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Cell wall response of bacteria *Serratia marcescens* LII61 – lipase and protease enzymes producer – in gram staining

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ABSTRACT

This study was aimed to reveal cell wall response of *Serratia marcescens* LII61 to Gram staining and its lipase and protease activities. Microscopic characters were obtained through the Gram staining procedure performed on variations in the age of bacterial cultures (16, 18, 24, 48, and 72 hours) which were grown on Luria Bertani Broth medium. Lipase activity assay was carried out using a spectrophotometer, with lipolytic selective media and observed every 4 hours for 24 hours. Protease activity was carried out using spotted method on proteolytic selective media and observed at 48 hours incubation time. The results of this study on Gram staining showed that *S. marcescens* LII61 has a Gram variable character, which was indicated by the change in color of the cell wall resulting from gram staining at the observed age of the culture. The highest lipase activity was produced at 16 hours after incubation (29.39 U/mL) and protease activity was indicated by hydrolysis index at 48 hours i.e 1.2. The cell wall response of the *S. marcescens* LII61 is an interesting matter to discuss because so far the bacterium *Serratia marcescens* is known as bacil Gram negative. The presence of gram variable properties shown in the results of this study can be attributed to the ability of bacterial cell wall to adapt in unsuitable nutrient condition, pH, temperature and electrolyte content in the environment.

Keywords: *Culture age, Gram variable, Serratia marcescens* LII61

Introduction

Indonesia is rich in biodiversity of microbes and biomass. Based on its geographical condition, Indonesian indigenous microbes have capability to produce superior enzyme, including lipase and protease, or better known as lipolytic and proteolytic microbes. At present, the use of lipase and protease enzymes in the food and non-food industries is increasing. In the food industry, lipase is widely used in the dairy industry, bread and cake industry, beer industry, spice industry, and processing meat and fish. Therefore, lipase is also used in the non-food industry, such as chemical and pharmaceutical industries, oleochemical industry, detergent industry, pharmaceutical industry, medicine, cosmetics industry and waste treatment industry (Momsia and Momsia, 2013; Farrokh *et al.*, 2014; Guerand, 2017).

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Meanwhile, protease play roles in protein breakdown reactions. The use of protease in industrial fields include the industries of detergent, leather, textile, food, protein hydrolysate, milk processing, pharmaceutical, beer, and waste processing industry (Moon and Parulekar, 1993).

Fatimah *et al.*, (2011) has succeeded in isolating bacteria producing lipase and protease from slaughterhouse waste in North Surabaya. One of the isolate is LII61 which were also investigated for their ability to degrade oil sludge (Ni'matuzahroh *et al.*, 2017), and produce bio surfactants (Renjana *et al.*, 2017).

Ni'matuzahroh *et al.*, (2017) has revealed the macroscopic character of colony, cell microscopy, and physiology of LII61 isolate. The study showed that LII61 isolate were identified as *Micrococcus* sp. LII61 uses a microbact 12A/12B identification kit. Microscopic character of LII61 isolate showed Gram-positive cocci (Renjana *et al.*, 2017) identified using 16S rRNA molecular marker; the result showed that LII61 isolate were identified as *S. marcescens* with 99% similarity degrees. So far, *S. marcescens* is classified in Gram-negative bacteria. Based on this background, this study aims to confirm the status of species from LII61 isolate molecularly, observe the response of this bacterial cell wall to Gram staining at different age cultures, and assay the activity of lipase and protease hydrolysis index.

Materials and Methods

Macroscopic Characterization

S. marcescens LII61 bacterial colonies from stock culture were stricked on Nutrient Agar medium using the quadrant method. Colony characterization was carried out on single colonies 24 hours culture age, which included shape, diameter, color, margin, colony texture, and elevation of the colonies.

Microscopic Characterization

Observation of cell microscopic characterization included cell shape and cell wall response to gram staining using HUCKER staining kit. Culture of *S. marcescens* LII61 bacteria was first cultured in Luria Bertani Broth medium and gram staining was carried out at 16, 18, 24, 48 and 72 hours of culture.

Lipase Activity Assay

The medium for lipolytic assay (100 mL) consist of 0.2 g yeast extract, 0.05 g $MgSO_4.3H_2O$, 0.23 g NaCl, 0.5 g K_2HPO_4, 1% cooking oil, and distilled water. Determination of lipase enzyme activity was carried out by the method of Pereira *et al.*, (1997) in modified Sumarsih, (2005). Lipase enzyme activity was determined by spectrophotometry using a *p*-nitro phenyl palmitate substrate (*p*-NPP). Lipase activity of *S. marcescens* LII61 was measured every 4 hours for 24 hours of incubation.

Protease Activity Assay

The medium for proteolytic assay with a mixture of Bussnell Hass Agar containing skim milk. The bacterium was inoculated as small spot into the medium and incubated at 37 ºC. The appearance of clear zones around the colonies after 48 hours of incubation show proteolytic activity. Measurement of protease activity was carried out according to the method performed by Melliawati *et al.*, (2015).

