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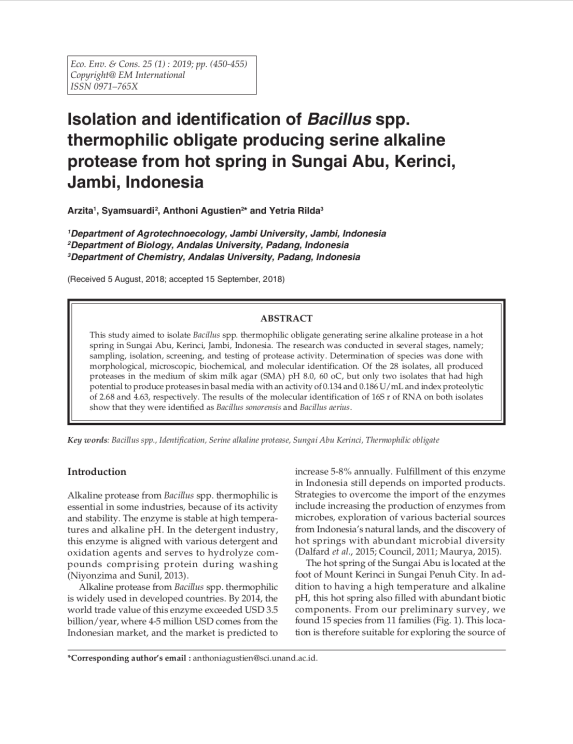

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Isolation and identification of *Bacillus* spp. thermophilic obligate producing serine alkaline protease from hot spring in Sungai Abu, Kerinci, Jambi, Indonesia

Arzita[1], Syamsuardi[2], Anthoni Agustien[2]* and Yetria Rilda[3]

[1]Department of Agrotechnoecology, Jambi University, Jambi, Indonesia
[2]Department of Biology, Andalas University, Padang, Indonesia
[3]Department of Chemistry, Andalas University, Padang, Indonesia

(Received 5 August, 2018; accepted 15 September, 2018)

ABSTRACT

This study aimed to isolate *Bacillus* spp. thermophilic obligate generating serine alkaline protease in a hot spring in Sungai Abu, Kerinci, Jambi, Indonesia. The research was conducted in several stages, namely; sampling, isolation, screening, and testing of protease activity. Determination of species was done with morphological, microscopic, biochemical, and molecular identification. Of the 28 isolates, all produced proteases in the medium of skim milk agar (SMA) pH 8.0, 60 oC, but only two isolates that had high potential to produce proteases in basal media with an activity of 0.134 and 0.186 U/mL and index proteolytic of 2.68 and 4.63, respectively. The results of the molecular identification of 16S r of RNA on both isolates show that they were identified as *Bacillus sonorensis* and *Bacillus aerius*.

Key words: *Bacillus spp., Identification, Serine alkaline protease, Sungai Abu Kerinci, Thermophilic obligate*

Introduction

Alkaline protease from *Bacillus* spp. thermophilic is essential in some industries, because of its activity and stability. The enzyme is stable at high temperatures and alkaline pH. In the detergent industry, this enzyme is aligned with various detergent and oxidation agents and serves to hydrolyze compounds comprising protein during washing (Niyonzima and Sunil, 2013).

Alkaline protease from *Bacillus* spp. thermophilic is widely used in developed countries. By 2014, the world trade value of this enzyme exceeded USD 3.5 billion/year, where 4-5 million USD comes from the Indonesian market, and the market is predicted to increase 5-8% annually. Fulfillment of this enzyme in Indonesia still depends on imported products. Strategies to overcome the import of the enzymes include increasing the production of enzymes from microbes, exploration of various bacterial sources from Indonesia's natural lands, and the discovery of hot springs with abundant microbial diversity (Dalfard *et al.*, 2015; Council, 2011; Maurya, 2015).

The hot spring of the Sungai Abu is located at the foot of Mount Kerinci in Sungai Penuh City. In addition to having a high temperature and alkaline pH, this hot spring also filled with abundant biotic components. From our preliminary survey, we found 15 species from 11 families (Fig. 1). This location is therefore suitable for exploring the source of

***Corresponding author's email :** anthoniagustien@sci.unand.ac.id.

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the alkaline serum protease producing bacteria, since *Bacillus* spp. thermophilic obligate live in the temperature range from 55 to 90 °C and pH 7.5-12 (Agustien *et al.*, 2015; Ebrahimpour and Kariminik, 2015; Canganella and Wiegel, 2014; Selim *et al.*, 2014).

