Isolation and identification of proteolytic bacteria from pig sludge and protease activity determination

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S Hamdani[1,2], N Asstiyani[1], D Astriany[1], M Singgih[2] and S Ibrahim[2]

[1] Sekolah Tinggi Farmasi Indonesia, Bandung, Indonesia
[2] School of Pharmacy, Bandung Institute of Technology, Bandung, Indonesia

E-mail: syarifhamdani@stfi.ac.id

Abstract. Proteolytic bacteria were found in many environment, some were found in soil or mud. This research was conducted to find proteolytic bacteria from pig sludge that is place for pig wallowing. Bacteria isolation was carried out by streak plate method on nutrient agar media. Qualitative test of proteolytic bacteria used skim milk agar medium. The results of isolation obtained 10 bacterias and 2 of them have potential as proteolytic bacteria. Identification of proteolitic bacteria used the 16s rRNA method, result showed that bacterias are Bacillus pseudomycoides and Staphylococcus sciuri. Protease isolation was carried out under 4^{o}C and under room temperature during centrifugation as separation process. Protease activity from Bacillus pseudomycoides was at 4^{o}C as much as 0.094 U/mL and was 0.082 U/mL at room temperature, while from Staphylococcus sciuri was at 4^{o}C as much as 0.120 U/mL and was 0.098 U/mL at room temperature.

1. Introduction

Indonesia is one of the countries that has the largest pig germplasm in the world, because five of the eight species of pigs in the world are in Indonesia [1]. This species has a natural behavior to smear the surface of their bodies with mud, this behavior is commonly called wallowing [2].

Wallowing activity has an important role in pig survival such as protecting the body from flies, regulating body temperature (thermoregulation), cleansing ectoparasites in the body, cleansing wounds on the skin, and sexual behavior such as showing competition between male animals [1, 3]. Various activities carried out at the time of this wallow pig allowed the pig mud pools to be rich in various microbial such as *Enterobacteriaceae, Streptococcus, Peptostreptococcus, Lactobacillus, Clostridium, Enterococcus, Escherichia, Bacteroides, Salmonella, Brucella, Mycobacterium, and Rickettsia* [4, 5], few of them including proteolytic bacteria [6,7].

Proteolytic bacteria are a type of bacteria that can produce protease enzymes, which are enzymes that can break down peptide bonds in protein molecules. In this study, protease is as an extracellular enzyme which is produced in cell and then released out of the cell [8]. Many proteolytic bacteria were found in soil, water, mud and certain strains of environmental. This makes proteolytic bacteria develope more than others enzimatic bacteria because of its abundance in nature and the surrounding environment [9].

According to The Ministries of Research, Technology, and Higher Education in Republic of Indonesia on 2017, currently almost 99% of enzyme (biocatalyst) needs for Indonesian industries

which are still imported from abroad such as from China, India, Japan and Europe. Enzyme needs tend to increase every year and global market demand for enzymes which is estimated to increase by around 7% in 2015-2020 per year. Enzyme consumption by industry in Indonesia is estimated at 2,500 tons with an import value of around 200 billion rupiah in 2017 which is an average volume growth rate of 5-7% per year. This value is large enough to be used as a basis for consideration in encouraging Indonesian's independence efforts in producing enzymes.

Therefore, this study was conducted to determine the presence of proteolytic bacterial isolates by utilizing potential Indonesian ingredients, and the research was conducted to increase enzyme production. In this study screening and isolation of proteolytic bacteria from pig sludge from several pig farms will be conducted. Isolation is carried out by growing proteolytic bacteria in a casein substrate. The protease enzyme produced will then determine the value of the activity [10].

2. Materials and Methods

2.1 Sampling

The containers and equipment used for sampling were previously sterilized. The samples were homogenized so that they were evenly distributed at the time of collection. Samples were taken as much as 100-500 ml, then placed in sterile bottles, after that they are taken to the laboratory and stored at 4^{o}C if not in use [11].

