Isolation of potential proteolytic bacteria from hospital wastewater

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Abstract. Wastewater needs to be processed before being disposed of. Generally, the wastewater that produced by a hospital contains a lot of bacteria, viruses, chemical compounds, and drugs which can be harmful to the health of the surrounding community. For this reason, it is necessary to process hospital wastewater by using effective and efficient wastewater treatment technology. One of the processes of wastewater treatment is biological processing that uses microorganisms. The aim of this study is to isolate microbial that produce proteolytic capabilities that are potential in treating hospital wastewater. This research is a descriptive study with a cross-sectional design. Samples were taken from the Wastewater Treatment Plant Installation of Kasih Ibu General Hospital in Denpasar. The screening of the proteolytic bacteria was done by growing the isolated culture on skim milk agar. The colonies that produce clear zone were identified as proteolytic bacteria. In this study, a total of 43 isolates were obtained and two isolates showed their proteolytic activity. Both isolates are Gram-positive cocci and Gram-negative bacilli.

1. Introduction
In the globalization era, progress in production and industry was growing rapidly. In production activities, various materials, water, and energy are needed to produce a product. However, in the production process, there is no perfect efficiency, so waste is still produced in the form of solid, liquid or gas. Wastewater is the residual liquid production process which is no longer used and must be managed before being discharged into the environmental drainage system so as not to cause pollution and a decrease in environmental quality [1-3]. Thus each waste produced must be managed properly based on its characteristics to reduce the quality of the pollutants contained therein and safely disposed of into the environmental drainage system [4,5].

Hospital activities also produce solid, liquid and gas waste with distinctive characteristics. Hospital waste generally comes from medical and non-medical activities, such as surgery, Emergency Care Unit, laboratory, radiology, kitchen, and laundry [6-8]. Hospital waste contains hazardous compounds, such as pathogenic microorganisms (bacteria and viruses), drug residues and chemicals from laboratories (antibiotics, phenol, chloroform), toxic chemicals, and organic materials that are biodegradable (protein, fat, and carbohydrates) [9-11]. By such characteristics, then hospital wastewater management requires a special plan and design covering efforts to minimize waste and wastewater treatment through a Waste Water Treatment Installation so that people are not contaminated with viruses, bacteria, and fungus from the waste. The waste management system is certainly required for every hospital to prevent such contamination from happening to the general public [12].
Based on the results of the 2002 Rapid Assessment conducted by the Directorate General of P2MPL, the Directorate of Water Supply and Sanitation involving the District Level Health Office, stated that as many as 648 hospitals from 1476 hospitals, which have 49% new incinerators and who have Waste Water Treatment Installation as much as 36%. From this amount, the quality of liquid waste that has been through processing that meets the new requirements reaches 52% [13].

Basically, hospital wastewater treatment can be done through 3 processes, namely physically-mechanic, biological and chemical [14-16]. Physical-mechanical and chemical processing is basically the same as wastewater treatment to get clean water. The interesting thing here is the biological treatment of wastewater by utilizing bacteria play a role in the aeration process so that they can reduce odor and pollution levels as well as produce activated sludge [17]. Activated sludge can be defined as a mixture of microorganisms which contact and digest biodegradable materials from waste water. These microorganisms growth in wastewater by consuming biodegradable materials such as proteins, carbohydrates, fats, and many other compounds. Bacteria that can degrade waste are expected to naturally live in the waste [18,19]. There are several previous studies about identification of indigene bacteria from hospital wastewater, one of them as done in Regional Public Hospital in Pacitan. They found Aeromonas hydrophilia as proteolytic bacteria [20]. Departing from this, the researchers were interested in identifying and isolating the types of bacteria present in hospital wastewater that have the potential to treat wastewater biologically. Based on the formulation of the problem above, the purpose of this study is to isolate proteolytic bacteria in hospital wastewater that have the potential to be developed in biological wastewater treatment.

2. Methods
This study is a descriptive study with a cross-sectional design with a type of laboratory exploratory research. The research was carried out for 8 months, on wastewater originating from Waste Water Treatment Installation of Kasih Ibu General Hospital in Denpasar. Research (microbial isolation and characterization) was carried out at the Udayana University Laboratory of Public Health and continued at the Biomedical Laboratory (Faculty of Medicine and Health Science, Warmadewa University). The population and sample in this study came from Wastewater Treatment Plant Installation (outlet) of Kasih Ibu General Hospital Denpasar. Sampling was carried out at five random points of 20 mL each using a sterile bottle. The five bottles are then mixed into 1 and followed by the culture process (by repeating the culture 3 times/triple).

