waste based on bacterial 16S rRNA genes had been reported by Mwaikono [2015] in Tanzania. However, the reported biodiversity study was focused on solid biomedical samples from dumpsite only. To date, there has been barely any report about the hydrolytic bacterial biodiversity from liquid hospital waste reservoirs. Ethica et al. [2018] reported how to obtain hydrolytic bacterial bioremediation agents. Nevertheless, the microbiology of liquid hospital wastewater remains poorly explored. Among the crucial steps to develop a bioremediation agent was to study the diversity of indigenous bacteria [Gagne-Bourgue et al. 2013; Dang et al. 2009]. The diversity of biomedical wastes-degrading bacteria could be identified by their ability to produce various hydrolytic enzymes. Besides, these bacteria could be examined and subsequently used as a bioremediatory agent to decrease the health risk of the biomedical waste from hospitals. Hence, it is urgently needed to investigate the diversity of culturable hydrolytic bacteria from liquid biomedical wastes at two primary reservoirs of different classes of hospitals. This work aimed to investigate the diversity of culturable hydrolytic bacteria with various hydrolytic activities from liquid biomedical wastes.

MATERIALS AND METHODS

Bacterial isolation and hydrolytic activity screening

Bacterial isolates were screened qualitatively for their hydrolytic activity, DNAase activity, phosphatase activity and Starch hydrolysis, according to the methods as described by Ethica et al. (2017).

DNA extraction and 16S rRNA gene amplification

The DNA from each isolate was extracted from cells by taking bacterial cells from an agar plate, suspended in sterile water (Sigma, Germany), and given the physical treatment of freezing (-70°C) and thawing (95°C). PCR amplification and purification of the 16S rDNA gene from the strain were carried out according to the method of Radjasa et al. [2007]. The PCR mixture consists GoTaq®Green Master Mix Promega (12.5 µL), primer 27 F (1 µL), primer 1492 R (1 µL), template DNA (1 µL), and ddH₂O (9.5 µL) so that the total volume was 25 µL. The primers used for 16S rDNA PCR were universal primers 27F (5'-AGAGTTTGTATCMTGGCTCAG-3') and eubacteria specific primers 1492R (5'TACGGY-TACCTTACGACTT-3'). PCR involved performing 30 cycles with denaturation at 94°C for 5 min as a hot start, then 30 cycles (denaturation at 94°C for 30 seconds, annealing at 54°C for 60 seconds (1 minute), extension at 72°C for 60 seconds), then extra extension for 2 minutes, and finally 4°C ~.

16S rDNA gene sequences

The results of PCR amplification of the 16S rDNA gene were sequenced at PT. Genetika Science, Jakarta. The results of the sequences were then analyzed for homology using the BLAST database and deposited to GeneBank to obtain the accession number.

Nucleotide sequence accession numbers

All bacterial sequences reported in this study have an accession numbers LC414157 - LC414181.

Phylogenetic analysis

CLUSTAL X and PAUP*4.0 software were used to construct a Phylogenetic tree [Swofford 1998].

RESULTS AND DISCUSSION

Bacterial hydrolytic activities

The bacterial isolates were screened for protease, amylase, lipase, and cellulase activities.

Table 1. Hydrolytic activities of hospital waste isolates (Ethica et al., 2018)

Σ active Enzyme	Hydrolytic activity				Σ Isolates	Total of isolates (%)
	Lipolytic	Proteolytic	Amylolytic	Cellulolytic		
1	x	v	x	x	4	4 (15.38)
2	v	v	x	x	5	7 (26.92)
	x	v	v	x	1	
	x	v	x	v	1	
3	x	v	v	v	11	13 (50.0)
	v	v	v	x	1	
	v	v	x	v	1	
4	v	v	v	v	2	2 (7.69)
Total						26

Note: v – active, x – no activity.

Table 1 showed that only two (7.69 %) of 26 of hydrolytic bacterial isolates are capable of producing all those types of hydrolase enzymes. Followed by 13, 7, and 4 isolates which can produce 3, 2, and 1 type of enzymes, respectively.