2.2 Isolation of bacteria

One ose of sludge was inoculated into TSB containing 0.1% nipasol, incubated at 37°C for 24 hours, than one ose of inoculum was streaked on the NA media. Bacterial isolation was carried out by taking separate colonies and then purified by the streak plate method repeatedly on NA media than followed incubated at 37°C for 24 hours respectivelly, until the pure isolates bacteria were obtained.

2.3 Identification of proteolytic bacteria

2.3.1. Qualitative test for proteolytic bacteria

The activity of *proteolytic* bacteria was tested qualitatively on skim milk media, indications that microbes are able to integrate protein (casein) which are shown through clear zones around the colonies [12].

$$\mathrm{IP} = \frac{\text{Clear zone diameter} - \text{colony diameter}}{\text{Colony Diameter}} \quad (1)$$

Pure colonies that gave a clear zone are calculated for their proteolytic index, bacterial as the highest proteolytic index (IP) is a bacteria that is choosen for protein isolation step.

2.3.2. Gram staining

One drop of distilled water is placed on a slide, one bacterial culture spreads as thin as possible to form a circle of diameter of 1cm. Gentian violet drops are allowed to stand for one minute. The dye was rinsed using lugol and allowed to stand for 30 seconds. The lugol solution is rinsed with 96% alcohol. Preparats are washed by distilled water and stained by Fuchsin, let stand 30 seconds and then rinsed by aquadest and allowed to dry. Preparations were observed under a microscope with a magnification of 100x objective lens using immersion oil [13].

2.3.3. 16s rRNA sequencing

Identification of bacteria was carried out molecularly used 16s rRNA gene analysis method [6]. Single colony that has been obtained is subsequently sequenced by Macrogen, Inc. South Korea to know the order of its base by using universal primers (Table 1).

Table 1. Primary universal 16S rRNA gene analysis.

Sequencing Primer	PCR Primer
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

2.4 Growth curves

The curve of growth is made by measuring optical density of cell suspension. Measurements are made within 6 hours until the stationary phase, measurement at wavelength of 660 nm [8].

2.5 Production and isolation of protease crude extracts

The bacterial inoculum in TSB was taken as much as 10% to be inoculated into 50 mL of liquid protease production medium, then shaked on orbital shaker at 160 rpm. The production mixture was centrifuged at 6000 rpm at 4°C for 30 minutes; supernatant is a crude protease enzyme [14].

2.6 Activity test of protease enzyme

Measurement of proteolytic activity was carried out according to Kunitz method [15], 1 mL of supernatant was inserted into a test tube than 1 mL substrate (1% casein in phosphate buffer pH 7) were added, then incubated at 37°C for 30 minutes. After that, 3 mL of 5% TCA solution was added, shaked and then allowed to stand at room temperature for ± 30 minutes, then centrifuged for 15 minutes.

The same treatment in the negative control is the mixture above with no addition of the substrate, while the positive control is the addition of tyrosine. The filtrate was measured with a uv-vis spectrophotometer at a wavelength of 288 nm. The linear tyrosine equation is used as a standard curve to be interpolated with the absorbance value obtained [15].

3. Results and Discussion

3.1. Results of bacteria isolation

Isolation of bacteria from 3 types of mud obtained 10 bacteria; the results of isolation can be seen in (Table 2). The isolation results were then identified as protease enzyme producing bacteria on next step.

Table 2. Bacteria isolated from pig sludge.

Origin of Mud	Amount of Bacteria
Farm I	6
Farm II	2
Wild pig	2

3.2. Identification of proteolytic bacteria

3.2.1. Qualitative test of proteolytic bacteria

Result of proteolytic bacteria qualitative test on skim milk medium from 10 bacterias gave 4 bacteria which provide clear zones with different activity levels (Table 3), 1 bacteria from farm I, 1 bacteria from farm II, and 2 bacterias from wild pig. Proteolytic activity of bacteria no. 4 from farm I had a vague clear zone or weak activity, so it is to low to have proteolytic index. Three others bacterias with strong activity (Figure 1) gave a different proteolytic index.

The results of the calculation of the proteolytic index of each bacterial isolate can be seen in (Table 3). The results showed that bacterial no. 9 and no. 10 which showed the biggest proteolytic index, so then this two bacterias were used as a starter in the protease enzyme production process.

