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Short Communication: Isolation and characterization of thermophilic actinobacteria as proteolytic enzyme producer from Ie Seuum Hot Spring, Aceh Besar, Indonesia

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Abstract. *Fitri L, Putri KA, Suhartono, Ismail YS. 2019. Short Communication: Isolation and characterization of thermophilic actinobacteria as proteolytic enzyme producer from Ie Seuum Hot Spring, Aceh Besar, Indonesia. Biodiversitas 20: 2802-2808.* Proteases are enzymes who catalyze the breakdown of peptide bonds in proteins. This enzyme could be produced from thermophilic bacteria that are able to grow at temperatures of 45-80ºC and are stable to heat. The samples were collected at Ie Seu'um hot spring, Aceh Besar. This study aimed to isolate, to characterize and to determine actinobacteria that were capable of producing protease enzymes. The sampling method in this study was conducted by purposive sampling at temperatures of 50, 60 and 70ºC. Isolation of thermophilic actinobacteria was carried out in Humic Acid Vitamin B (HV) Agar medium and morphological characterization was carried out in Yeast Malt Agar (YMA), Yeast Starch Agar (YSA), Oatmeal Agar (OA) media. Microscopic characterization and measurement of clear zone diameter formed were carried out in skim milk medium. The results showed that one actinobacteria isolate was obtained at a temperature of 50ºC and five isolates at a temperature of 60ºC, meanwhile, no actinobacteria could be obtained at a temperature of 70ºC. A total of 4 isolates obtained were able to produce protease enzymes. The highest Proteolytic Index (IP) value was obtained from IS01 which was 3.8.

Keywords: Actinobacteria, hot spring, Ie Seu'um, protease

INTRODUCTION

Protease is an enzyme that catalyzes the breakdown of peptide bonds in protein. Protease is required physiologically for organisms life such as plant, animal and microorganism. Protease is an important enzyme that has wide applications in pharmaceutical industry, leather tanning, food, waste treatment (Vonothini et al. 2008), development of cancer cell treatment (Balachandran et al. 2012) and laundry (Ghorbel et al. 2014).

Protease could be produced by plant, animal, and microorganism. Microorganism was the most potential source of enzyme compared to plant and animal. The use microorganism was the most beneficial way due to their rapid growth, could grow on the substrate, the products could be increased through adjustment of growth conditions and genetic engineering. Thermophilic bacteria was one of microorganism that capable of producing protease (Said and Likadja 2012).

Thermophilic bacteria are bacteria that can grow at temperatures of 45-60ºC. Thermophilic bacteria are also a potential bacteria to produce protease that is stable towards heat, so it is highly required in food, non-food industries, and biotechnology application because it could reduce the possibility of contaminants. This microorganism is not only tolerant of extreme environmental temperatures, but also able to survive and reproduce in extreme temperature conditions. One of bacteria classified as thermophile is actinobacteria (Akhdiya 2003).

Actinobacteria is a group of Gram-positive bacteria, having a characteristic filamentous morphology. Actinobacteria could be found either in terrestrial or aquatic environment. Actinobacteria has a way to survive in very dry conditions by producing spores. The spores have different forms, such as spherical, cylindrical or oval (Chaudhary and Shradda 2016). Actinobacteria can live in various habitats such as soil, compost (Dilip et al. 2013) and waters (Ambarwati 2007).

Hot spring has a high temperature so that living creatures are very rare in that place. Actinobacteria is one of the bacteria that can survive at high temperatures so that it can be classified as thermophilic bacteria (Dilip et al. 2013). Indonesia has many hot springs which until now continue to be explored to find important bacteria for science, one of them is in Aceh.

Aceh is one Indonesia regions that have hot springs. One of the hot spring found in Aceh is Ie Seuum which is located in Ie Seuum Village, Masjid Raya Subdistrict, Aceh Besar District or ± 45 km from Banda Aceh, the capital city of Aceh Province, Indonesia. Ie Seuum has a hot water temperature ranging from 70-80ºC, which has the potential

to obtain thermophilic bacteria. Therefore, it was necessary to do research on the potential of hot water sources to discover actinobacteria especially thermophilic actinobacteria which are capable of producing protease enzymes.

The purpose of this study was to isolate, to characterize and to screen actinobacteria which were capable of producing protease from hot spring Ie Seuum, Aceh Besar, Indonesia.