Molecular Identification of *Serratia marcescens* LII61

Isolation of genomic DNA from *S. marcescens* LII61 using the *Wizard® DNA Purification Kit* (Promega). Measurement of purity and DNA concentration was carried out using *Thermo Scientific TM Multiskan GO Microdroplet Spectrophotometer* at wavelengths of 260 nm and 280 nm. The gene amplification procedure is adjusted to the direction of the PCR Master Mix Protocol (Promega) manufacturer. Molecular marker of the 16S rRNA gene determined bacterial identification, primers used to amplify the 16S rRNA gene were universal primers, namely: P0F-5'-GAG AGT TTG ATC CTG GAG CAG-3 'and P6R-5'- CTA CGG CTA CCT TGT TAC GA-3 ' (Chakraborty *et al.*, 2015).

The PCR conditions used were pre-denaturation temperature of 95 ºC for 1 minute, denaturation temperature of 95 ºC for 30 seconds, annealing 55 ºC for 45 seconds, elongation of 72 ºC for 1 minute, and final extension 72 ºC for 5 minutes. Confirmation of genomic DNA and PCR product was carried out using agarose gel electrophoresis with a concentration of 1%, at 135 volts for 25 minutes. Electrophoresis results were visualized at UV-*Transluminator* then documented with a *geldoc* camera.

Amplicon DNA was sequenced at *First Base Laboratories* using the *Bigdye® Terminator v3.1 Cycle Sequencing kit* and analyzed using the ABI PRISM *3730xl Genetic Analyzer* (Applied Biosystems, USA). The DNA sequencing results were edited using the

BioEdit Sequence Aligment Editor version 7.2.5 software. Then analyzed using the BLAST program in NCBI (https://www.ncbi.nlm.nih.gov/).

Results and Discussion

Macroscopic Characteristics of Colonies

S. marcescens LII61 bacterial colonies on Nutrient Agar media with a 24 hours culture age can be found in Figure 1 and colony characters showed in Table 1.

Fig. 1. Colonies of *Serratia marcescens* LII61 bacteria on Nutrient Agar media at 24 hours culture age

Table 1. Characteristics of *Serratia marcescens* LII61 colonies

No	Colony Character	Colony Character *Serratia marcescens* LII61
1	Colony Diameter	3.3 mm
2	Colony colors	Red
3	Margin	Entire
4	Colony texture	Mucoid
5	Elevation	Umbonate

S. marcescens LII61 has red colonies that are not always found in some other *Serratia* species (Barrow and Feltham, 2003). It can produce specific secondary metabolites that is prodigiosin (Elkenawy *et al.*, 2017).

Microscopic Characteristics

The cell of *S. marcescens* bacteria LII61 was a short rod at 16 and 18 hours and change into cocci at a culture age above 24 hours. The results gram staining for *S. marcescens* LII61 at 16, 18, 24, 48, and 72 hours can be found in Figure 2.

Based on the result changing in response to gram staining of *S. marcescens* LII61 bacteria can be categorized into gram variable, gram-negative properties were shown at 16 to 18 hours of age culture, and begin to show gram positive at the age above 24 hours. Smolin *et al.*, (2005) also express the gram variable nature of this bacterial species.

In various literature, it is mostly explained that *S. marcescens* include gram negative bacteria (Elkenawy *et al.*, 2017; Lee *et al.*, 2007; Li *et al.*, 1995; Panizza *et al.*, 2013), but in this study indicates that this species belongs to the gram variable. The difference in the result of gram staining can be influenced by various environmental conditions, which cause the structure of the cell wall to change. According to Beveridge, (1990), in certain eubacterial groups; the response of gram staining is erratic caused by unsuitable nutrient, temperature, pH or other electrolyte content. However, in spesific bacterial, the nature of gram variable will be different in under optimal condition. Based on description, the result of gram staining in *S. marcescens* LII61 species are caused by change in the formation of the peptidoglycan layer on the cell wall structure. This is influenced by the environmental conditions of the bacteria. This bacteria species is found in slaughterhouse wastes, which contains nutrition, pH and electrolytes can affect the mechanism of peptidoglycan layer formation in the structure of cell wall.

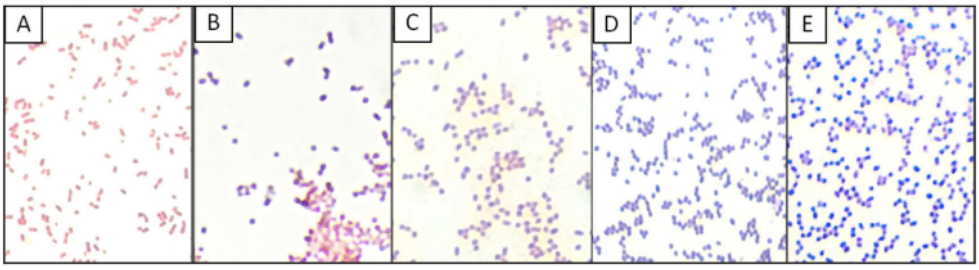

Fig. 2. Gram staining of *Serratia marcescens* LII61. (A, B, C, D, E: 16, 18,24, 48, 72 hours M 1000x)

Lipase Activity Assay

Lipase activity of *S. marcescens* LII61 bacteria from each time of observation during 24 hours incubation is shown in Fig. 3.

