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Identification of indigene bacteria from waste water of Regional Public Hospitals in Pacitan

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Abstract: Hospital wastes has a huge impact and harmful to the living creatures around the disposal area, although proper treatment has been performed by using an appropriate waste water treatment plant (WWTP). Ineffective use of an integrated WWTP on wastewater is not be able to fulfill the environmental constitution. It was required more efficient and eco-friendly invention of bioremediation by using indigenic bacteria. The purpose of this study was to identify bacteria species and characteristic of bacteria candidates potentially as bioremediation agent. Indigenic bacteria are obtained from wastes of Dr. Darsono Hospital in Pacitan Regency, which becoming a source of organic nutritions (amylum, protein and lipid) for indigene bacteria. The potency of indigene bacteria in decomposing wastes by degrading organic pollutant substances waste can be determined by calculating the hydrolysis index result. In sequence, four species of bacteria potentially as candidates for bioremediation are *Acinetobacter baumannii*, *Enterobacter agglomerans*, *Aeromonas hydrophila*, and *Burkholderia cepacia*. There were three isolates classified as amylytic bacteria (*Acinetobacter baumannii*, *Enterobacter agglomerans*, and *Aeromonas hydrophila*), *Aeromonas hydrophilla* clasified as proteolytic bacteria and two isolates as lipolytic bacteria (*Burkholderia cepacia* dan *Aeromonas hydrophilla*). *Enterobacter agglomerans* having the highest hydrolysis of amylum with amylytic index = 2.94, *Aeromonas hydrophila* has the highest protein hydrolysis with proteolytic index = 7.80, and *Burkholderia cepacia* has the highest lypid with hydrolysis index = 7.00.

1. Introduction

Hospitals are public facilities for health services that produce waste. The unsolved problems of hospital waste could exhibit danger to the environment and disrupt the lives of people and living things around. In addition, the hospital management must obey the environment constitution regarding waste water disposal management. It should be fulfilled the required quality standards in accordance with the Law of the



Republic of Indonesia Number 44 of 2009 concerning Hospital Article 11 and Government Regulation of the Republic of Indonesia Number 101 of 2014 [1]. However, almost all wastewater treatment technologies are not effective. The main reason is that because of the high costs operational and complexity of the operating system, so that a more effective processing and management system is needed.

Mostly, hospital wastewater contains organic materials. However, alternative waste treatment systems such as biodegradation are considered more appropriate to be used as alternative. The biological use of wastewater treatment process is directed to reduce the organic content in wastewater. Use the activity of microorganisms that should be able to decompose the substrate into a simpler form. Microorganisms decompose organic waste into simple organic compounds by converting them into the forms of carbon dioxide (CO₂), methane (CH₄), hydrogen (H₂) and hydrogen sulfide (H₂S), as well as water (H₂O) and energy destined for their growth and reproduction processes [2].

The use of microbial processes to degrade and change environmental pollutants is known as bioremediation. In its application in the environment, and modification system also needs to be developed so that bioremediation methods can show optimum results [3]. Bioremediation methods can be applied to degrade hospital waste by using its various indigenous microbes. Biological agents such as indigene bacteria are microbes that originally living in the environment. Indigene bacteria play an important role as decomposers in the ecosystem. Indigene bacteria are able to adapt to the environment because they are able to synthesize enzymes for their growth. Exo-enzyme secreted by bacteria can decompose complex molecules to be simpler [4]. The combination of several types of microbes that called the consortium is usually more effective in reducing pollutant levels compared to only one type of microbe. Generally, the degradation process in the environment is carried out by microbial consortiums not just only one type of microbes that remains unknown [4]. Therefore, this paper discusses microbial selection methods that have the potential to degrade hospital waste as candidates for bioremediation.

2. Materials and Methods

2.1. Isolation of indigene bacteria from public hospital Dr Darsono waste in Pacitan Regency

Sample collection was carried out using randomly method. It was taken from the waste disposal channel of Public Hospital Dr. Darsono Pacitan Regency. The sample area was divided into fifteen spots. Each sample was taken using sterile bottles and stored in the cool box. Total volume of 10 ml sample was taken from each point, and then dissolved in 90 ml 0.1% peptone solution for dilution. Final dilution is obtained at levels 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶. Bacteria suspension was incubated on NA medium using pour plate method, then incubated at 37°C, for 24 hours. Each colony of bacteria was then isolated on another NA medium and incubated at 37°C for 24 hours to get a single colony.