Samples that have been taken will then be cultured and isolated to obtain a pure culture. Started by the diluting the waste samples with sterile aquades in stages to \(10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}\) and \(10^{-6}\). Microbes are then bred by inoculating microbial wastes resulting from dilution \(10^{-4}, 10^{-5}\) and \(10^{-6}\) into Plate Count Agar (PCA) [21]. The incubation process is carried out in aerobic conditions at 37°C for 24 hours. Then purification was carried out until pure isolates are obtained. Then each of the pure isolates were stained (Gram stain) to determine the characteristics of each isolate. In addition, observations was made regarding the characteristics of microbes in breaking down proteins (proteolytic). Proteolytic characteristics were tested using skim milk agar with a composition of 3% skim and 3% agar. Inoculation was carried out by inserting isolates in the skim milk agar with sterile toothpicks, then incubated at 37°C for 24 hours. Proteolytic isolates are characterized by the formation of clear zones in skim milk agar [22].

3. Results and discussion
In this study, a total of 43 pure isolates were obtained (7 pure isolates in first replication, 24 in second replication, and 12 in third replication). Most of the pure isolates (67.44%) are gram-positive coccus bacteria. This is consistent with each replication, which in the gram-positive coccus bacteria at first replication at 57.14%, second replication at 76.17%, and third replication at 50%. These results are different from the results found in research on Sanglah General Hospital waste, which microbes found mostly are Enterobacteriaceae class bacteria. Enterobacteriaceae is a family of Gram-negative bacilli bacteria [23].
Microbial screening with proteolytic characteristics begins with growing pure isolates in Nutrient Broth (NB) media. The growth of pure isolates on NB media was characterized by the appearance of turbidity after overnight incubation. Each pure isolate that is grown is then planted in skimmed milk agar. Proteolytic isolates are characterized by the formation of clear zones in skim milk agar. From 43 pure isolates inoculated on skim milk agar, 2 pure isolates were obtained which had proteolytic activity, they are RS2 $10^5$ 7 and RS2 $10^6$ 6A codes that shown in figure 1. Based on figure 1, it was found clear zone around isolates. Clear zone was formed because bacteria digest proteins that contained in skim milk agar.

![Figure 1. Formation of clear zones in skim milk agar.](image)

Level of proteolytic activity can be seen from ratio of diameter of clear zone and diameter of colony. The result of the ratio were stated with “R” in table 1. Based on table 1, it was found that isolates with RS2 $10^5$ 7 code (a gram-positive coccus bacteria) had higher diameter of clear zone (1.9 cm) than RS 2 $10^6$ 6A (a gram-negative bacilli bacteria).

| Isolate Code | Diameter of Colony (cm) | Diameter of Clear Zone (cm) | R  |
|--------------|-------------------------|-----------------------------|----|
| RS2 $10^5$ 7 | 1.0                     | 1.9                         | 1.9|
| RS 2 $10^6$ 6A | 1.2                  | 1.7                         | 1.4|

The highest proteolytic activity was produced by isolates of RS2 $10^5$ 7 code (table 1). Other studies on proteolytic testing in lactic acid bacteria categorized high proteolytic activity when $R \geq 1.5$ [24]. Based on table 1, it was found that isolates with RS2 $10^5$ 7 code (a gram-positive coccus bacteria) had high proteolytic activity ($R=1.9$) when viewed from the size of the ratio of clear zone/colony diameter ($R$). These result is similar with research on proteolytic bacteria from soil samples. In that study, the highest proteolytic activity was obtained by gram-positive coccus bacteria [25]. Another study about proteolytic bacteria associated with seagrass *Enhalus acoroides* also has same result with this study. That study obtained the highest proteolytic activity produced by gram-positive cocci bacteria [26]. Other study about isolation of proteolytic bacteria selected from civet feces also found gram-positive bacteria which have the highest proteolytic capabilities [27].

4. Conclusion
In this study, a total of 43 pure isolates were obtained (7 pure isolates in replication I, 24 in replication II, and 12 in replication III). Most isolates (67.44%) are Gram-positive coccus bacteria. There were found two isolates that had proteolytic properties (RS 2 $10^5$ 7 isolates which were Gram-positive cocci bacteria and RS 2 $10^6$ 6A code isolates which were Gram-negative bacilli bacteria). The highest proteolytic activity was produced by isolates of RS2 $10^5$ 7 code. From the results of this study, it was
suggested that the study is continued with the microbial identification process using the PCR technique and optimizing the growth of protease-producing bacteria to maximize protease production.

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