Table 3. Results of qualitative test of protease-producing bacteria.

Origin of Mud	Bacteria Number	Clear Zone	Proteolytic Index
Farm I	1	-	-
	2	-	-
	3	-	-
	4	+	-
	5	-	-
	6	-	-
Farm II	7	-	-
	8	+ +	0.075
Wild pig	9	+ + +	0.625
	10	+ +	0.561

Bacteria no. 8 Bacteria no. 9 Bacteria no. 10

Figure 1. Proteolytic index on skim milk medium.

3.2.2. Gram staining results

The results of gram bacterial staining No. 9 were in the form of purple coccus while bacterium No. 10 was in the form of purple bacillus, which showed that both bacteria were classified as gram-positive bacteria of different genus.

3.2.3. 16s rRNA full sequencing

Identification of 3 proteolytic bacteria through DNA sequencing using 16s rRNA method showed that bacteria number 9 was *Staphylococcus sciuri* (Figure 2), while bacteria of number 10 was *Bacillus pseudomycoides* (Figure 3), with each homology level at 99%. According to Janda and Abbot [16] a bacterial species is said to be the same if it has homology more than or equal to 97%.

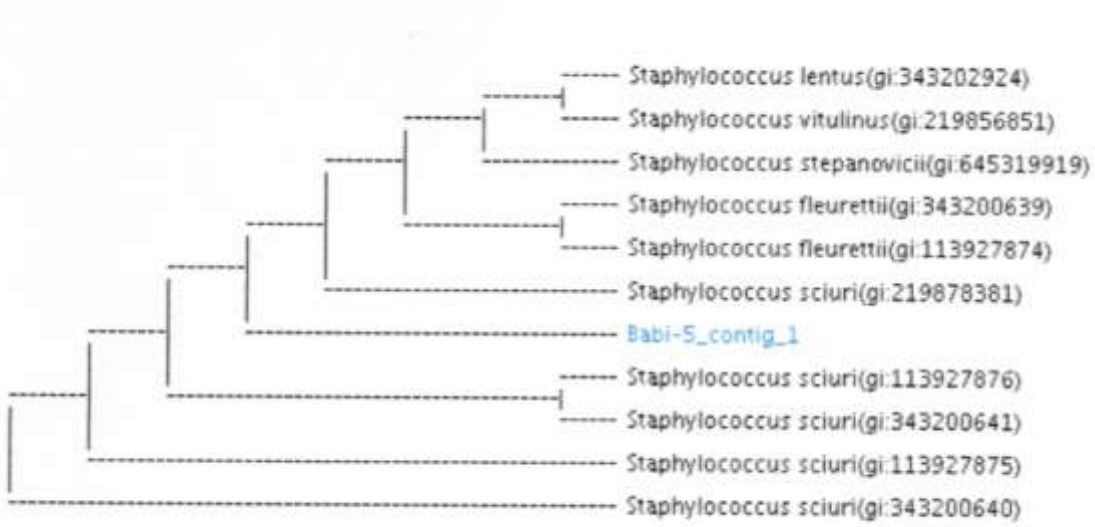

Figure 2. Phylogenetic Trees Results of alignment of 16s rRNA bacteria no.9.

Staphylococcus sciuri strains isolated from several animal, human skin whom frequent contact with farm animals [17, 18] and various food products of animal origin [19], this bacteria is patogenic for newborn pig [20] but found as proteolitic bacteria [21].