2.2. Amylolytic bacteria selection

Amylase activities was measure by inoculated bacteria on Amylum Agar Medium, then incubated at 37°C for 24 hours. Furthermore, the selection of bacterial isolates that form a clear zone around bacterial colonies is carried out. An iodine solution was added to the bacterial growth medium so that clear zone around bacteria colonies is showing.

2.3. Screening of proteolytic bacteria

Bacteria was inoculated in Skim Milk Agar (SMA) medium, and then incubated at 37°C for 48 hours. The selection of bacterial isolates that form clear zones around bacterial colonies is carried out. Bacteria that can hydrolyze proteins (proteolytic bacteria) will form clear zones in the medium around bacterial colonies.

2.4. Lipolytic bacteria assay

Bacteria were then inoculated on NA + lipid medium (supplemented with olive oil). This selective medium is containing peptone, olive oil, Tween-80, NaCl, CaCl₂.2H₂O, methyl red and agar. The inoculation process was carried out aseptically at Laminar Air Flow (LAF) by zigzag streak method. Selection of lipolytic bacteria are based on clear zones that formed around bacterial colonies.

2.5. Morphology and identification of selected bacteria

Morphological descriptions of bacterial colonies include color, shape, diameter, edge, elevation, and density of bacterial colonies are measured. Further identification on morphology was supported by SEM (Scanning Electronic Microscope). Description of bacterial microscopic and physiological characteristics including observation of Gram properties, cell shape, spores, capsules, motility and type of bacterial respiration supported using a 1000x light microscope. Indigene bacteria that are able to hydrolyze amylum, protein and lipid were identified using *Microbact TM GNB 12 A / B / E, 24E Identification Kits*, then referenced to the bacterial identification book, "*Bergey's Manual of Determinative Bacteriology* (8th Ed.)" [5].

2.6. Hydrolysis index of amylum, protein, and lipid measurement

The test was carried out by growing amylolytic bacteria in AA medium using quadrant scratch plate method. The hydrolysis index is determined based on the results for the diameter of the clear zone and the diameter of the bacterial colony.

2.6.1 Hydrolysis index

Hydrolysis index was calculated based on equation (1) Melliawati et al. [6]:

$$\text{Index of starch hydrolysis} = \frac{\text{diameter of bacterial clear zone}}{\text{diameter of bacterial colonies}} \quad (1)$$

The hydrolysis index can be used as a parameter for the strength or weakness of the ability of bacteria to hydrolyze complex compounds into simpler compounds.

3. Results and Discussion

A total of 28 isolates was found in the wastewater from the Public Hospital Dr. Darsono Pacitan Regency. All bacterium was tested for their ability to hydrolyze amylum, protein and lipid. The results of the test are shown in Table 1.

Table 1. Enzym activities of indigene bacterial selected from wastewater.

No	Isolate Code	Amylolytic ^a	Proteolytic ^a	Lipolytic ^a
1.	A	-	-	+
2.	B	-	-	+
3.	C	-	-	+
4.	D	-	-	+
5.	E	-	-	+
6.	F	+	+	+
7.	G	-	-	+
8.	H	-	+	+
9.	I	+	+	+
10.	J	+	+	+
11.	K	+	-	+
12.	L	+	+	+
13.	M	+	+	+
14.	N	-	+	+
15.	O	+	-	+
16.	P	+	+	+
17.	Q	+	+	-
18.	R	+	+	-
19.	S	+	+	+
20.	T	+	+	+
21.	A ₁	+	+	+
22.	B ₁	+	+	+
23.	C ₁	+	+	+
24.	D ₁	+	+	+
25.	E ₁	+	+	+
26.	F ₁	+	+	+
27.	G ₁	+	+	+
28.	H ₁	+	+	+

^aInformation (+) = amylolytic, proteolytic and lipolytic bacteria,
 (-) = not amylolytic, proteolytic and lipolytic bacteria.

Amylolytic bacteria were determined by the ability to hydrolyze amyllum in AA medium marked with clear zones around bacterial colonies (**Figure 1**). According to Wahyudi et al. [7] that blackish blue color occurs when iodine molecules enter a spiral amyllum molecule (α -helix) in the medium. The iodination process occurs when amyllum has been converted by amylase enzyme into maltose and glucose.