The protease was produced by all of these strains. Most isolates (50%) possessed multiple hydrolytic activities, including protease, amylase, lipase, and cellulase. The R1-17 and R2-6 isolates were selected for further studies.

Several previous studies of hydrolytic bacteria on the taxonomy and biotechnological applications have been conducted [Sanchez-Porro et al. 2003; Rohban et al. 2009; Baati et al. 2010]. However, limited information on the understanding of the phylogenetic composition of bacterial hydrolytic communities associated with hospital wastewater is available. In this study, we reported that the bacteria isolated from hospital wastewaters possessed multiple hydrolytic activities, including protease, amylase, lipase, and cellulase (Table 1). Ethica et al. [2017] and Ferreira et al. [2011] reported that the primary inlet of hospitals is a promising source of cultivable hydrolytic bacteria. Similar results were also reported on extensively exploited hydrolytic activities of bacteria from various environmental sources, such as hypersaline soils [Sorokin et al. 2017; Sanchez-Porro et al. 2003] deep-sea sediment [Dang et al. 2009], Sea Arctic ice [Groudieva et al. 2004] and lake [Rohban et al. 2009].

16S rRNA gene sequence identification

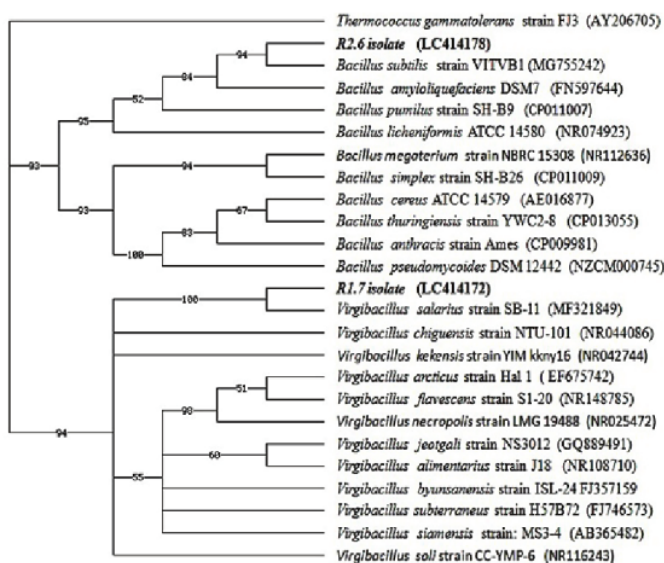
Table 2 showed that all strains belong to the *Bacillus* genera, except 3 isolates (R1-11, R1-17, and R2-1) which are identified as the *Virgibacillus*

genera. The phylogenetic tree R2-6 and R1-17 isolates represent the *Bacillus* and *Virgibacillus*, respectively, and the related members from these genera were constructed as shown in Figure 1.

All sequences reported in this study have the Accession Numbers LC414157 to LC414181. In general, this study showed the presence of an abundant hydrolytic bacterial community from the *Bacillus* group in liquid biomedical waste of hospitals. A total of 26 isolates can produce one to three types of enzymes. Four hydrolytic enzyme activities were achieved by the R1-17 and R2-6 isolates. Phylogenetic analyses showed that the R1-17 and R2-6 isolates are closely related to the *Virgibacillus salarius* strain B-11 and the *Bacillus subtilis* strain VITVB1, respectively (Figure 1). Different results from some previous studies showed that most of the poly-hydrolytic bacteria isolated from hypersaline environments belonged to Firmicutes and Actinobacteria [Sorokin et al. 2017], *Salinivibrio* [Sanchez-Porro et al. 2003], and *Halomonas* [Ruginescu et al. 2019]. These isolates might be explored further for the production of bioremediation agents. Cammarota and Freire [2006] reported the use of hydrolytic enzymes to treat wastewater with high oil and grease content.