MATERIALS AND METHODS

Isolation and purification of actinobacteria

This study used purposive sampling method by conducting isolation of thermophilic actinobacteria taken at temperatures of 50, 60 and 70ºC from hot springs Ie Su'um, Aceh Besar, Indonesia. Water samples were taken using a long dipper then put in a thermos. Then the thermos was tightly closed and immediately brought to the laboratory (Chaudhary and Shradda 2016).

A total of 0.1 mL of water samples were taken using a micropipette and then transferred into a Petri dish containing HV medium and spread to the entire medium surface using a spreader rod. Then incubated at 45°C for 7-30 days. Furthermore, purification was conducted in YMA medium (Chaudhary and Shradda 2016).

Characterization of actinobacteria

Morphological characterization of actinobacteria isolates was observed in YMA, YSA, and OA media. Observation of colony morphology from each medium included colony form, elevation, color of aerial mycelium, and color of substrate mycelium. Microscopic observations were carried out using an optical microscope at 400 x magnification. Observations which was conducted microscopically was observation of hyphae forms (Astuty 2017).

Screening of protease producing actinobacteria

Screening of thermophilic actinobacteria was carried out according to the method of Benerjee et al. (1999). Actinobacteria isolates were inoculated in skim milk medium then incubated at 45ºC for 24-48 hours. The bacteria colonies and clear zones formed around the colonies were measured in diameter. The Proteolytic Index was counted to find out which isolate had the highest IP value.

$$IP = \frac{Clear\ zone\ diameter - Colony\ diameter}{Colony\ diameter}$$

IP = Proteolytic Index

Data analysis

Data analysis was carried out descriptively and displayed in the form of tables and images.