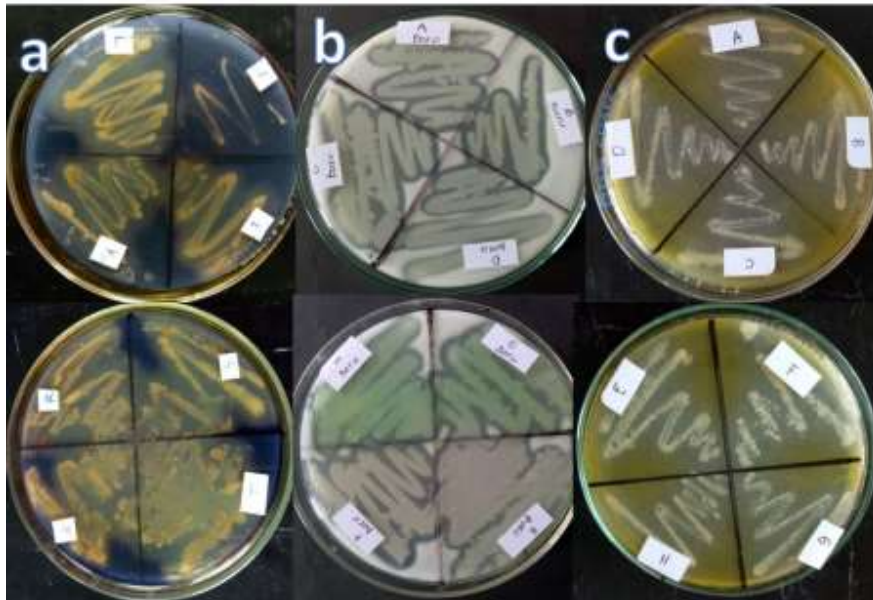


Figure 1. Bacterial assay on different media, (a) (above and below) shows amylolytic activity of several isolate, (b) (above and below) the clear zone around colonies that shows proteolytic activity, and (c) (above and below) shows an isolates that have lipolytic activity.

The proteolytic activity of bacteria indicated by the presence of clear zones around bacterial colonies as the result of breaking the protein peptide bonds into smaller peptides. Casein in skim milk is protein consist of phosphoproteins which bind to calcium to form calcium calceinate salt that made white color on the medium. Proteases produced by bacteria that cause casein to be hydrolyzed to soluble amino acids that made the white color disappears and a clear zone is formed around bacterial colonies [8].

Lipolytic bacteria can hydrolyze the lipid contained in the modified NA + L medium into forming fatty acids and glycerol. Olive oil was added to the medium which functions as a substrate that will be hydrolyzed by lipolytic bacteria. Lipolytic bacteria isolates will produce clear zones around bacterial colonies. The clear zone formed is due to the degradation of lipid in the media into simpler molecules. The formation of fatty acid deposits by the bacteria shows that these bacteria can produce lipases. Lipid (olive oil and Tween-80) that has been hydrolyzed into fatty acids, where free fatty acids are bound to Calcium ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) which has fused with the media. Calcium complexes with these fatty acids can be observed as turbid white deposits around colonies. In bacteria these enzymes are secreted by cells into their outer environment. The ability of bacteria to secrete extracellular enzymes is a mechanism of adaptation to the environment in which they grow. The presence of lipid in the waste water of Dr. Darsono Hospital was triggered the ability of secretion extracellular lipase to break down lipid into fatty acids and glycerol so that it can be used by these bacteria as a carbon sources [9].

The morphological description of each isolate showed that each bacterial isolate did not have differences in colony morphology based on color, diameter, density, elevation, shape, and shiny or bleak surfaces. Seven isolates including gram negative bacteria was coded L, R, S, F, C1, and E1. Species identification of the highest amylolytic, proteolytic, and lipolytic bacteria found in Dr. Darsono's Hospital was using *Microbact TM GNB 12 A / B / E, 24E Identification Kits*. The data are presented in Table 2.

Table 2. Bacterial species found in waste Dr. Darsono Hospital.