CONCLUSION

The results of this study demonstrate that the biodiversity of bacteria isolated from the hospital wastewater has hydrolytic activity. All bacterial strains showed positive activity for hydrolytic enzymes like protease. *Bacillus* was the dominating genus from the hospital liquid



27 **Figure 1.** A phylogenetic tree based on the 16S rRNA gene sequences between R2-6 and R1-17 isolates belonging to the genus *Bacillus*, *Virgibacillus*, and the related members from these genera. *Thermococcus gammatolerans* strain FJ3, taken as an outgroup, was used to root the tree

Table 2. The results of homology BLAST analyses of 26 bacterial isolates

No.	Isolate	Acc. No.	Closely related	Similarity, %	Acc. No.
1	R1-1	CP029070-1	<i>Bacillus amyloliquefaciens</i> strain ALB69	100	LC414157
2	R1-2	CP029070-1	<i>Bacillus amyloliquefaciens</i> strain ALB69	100	–
3	R1-3	CP029070-1	<i>Bacillus amyloliquefaciens</i> strain ALB69	100	LC414158
4	R1-4	CP021888-1	<i>Bacillus velezensis</i> strain SRCM100072	100	LC414159
5	R1-5	MF612168-1	<i>Bacillus anthracis</i> strain LOS6	99	LC414160
6	R1-6	CP029070-1	<i>Bacillus amyloliquefaciens</i> strain ALB69	99	LC414161
7	R1-7	MF083049-1	<i>Bacillus anthracis</i> strain USW-ERY-2	100	LC414162
8	R1-8	KX369292-1	<i>Bacillus</i> sp- M2-12(2016)	100	LC414163
9	R1-9	MF083049-1	<i>Bacillus anthracis</i> strain USW-ERY-2	100	LC414164
10	R1-10	MG027637-1	<i>Bacillus cereus</i> strain VBE41	99	LC414165
11	R1-11	LC385622-1	<i>Virgibacillus</i> sp- TKC 166	99	LC414166
12	R1-12	CP029070-1	<i>Bacillus amyloliquefaciens</i> strain ALB69	99	LC414167
13	R1-13	MF079281-1	<i>Bacillus pumilus</i> strain NES-CAP-1	100	LC414168
14	R1-14	CP029070-1	<i>Bacillus amyloliquefaciens</i> strain ALB69	99	LC414169
15	R1-15	MH362700-1	<i>Bacillus subtilis</i> strain ZGL14	100	LC414170
16	R1-16	KX129756-1	<i>Bacillus</i> sp- CBE330-AF_5	99	LC414171
17	R1-17	LC385622-1	<i>Virgibacillus</i> sp- TKC 166	100	LC414172
18	R2-1	LC385622-1	<i>Virgibacillus</i> sp- TKC 166	100	LC414173
19	R2-2	KY750686-1	<i>Bacillus toyonensis</i> strain DFT-2	99	LC414174
20	R2-3	JX854556-1	<i>Bacillus amyloliquefaciens</i> strain MG1	99	LC4141675
21	R2-4	KY750688-1	<i>Bacillus cereus</i> strain DFT-4	99	LC4141676
22	R2-5	MG988279-1	<i>Bacillus</i> sp- (in: Bacteria) strain PC-3	100	LC414177
23	R2-6	CP020915-1	<i>Bacillus subtilis</i> strain 50-1	100	LC414178
24	R2-7	MF280167-1	<i>Bacillus velezensis</i> strain JS25R	99	LC414179
25	R2-8	LC055679-1	<i>Bacillus</i> sp- GG3(2015)	99	LC414180
26	R2-9	MF280167-1	<i>Bacillus velezensis</i> strain JS25R	99	LC414181

wastewater. The *Virgibacillus salarius* strain R1-17 and *Bacillus subtilis* strain R2-6 have four hydrolytic enzyme activities. Therefore, further studies will be focused on the hydrolase enzyme characterization produced by these bacteria for their potential application as a bioremediation agent for hospital wastewater.

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