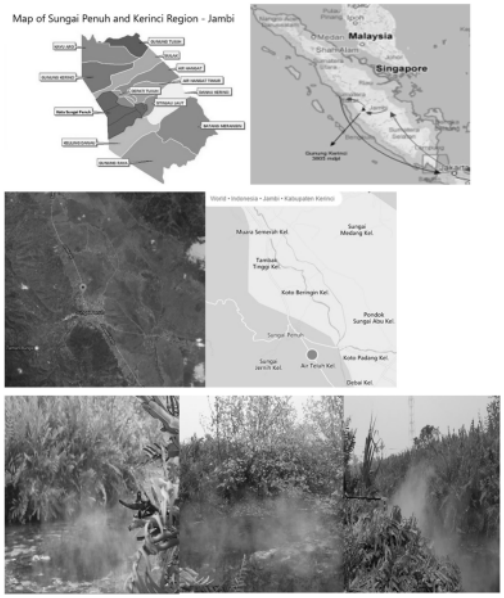

Fig. 1. Location of the Sungai Abu hot spring, Air Hangat Region, Kerinci, Jambi Indonesia

One of the methods to obtain *Bacillus* spp. thermophilic that can produce potential and high-activity proteases is by isolating bacteria from thermal environments such as the Sungai Abu hot springs. The isolate can be screened in selective mediums, and then the produced enzymes are tested for their activity and stability in high temperatures and alkaline pH. Identification of bacterial species can be made morphologically, microscopically, biochemically and molecularly. Molecular technology with 16S rRNA method can precisely and accurately identify the type of cultured bacteria up to species level. This method can also determine the characteristics, phenotypic, genotypic and classification of various types of bacteria. Isolation and screening of *Bacillus* spp. thermophilic obligate from hot springs is essential to discover the potential *Bacillus* spp that can produce serine alkaline protease with desired characteristics (Ebrahimpour and Kariminik, 2015; Ha *et al.*, 2013; Selim *et al.*, 2014).

Experimental

Hot water sampling

Hot water samples were taken from 10 cm below the surface using sterile bottles (100 mL) and flasks (500 mL) at four points of sampling in the pond and in the flow of the Sungai Abu hot spring, which has a temperature of 67-92 °C (Fig. 2). The temperature of water sample was kept stable by placing the sample on a box containing hot water that was changed every two hours. Inoculation was done within 24 hours of sampling at 60 °C. Physical (temperature and turbidity), chemical (pH and mineral content), and biological (plant species) properties were also observed in hot springs and surrounding areas.

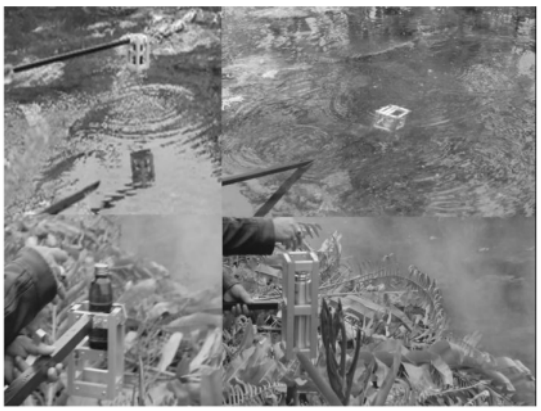

Fig. 2. Water sampling technique

Isolation of *Bacillus* spp. thermophilic obligate

Isolation of *Bacillus* spp. thermophilic was carried out through the following steps; first, the water sample was heated to 67-92 °C, and then 1 mL of it was transferred into a Petri dish containing 15 mL of *Bacillus* Thermophilic (TB) medium pH 8.0. Then, it was coated with wrap and incubated at 60 °C for 24 hours. Round and white colonies are colonies of *Bacillus* spp. thermophilic. The colonies were purified using the quadrant method and reproduced and stored in tilted TB medium. This medium was used as *Bacillus* spp stock. (Agustien *et al.*, 2015; Mothe and Sultanpuran, 2016).