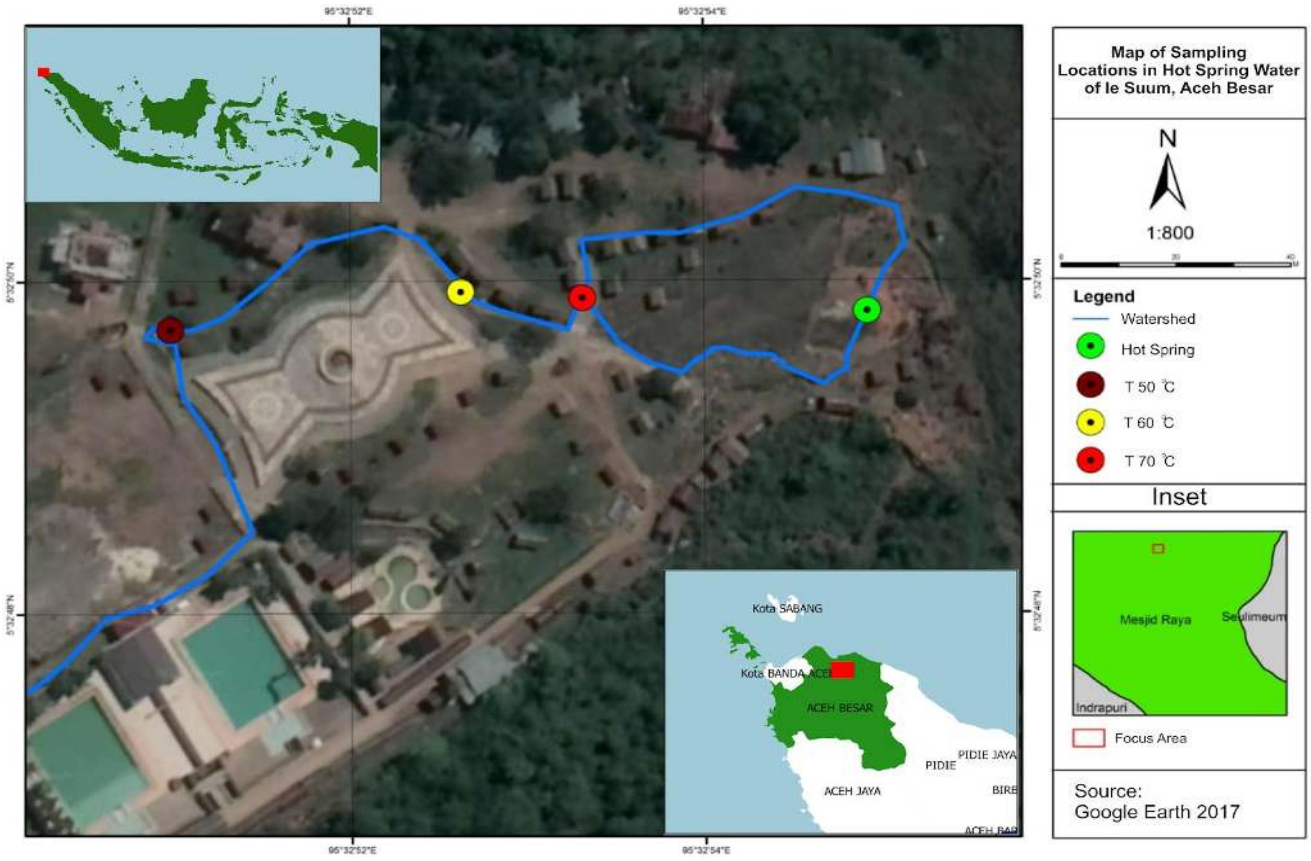

Figure 1. The map of sampling location in Ie Su'um Hot Springs, Aceh Besar, Indonesia

RESULTS AND DISCUSSION

Isolation of actinobacteria of thermophiles

Actinobacteria which were purified in YMA medium were six isolates. One isolate was from 50ºC and five isolates were from 60ºC, meanwhile, no actinobacteria were found at 70ºC. This was presumably due to incubation temperature of 45ºC is too low so that it inhibits the growth of actinobacteria, while the incubation temperature that is too high could damage nutrients in the media. Li et al. (2016) stated that nutrients in the media would be degraded and damaged at temperatures above 60ºC. Astuty (2017) said that actinobacteria grew well in the media because they were able to utilize the nutrients contained in the media and were able to break down carbon contained in the media. Jiang et al. (2012) successfully isolated 68 thermophilic actinobacteria from Central-Eastern Tibet river, China. A study by Valverde (2012) regarding biogeography of bacteria communities in hot water focused on actinobacteria succeeded in obtaining 12 thermophilic actinobacteria. According to Kokare et al. (2004); Bredholt et al. (2008) differences in results obtained could be caused by several factors such as environmental conditions, temperature, growing media and differences in pretreatment before the isolation.

Characterization of actinobacteria

Morphological characterization is basic information in describing actinobacteria included the formation of substrate mycelium, aerial mycelium, and the production of dissolved pigments (Wink 2011). Morphological character of actinobacterial isolates could be seen in Figure 2.

Actinobacteria colony form showed that ATIS51, ATIS62, ATIS63, and ATIS64 had a rough and irregular structure, whereas ATIS61 and ATIS65 had a rough and velvety structure. According to Lechevalier and Lechevalier (1967); Kanti (2005) based on its ability to form surface mycelium, actinobacteria are divided into two groups, namely groups that are able to produce mycelium which usually looks rough on the surface and groups that are unable to produce mycelium usually has a slippery colony surface.

Actinobacteria characteristics in YMA medium showed that actinobacteria were belonging to the genus *Streptomyces*. This result was in line with what was stated by Dhanasekaran et al. (2009) that *Streptomyces* sp. had a variety of aerial mycelium colors, ranging from white, gray, to brown.

According to Anandan et al. (2016), *Streptomyces* had thicker aerial mycelium than substrate mycelium. Aerial mycelium was able to show sufficient differentiation, so that different types of isolates could be separated into several groups that have similar aerial mycelium characteristics. This was one of the most important criteria for the classification of the genus *Streptomyces*. Aerial mycelium characteristics consist of structures (cotton, velvet, or powder), concentric zone formation, and pigmentation. The results of actinobacteria morphological characterization could be seen in Table 1.