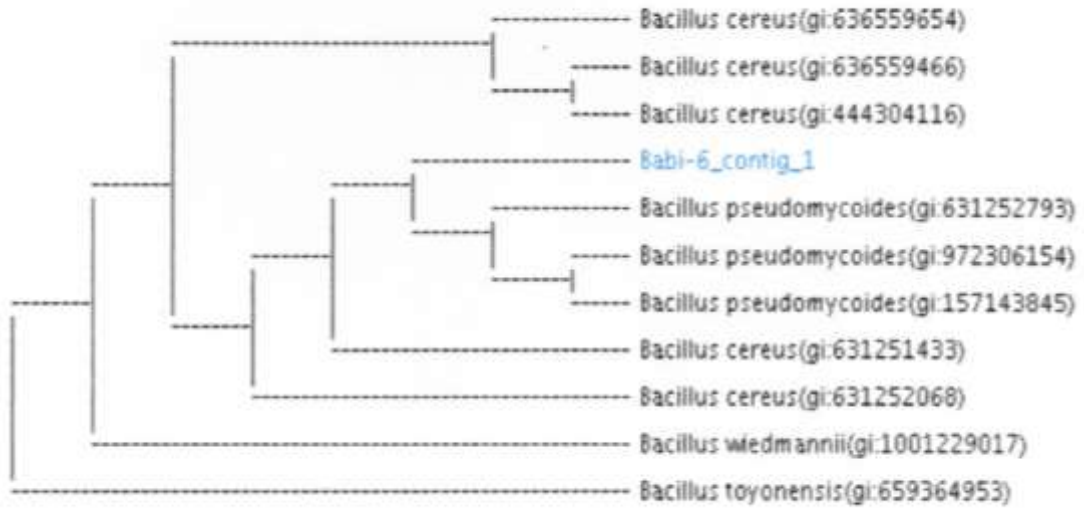

Figure 3. Phylogenetic Tree Result of alignment of 16s rRNA bacteria no. 10.

Bacillus pseudomycoides is a proteolytic bacteria with the ability to produce lantibiotic as a new antibiotic. Protease excreted during antibiotic production by the still unknown mechanism [22].

3.3 Growth curve

Staphylococcus scuiri and *Bacillus pseudomycoides* growth curves have different log phases. *Staphylococcus scuiri* shows a longer lag phase compared to *Bacillus pseudomycoides*. The results of the determination of the growth curve are used as the optimal time in the production of enzymes in the next stage, which is in the exponential phase.

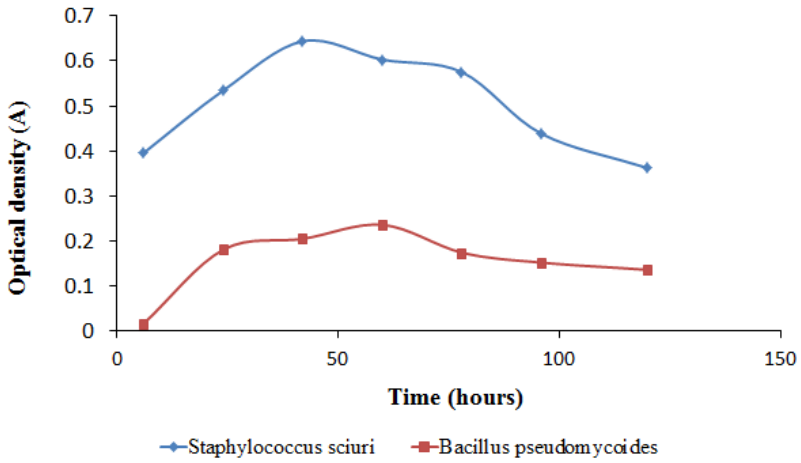

Figure 4. Growth curves of *Staphylococcus scuiri* and *Bacillus pseudomycoides.*

3.4. Protease enzyme activity test results

Staphylococcus scuiri enzyme activity through separation at 4^0C showed the highest activity with a considerable difference in activity compared to separation at room temperature (Table 4). The same condition occurs in *Bacillus pseudomycoides* which shows a difference in activity between the use of 4^0C temperature compared to room temperature.

Table 4. Test results of protease enzyme activity.

Bacteria	Protease enzyme activity (U/mL)	
	Centrifugation (4^oC)	Centrifugation (Room temp)
Staphylococcus scuiri	0.120	0.098
Bacillus pseudomycoides	0.094	0.082

4. Conclusion

Protease producing bacteria from pig sludge were *Bacillus pseudomycoides* and *Staphylococcus sciuri*, with protease activity from *Staphylococcus sciuri* was 0.120 U/mL at 4^oC U/mL and 0.098 U/mL at room temperature, while *Bacillus pseudomycoides* of 0.094 U/mL was at 4^oC and 0.082 U/mL was at room temperature.

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