No	Isolate Code	Has the ability of hydrolysis (amylum / protein / lipid)	Species Name
1	L	Amylum	<i>Acinetobacter baumannii</i>
2	R	Amylum	<i>Enterobacter agglomerans</i>
3	S, E ₁ , C ₁ , E ₁	Amylum, Protein, Lipid, Protein	<i>Aeromonas hydrophila</i>
4	F	Lipid	<i>Burkholderia cepacia</i>

Acinetobacter baumannii known has characteristics as gram-negative, in the form of bacilli or kokobasil [10]. Research on *Acinetobacter baumannii* as amylolytic bacteria still not revealed yet. Some species belonging to the genus of *Acinetobacter* much known have a hydrolysis activity of amyllum, for example *Acinetobacter calcoaceticus* a research conducted by Pascon et al.[11]. Onishi & Hidaka stated that *Acinetobacter* sp. was able to produce *dextrinogenic amylase*, thus supporting the results of the study that *Acinetobacter baumannii* was classified as amylolytic bacteria [12]. The results of these studies indicate that *Acinetobacter baumannii* have the ability to break down amyllum into simpler compounds. *Enterobacter agglomerans* is a rod-shaped group of gram-negative bacteria that have a length of 1.2-1.3 μm and a width of 0.6-1.0 μm . *Enterobacter agglomerans* in this study was clasified as amylolytic bacteria because of its ability to form a clear zone on the medium.

Aeromonas hydrophila is a gram negative bacteria with short stem morphology of varying size (width: 0.8-1.0 μm and length: 1.0-3.5 μm), has no spores, and motile. It has protruding surface, round shape, shiny, cream color until edges, and it has 2-3 μm in diameter [13]. *Aeromonas hydrophila* in this study was clasified as amylolytic, proteiolytic and lypolytic bacteria because of its enzyme activity. Furthermore it was stated that *Aeromonas hydrophila* is able to ferment carbohydrates into acids [13]. These bacteria can use sugar such as fructose, glucose, sucrose, maltose and trehalose as source of energy. In many research, bacterium *Aeromonas hydrophila* are capable to hydrolyzing proteins by its exoenzyme. *Aeromonas hydrophyla* produces enzyme that encoded by the lipase gene, nuclease, aerolysin and serine protease and contain exotoxins in the form of aerolysins [14].

Burkholderia cepacia is a gram-negative bacterium, has a stem shape, not producing spores, bacteria are motile because it has only one flagellum or monotrichous flagella [15]. *Burkholderia cepacia* has the ability of lipid hydrolysis because of lipases that produced by lipolytics bacteria [14]. Lipolytic bacteria are able to produce extracellular lipase enzymes that can hydrolyze lipid into fatty acids and glycerol. Lipids or triglycerides are molecules with long carbon chains that can be broken down into fatty acids and glycerol which have short carbon chains (C-8 to C-18) by lipase enzymes. This finding is also supported by Galbraith et al. [16]. *Burkholderia* spp. produces two compartmental forms of amnithine lipid amide (OL) where 3-hydroxy acid is bound to amide itself by non-hydroxy acid and 2-hydroxy acid (OL2). These ions have a function in some Gram negative bacteria where hydroxy acid is derived from lipopolysaccharide [17]. SEM analysis result was shown in Figure 2.

Hidrolitic index was indicated how strong the ability of amylolytic bacteria in hydrolyzing amyllum, as well as the calculation of protein and lipid hydrolysis index in each bacterium. The determination was made by the Quadrant Streak Plate method. This method is used to measure the diameter of the colony and diameter of the clear zone formed by bacterium.

The results of the amyllum hydrolysis index calculation showed that of the three isolates had different amyllum hydrolysis index. *Enterobacter agglomerans*, 2.94 while *Achinobacter baumannii* showed the lowest amyllum hydrolysis index, 1.87. The results of the protein hydrolysis index calculation showed that the *Aeromonas hydrophila* isolate had protein hydrolysis index as much as 7.80. The results of the lipid hydrolysis index calculation showed that two isolates had a different lipid hydrolysis index, it is *Burkholderia cepacia* with hydrolysis index 7.00 while *Aeromonas hydrophila* showed the lowest lipid hydrolysis index is 3.37.