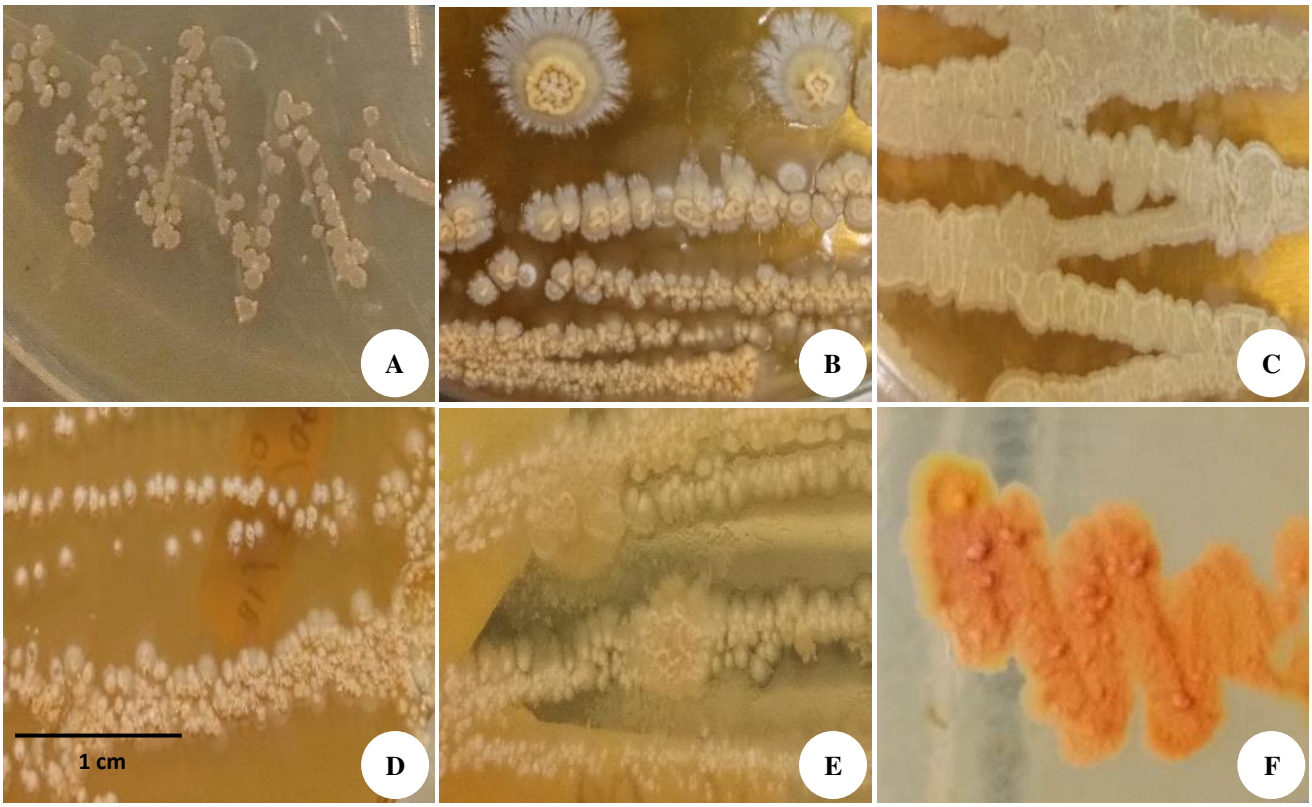

Figure 2. Characterization of thermophilic actinobacteria in YMA medium. A. ATIS51, B. ATIS61, C. ATIS62, D. ATIS63, E. ATIS64, F. ATIS65. Note: ATIS: Theemophilic Actinobacteria from Ie Seu'um

Table 1. Characterization of actinobacteria in YMA, YSA, and OA media

Media	Isolate	Color of aerial mycelium	Color of substrate mycelium	Diffused pigment	Elevation
YMA	ATIS51	Cream Colored	Cream Colored	-	Flat
	ATIS61	White in the margin and cream-colored in the middle	Light brown	Ivory yellow	Umbonate
	ATIS62	White	Light cream-colored	Cream-colored	Flat
	ATIS63	Dark cream-colored	Cream-colored	Cream-colored	Flat
	ATIS64	Cream-colored	Cream-colored	Yellow	Flat
	ATIS65	Orange	Orange	-	Umbonate
YSA	ATIS51	White	Yellow	-	Flat
	ATIS61	White	White bone	-	Flat
	ATIS62	Cream-colored	Yellow	Cream-colored	Flat
	ATIS63	Cream-colored	Yellow	-	Flat
	ATIS64	White	White	-	Flat
	ATIS65	White bone	White bone	-	Flat
OA	ATIS51	-	-	-	-
	ATIS61	White bone	White	-	Flat
	ATIS62	White	White	-	Flat
	ATIS63	White	White	-	Convex
	ATIS64	White	White	-	Flat
	ATIS65	White	White	-	Flat