Screening of the alkaline protease producing *Bacillus* spp. Thermophilic obligate

The medium used for the identification and produc-

tion of bacterial proteases was plate count agar (Merck) with skim milk (10 g/l). Screening of *Bacillus* spp. thermophilic that produced alkaline protease was developed from Irfan *et al.* (2012) method. Each stock of *Bacillus* spp. was inoculated into a medium Milk Skim Agar (MSA) pH 8.0 in a Petri dish and incubated at 60 °C. A clear zone formed around the colony was an indication of proteolytic activity. The formation of the clear zone was observed, and the diameter was measured to determine proteolytic index using the formula:

$$P = \frac{\emptyset c_i \quad z_i \quad - \emptyset b_i \quad c_i}{\emptyset b_i \quad c_i}$$

Production and test of serine alkaline protease activity of *Bacillus* spp. thermophilic obligate

Production and protease activity of *Bacillus* spp determined using method developed from Agustien *et al.* (2015) and Dalfard *et al.* (2015). Five percent of the potential isolates were fermented on basal medium pH 8.0 at temperature 60 °C with agitation 150 g for 24 hou Subsequently, 0.5 mL of fermented supernatant was added to a 1.5 ml tube containing 0.25 mL of casein 2%, which was dissolved in 0.25 mL Tris-HCl buffer 50 mM pH 8.0. The tube was incubated at 60 °C for 10 mins. The reaction as stopped with 0.5 mL Trichloro Acetic Acid 10%. The mixture was incubated at room temperature for 20 minutes and then centrifuged at 6000 g for 20 mins. 0.375 mL supernatant was transferred into new tubes and was added with 1.25 mL of Na_2CO_3 0.5 M and 0.25 mL of Folin Ciocalteus 1N. The absorbance of the mixture was read at 578 nm. The enzyme activity was determined by using the formula:

$$a \quad \left(\frac{U}{m}\right) = \frac{A.sc \quad - A.b}{A.s_i \quad - A.b} \times \frac{D \quad f}{r_i \quad ti}$$

A unit of enzyme activity was defined as the number of enzymes that produce color equivalent to 1 µg of tyrosine per minute at pH 8.0 and temperature of 60 °C. Serine protease was determined by testing phenylmethylsulfonyl fluoride (PMSF) inhibitors on enzyme production (Ghareib *et al.*, 2014; Mothe and Sultanpuran, 2016).

Identification of serine alkaline protease producing *Bacillus* spp. thermophilic obligate

Identification of *Bacillus* spp. thermophilic obligate that potentially produced serine alkaline protease was carried out in three stages; macroscopic (morphological colonies aged ≥ 24 hours), microscopic (Gram cell and spore staining), and biochemistry (sugar series test, oxidase catalase, gelatin, amylase hydrolysis, gas formation, and motility) (Ghati *et al.*, 2013). Molecular identification was made to the potential isolate, which had IP > 2, using a modification of Panda *et al.* (2013) method. The treatment was initiated by isolating the isolate DNA from the TB medium inoculated at 60 °C, pH 8.0, for 8 hours using lysozyme. The DNA from Polymerase Chain Reaction (PCR) product was electrophoresed with 1% agarose gel and purified with QIA quick PCR (Qiagen). Measurements were done with A260/A280 spectro-photometry, 16S rDNA isolate sequence amplified with Big Dye ABI PRISM Chemistry, Biosystems, USA, with 1492R primers (5'-TAC TACGGYCTTGTTACGACT-3 ') and 27F (5'-AG AGTTTGATCMTGGCTCAG-3') on a PCR machine. The PCR protocol performed one denaturation cycle at 94 °C for 1 min, annealing at 48 °C for 1 min, and elongation at 72 °C for 2 mins for 30 cycles, and additional elongation 10 min after amplification process (Selim *et al.*, 2014). The pure reaction mixture was sequenced electrophoretically with DNA sequencer machine Biosystems Applied Model 310 (Perkin Elmer, USA). PCR fragments were analyzed by a Macrogen sequencer machine, and then the resulting nucleotide sequence was compared to Gen Bank's base data using the BlastN program. Phylogenetic analysis of isolates was done with MEGA (molecular evolutionary genetic analysis) version 7 (Kumar *et al.*, 2016). A phylogenetic tree was constructed based on genetic kinship distance by the neighbor-joining method (Tamura *et al.*, 2004). The strength of phylogenetic trees was tested using bootstrap (Felsentein, 1985) with 1000 repetitions.