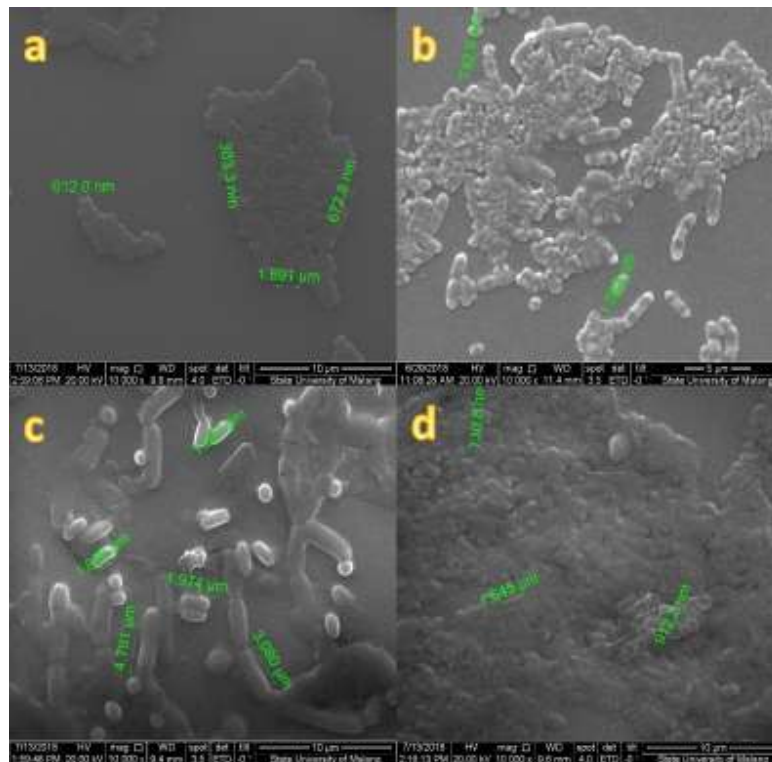


Figure 2. SEM result of selected bacteria found in wastewater Dr. Darsono hospital, (a) *Acinetobacter baumannii*, (b) *Enterobacter agglomerans*, (c) *Aeromonas hydrophila*, and (d) *Burkholderia cepacia*.

Enterobacter agglomerans has a higher ability to hydrolyze amylum compared to *Escherichia coli*. Whereas, in bacterium *Acinetobacter baumannii* and *Aeromonas hydrophila*, the amylum hydrolysis activity remains unknown. Bacteria that produce amylase enzyme usually can hydrolyze amylum into maltose, glucose, and dextrin molecules [7].

Various species of amylolytic bacteria have been isolated in this study, possibly for being used in industry or environmental management process such as bioremediation. Amylase enzyme has been applied in a number of industrial processes such as the food industry, fermentation, pharmaceutical / health and environment application.

In the study of indigene bacteria from Dr. Darsono Hospital wastes *Aeromonas hydrophila* has the highest protein hydrolysis index, based on the enzyme activity result. This finding was in line with research conducted by Shotts *et al.* which states that *Aeromonas hydrophila* are be able to hydrolyzes complex albumin, casein, and hibrinogen was higher 50% than other bacterium, these bacteria mostly found in aqueous environments with environmental function is to hydrolysis the protein in fish and other animals waste [18]. Decomposition of proteins that hydrolyse peptide bonds to release each amino acid so that amino acids can be absorbed into the cell. Various species of proteolytic bacteria isolated from Dr. Darsono Hospital wastes have the prospect of being use in the processing of immune stimulation health and biofilm agents as bioremediation as well as described by Merino *et al.* [20].

Burkholderia cepacia has the highest in lipid hydrolysis index compared to *Aeromonas hydrophila* in this research. However, the antimicrobial agent produced by *B. cepacia* showed that it was influenced by different nutritional, experimental and environmental factors both *in vitro* and *in vivo*. Recently a study on the effectiveness of the role of *B. cepacia* compared to *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalli* using various phenol sequences are basically formed from lipids [20]. In this research, *B. cepacia* was higher in use of lipid than other bacterium. According to Gaman *et al.* [21] it was supported

by lipase enzymes are able to hydrolyze lipid and solve it into three fatty acid molecules and one glycerol molecule. *B.cepacia* bacteria which are ubiquitous with potential use for biological treatment and also found in natural sources including soil, water and plants, or from hospitals, food stores, restaurants and hospitalizations.

4. Conclusion

The results of identification using identification kit show that four isolates are belong to species *Acinetobacter baumannii*, *Enterobacter agglomerans*, *Aeromonas hydrophila*, and *Burkholderia cepacia*. Identification and measurement on morphological features and the highest hydrolysis index score was classified *Acinetobacter baumannii* and *Enterobacter agglomerans* as amyolytic bacteria, *Aeromonas hydrophila* as proteolytic bacteria, and *Burkholderia cepacia* as lypolytic bacteria that made all four species are classified as bioremediation agent potential.

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