The results of actinobacteria characterization (Table 1) showed that there were differences in colony form, color of aerial and substrate mycelium, and pigment produced by each isolate. The color of mycelium in YMA medium tended to be brighter, such as white to orange and most isolates were able to produce cream-colored and yellow pigments. Whaitaka et al. (2017) in his study of volcanic crater thermophilic actinobacteria succeeded in obtaining actinobacterial isolates which had cream-colored, brown and purple aerial mycelium. Valanarsu et al. (2010) revealed that YMA medium could be used to observe actinobacteria morphology because actinobacteria were able to grow well and produce pigments in this medium. According to Arasu et al. (2008), diffuse pigments produced by actinobacteria in YMA medium were usually cream-colored to red. A study conducted by Nurkanto and Agusta (2015) showed that actinobacteria that had different aerial mycelium colors were from different species after being identified molecularly. According to Shirling and Gottlieb (1996), YMA medium was suitable to be used to observe macroscopic morphology of actinobacteria colonies.

YSA and OA media were comparative media used to see differences in colony form, mycelium color, and diffused pigments. The color of actinobacteria colonies in YSA and OA media tended to be cream-colored and white, and only one isolate was capable of producing diffuse pigment. This occurred probably due to composition differences between YMA, YSA and OA media. Astuty (2017) in his research also obtained differences in mycelium color between YMA, YSA and OA media. The most varied mycelium colors were found in YMA medium. Wulandari and Nanik (2016) added that OA medium was a nutrient-poor media, contained a lot of minerals and starch as the carbon sources. Starch contained in OA medium had to be degraded into shorter-chain compounds by producing amylase to degrade starch into simpler saccharide compounds, so that it would be more easily utilized by actinobacteria.

According to Arasu et al. (2008), actinobacteria ability in producing aerial mycelium and pigmentation was strongly influenced by conditions, types of growing media and differences composition of each medium. According to Abdulla et al. (2008), the different mycelium colors were caused by the formation of special metabolites called pigments. Li et al. (2016) stated that aerial mycelium was a mycelium that developed on certain substrates and grew on the surface. Aerial mycelium was characterized by fibrous sheath, except the genus *Pseudonocardia* and *Amycolata*. Actinobacteria aerial mycelium types depended on species characteristics, nutritional conditions, or environmental factors.

Microscopic characterization was carried out with the aim of seeing differences hyphae form of actinobacteria isolates. Microscopic characterization results of actinobacteria isolates could be seen in Figure 3.

The results of microscopic characterization (Figure 3) showed that isolate ATIS51, ATIS62, ATIS63, ATIS64 had flexuous (rectiflexibilis) hyphae, ATIS61 had stright (rectis) hyphae, and ATIS65 had open spirals hyphae. Characterization of hyphae form was carried out based on Anandan et al. (2016) (Appendix 4). Gurung et al. (2009) also succeeded in obtaining bacteria isolates which had rectiflexibilis hyphae and were believed belong to the genus *Streptomyces*. Based on the characterization of hyphae form, all six isolates were believed belong to the genus *Streptomyces*.