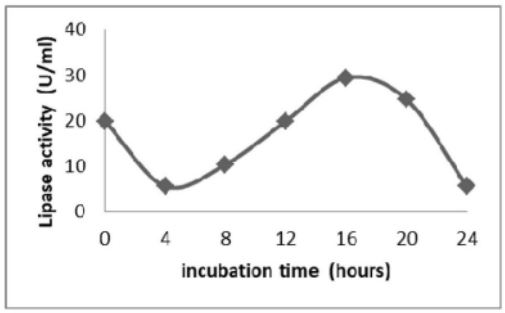

Fig. 3. Lipase activity of *S. marcescens* LII61 bacteria at different incubation times

The highest activity was registered at 16 hours (29.39 U/mL). The activity of lipase enzyme can be related to the phase of bacterial growth. Maximum lipase activity was obtained at 16 hours of incubation. This result is appropriate with Gururaj *et al.*, (2016) which works with *Acinetobacter* sp. and obtain maximum lipase activity at 16 hour of incubation. Based on this result, *S. marcescens* LII61 shows important characteristic for industries because this strain will be prefered if it has higher activity and shorter incubation time (Niyonzima and More, 2014).

Protease Activity Assay

The results showed that *S. marcescens* LII61 can produce protease enzyme. The clear zone formed around the colononies is caused by the casein substrate in the media has been hydrolyzed into peptide and amino acid compounds (Nurkhasanah and Widodo, 2015). Casein as a substrate will induce protease enzyme activity and bacteria that hydrolyze casein are characterized by the presence of clear zones around the colonies (Atlas, 2010). The results of the protease activities assay were shown in Figure 4.

The ability of protease activity can be seen based on the proteolytic index. Observations on incubation 48 hours showed that the average proteolytic index is 1.20.

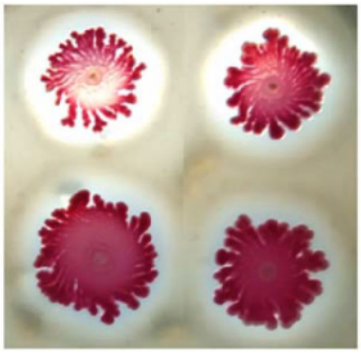

Fig. 4. Proteolytic activities of *Serratia marcescens* LII61 at 48 hours.

Molecular Identification of *Serratia marcescens* LII61

The purity and concentration of DNA is categorized as good because it has a purity value between 1.8-2.0, which is 1.93 and a concentration of 35.1 ng/µL. Amplification of the 16S rRNA gene from *S. marcescens* LII61 using the PCR method visualized in Fig. 5. The DNA band obtained had a size of ± 1300 bp.

Based on the confirmation results of the 16S rRNA gene by electrophoresis, a single band was obtained, and then it was followed by sequencing at the *1st Base Sequensing Service*, Singapore. Sequencing data were analyzed using the BioEdit Sequence Aligment Editor version 7.2.5. The sequence of *S. marcescens* LII61 16S rRNA gene has a size of 1045 base pairs. Based on BLAST analysis, the 16S rRNA gene from *S. marcescens* LII61 has similarity (98.56%)

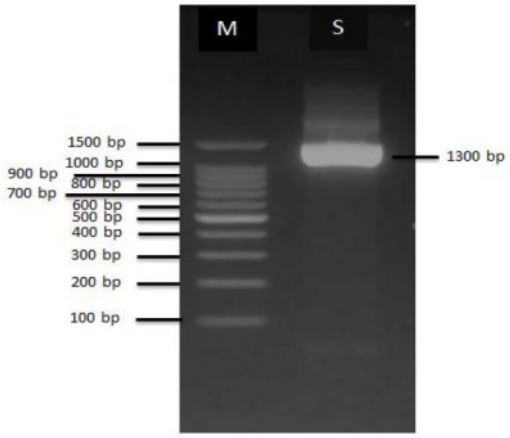

Fig. 5. 16S rRNA gene amplification of *Serratia marcescens* LII61 bacteria in 2% agarose gel. M: DNA Marker 100 bp; S: sample.

with *S. marcescens* strain ADY09 (acc. number MH084798.1). So the results can be concluded that the species used in this study is *S. marcescens* strain LII61 has a red colony character and gram variable.

Conclusion

Based on this research, *S. marcescens* LII61 is gram variable, has red colony and similarity (98.56%) with *S. marcescens* ADY09. This bacterium can produce lipase (29.39 U/mL) at 24 hours after incubation and protease with hydrolytic index is 1.2 at 48 hours.

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