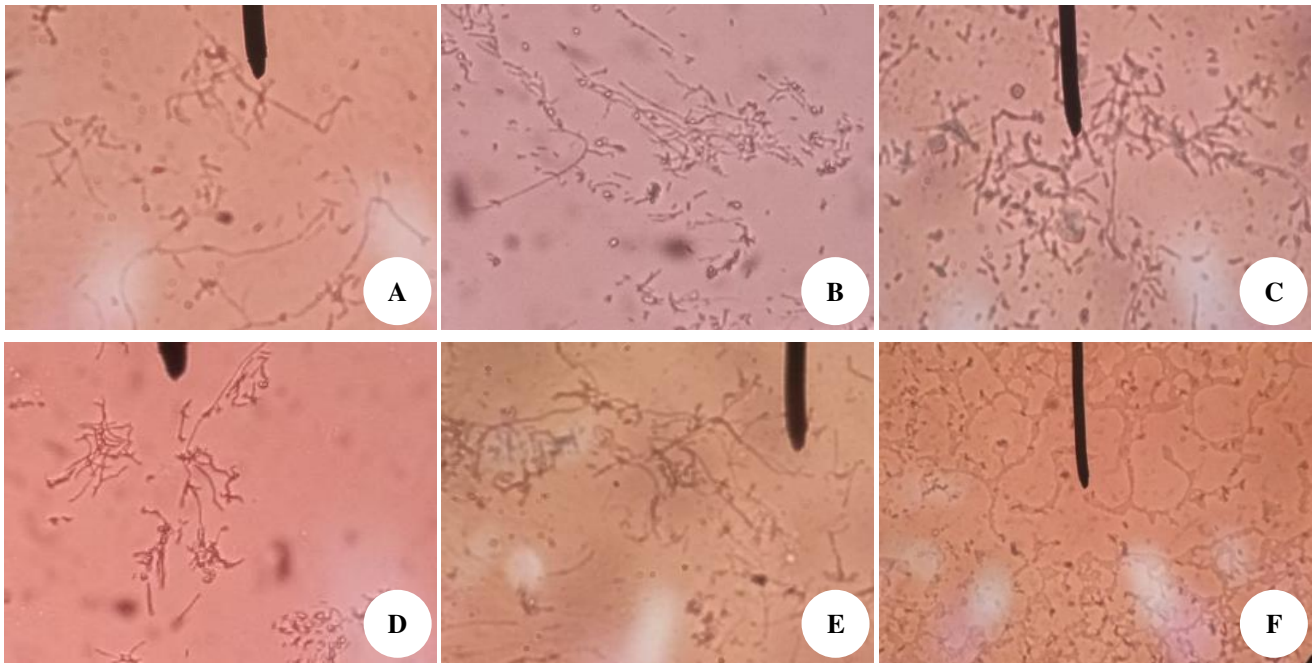

Figure 3. Microscopic characterization of thermophilic actinobacteria at 400x magnification. A. ATIS51, *flexuous*, B. ATIS61, *straight*, C. ATIS62, *flexuous*, D. ATIS63, *flexuous*, E. ATIS64, *flexuous*, F. ATIS65, *open spiral*

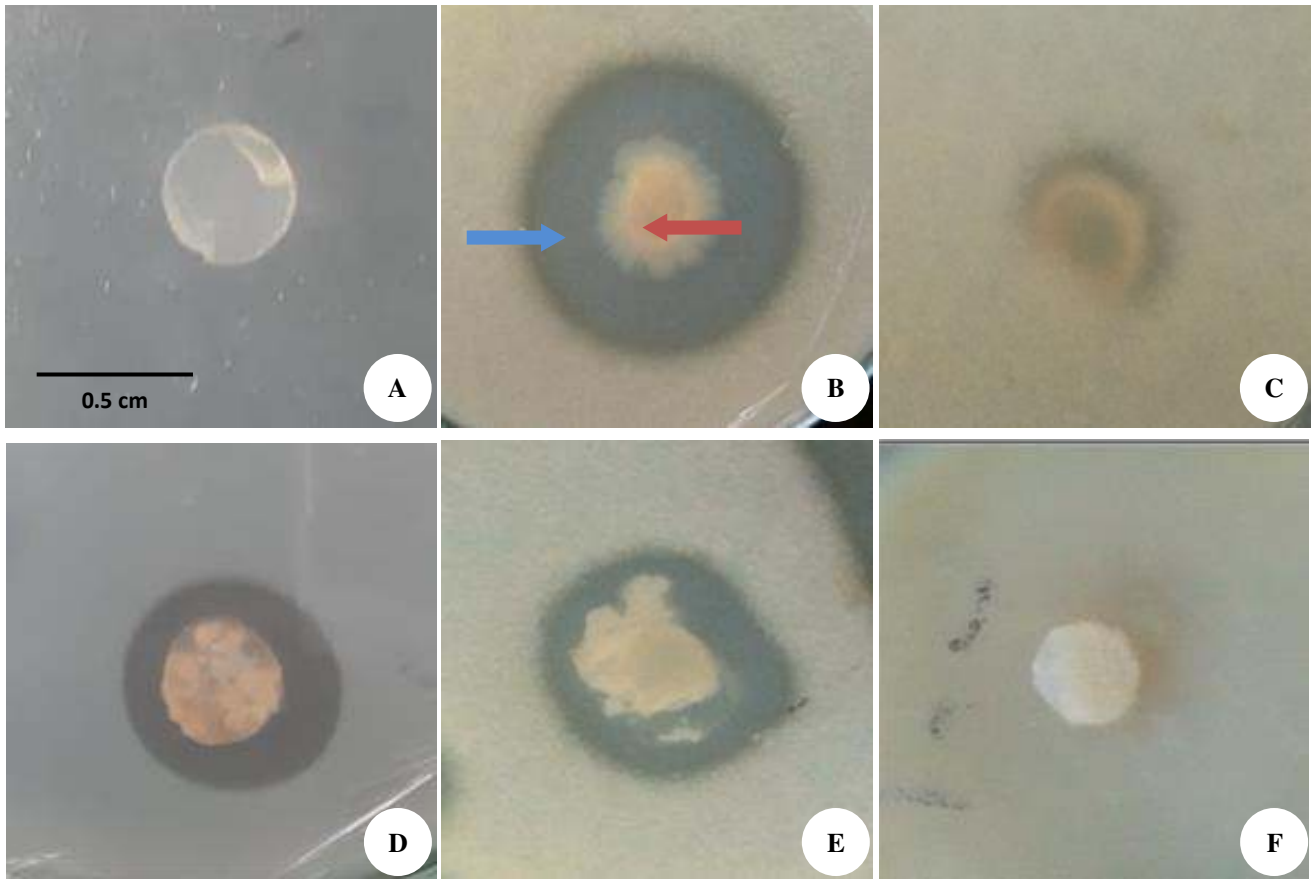

Figure 4. The results for screening of protein producing actinobacteria. A. ATIS51, B. ATIS61, C. ATIS62, D. ATIS63, E. ATIS64, F. ATIS65. Note: → : Clear zone; → : Bacterial colony

Kalakoutskii and Agre (1976) stated that the genus *Streptomyces* could have straight (rectis), flexuous (rectiflexibilis), curved (looped/retinaculiperti) and helicoidal (spirales) hyphae forms. Jeffrey (2008) added that various species that belong to the genus *Streptomyces* could have rectiflexibiles (RF), retinaculiaperti (RA) and spirales (S) spore chains. Flardh et al. (2012) stated that *Streptomyces* colony was not an accumulation of single cells like in other bacteria, but an accumulation of branching filaments. Colony formed complex hyphae branching where the aerial hyphae would later form a sporophore or an aerial spore chain. *Streptomyces* aerial spores were arranged in a long and curled chain.

Screening of protease producing actinobacteria

Measurements of actinobacteria isolates that were capable of producing protease were characterized by the formation of clear zone around the colony (Yuratmoko et al. 2007). The results of actinobacteria that were capable of producing protease could be seen in Figure 4.

Observation of protease producing actinobacteria was carried out on the fourth day of incubation. Based on Figure 4. Actinobacteria isolates that were capable of producing protease enzymes were isolated ATIS61, ATIS62, ATIS63, and ATIS64. Clear zone diameter obtained were 2.4; 1.5; 1.3 cm. Proteolytic Index (IP) value of ATIS61, ATIS63, and ATIS64 isolates were 3.8, 2, and 1.6 respectively. While IP value of isolate ATIS62 could not be calculated because the diameter of the clear zone formed was too small.

According to Linda et al. (2016), actinobacteria that were capable of producing protease were characterized by the formation of clear zone around the colony in media, which means that actinobacteria were able to hydrolyze proteins by producing protease. Vonothini et al. (2008) managed to select 6 actinobacteria isolates from Vellar river, India which had clear zones in gelatin agar medium. Clear zone was the indicator that actinobacteria isolates were able to utilize protein in the media as nutrition sources.

According to Kurniawan (2011), if there was a clear zone ≥ 2 cm around the colony, it could be concluded that the bacteria were included into strains that were good for producing protease. The clear zone could be formed because bacteria secreted protease to their environment so that milk proteins would be hydrolyzed and caused the surrounding colony became clear.

In conclusion, the conclusion that could be taken based on this study was that the number of actinobacteria isolates obtained from Ie Seu'um hot springs were six isolates. One isolate obtained from 50ºC and 5 isolates from 60ºC, while no actinobacteria isolate was obtained at 70ºC. Based on microscopic characterization results, all six isolates obtained were believed to belong to the genus *Streptomyces*. Actinobacteria isolates that were capable of producing protease were isolated ATIS61, ATIS62, ATIS63, and ATIS64. Isolate ATIS61 had the highest Proteolytic Index (IP) value which was 3.8.

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