Results and Discussion

The hot spring of the Sungai Abu, Kerinci, Jambi, Indonesia is located on 20 'S 02 02' 18.3 ', E 101 26' 39.8 'and 812 m asl (GPS data). 28 strains of *Bacillus* spp. thermophilic were isolated from this location, and all strains produced proteases, which was characterized by proteolytic index ranging from 0.10-4.63 (Table 1). Morphological, microscopic, and biochemical characteristics of all isolated strains, are shown in Table 1, and Figs. 3, 4, 5.

Types of *Bacillus* thermophilic alkaline protease producers in hot spring of the Sungai Abu were di-

Table 1. Proteolytic index and strain observation of thermophilic obligate *Bacillus spp.* producing alkaline protease

Code of isolate	PI	Colony morphology (color, shape, edge, surface, and diameter (mm))	Microscopic (cell shape, Gram, spore location)	Biochemical																				
				CATALASE	OXYDASE	GAS	SULFIDE	TSIA	MORTILITY	INDOL	UREA	CITRATE	LACTOSE	GLUCOSE	SUCROSE	MANNITOL	MR	VP	OF	ARABINOSE	XYLOSE	NITRATE	GELATIN
SA-1	0.66	White, Circular, Erose, Flat, 3.08	Basil, G+, Subterminal	+	+	-	-	M/K	+	-	+	-	-	-	+	+	+	+	-	-	-	+	+
SA-2	0.84	Dark white, Circular, Erose, Umbellate, 2.82	Basil, G+, Subterminal	+	-	-	-	M/K	+	-	-	-	-	-	+	-	-	+	-	+	-	+	+
SA-3	0.37	Pale white, Irregular Filament, Convex, 2.82	Basil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	-	+	-	+	+	-	+	-	-	+
SA-4	0.64	White, Circular, Entire, Raised, 3.36	Basil, G+, Central	+	+	+	-	M/K	+	-	+	-	-	-	+	+	+	+	-	-	-	+	+
SA-5	0.72	Dark white, Irregular, Filament, Flat, 5.19	Basil, G+, Terminal	+	-	-	-	M/K	+	-	-	-	-	+	+	-	+	+	-	+	-	+	+
SA-6	1.23	White, Circular, Entire, Raised, 1.84	Basil, G+, Central	+	+	-	-	M/K	+	-	+	-	-	-	+	+	+	+	-	-	-	+	+
SA-7	1.06	Dark white, Irregular, Filament, Convex, 4.22	Basil, G+, Terminal	+	+	+	-	M/K	+	-	+	-	-	-	+	+	-	+	-	-	-	+	+
SA-8	0.10	White, Irregular, Filament, Convex, 5.58	Basil, G+, Tentral	+	+	+	-	K/K	+	-	+	-	-	+	+	+	+	+	-	-	-	+	+
SA-9	0.18	Dark white, Irregular, Lobate, Raised, 2.84	Polymorph, G+, Subterminal	+	+	-	-	M/K	+	-	+	-	-	-	+	+	+	+	-	-	-	+	+
SA-10	0.80	Dark white, Circular, Entire, Raised, 1.93	Diplobasil, G+, Central	+	-	-	-	M/K	+	-	+	-	-	-	+	+	-	+	-	-	-	-	+
SA-11	**2.68**	**White, Rhizoid, Filament, Flat, 1.69**	**Diplobasil, G+, Central**	+	+	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	-
SA-12	0.51	White, Irregular, Filament, Convex, 2.11	Basil, G+, Subterminal	+	+	-	-	M/K	+	-	-	-	-	+	+	+	-	+	-	-	-	+	-
SA-13	1.04	White, Circular, Entire, Raised, 1.84	Basil, G+, Terminal	+	+	-	-	M/K	+	-	+	-	-	-	+	+	-	+	-	-	-	+	-
SA-14	0.49	White, Irregular, Entire, Raised, 2.52	Basil, G+, Subterminal	+	-	-	-	M/M	-	-	-	-	-	-	+	-	+	+	-	+	-	+	+
SA-15	0.33	White, Circular, Filament, Convex, 3.61	Basil, G+, Central	+	+	-	-	M/K	+	-	+	-	-	-	+	+	-	+	-	-	-	+	-
SA-16	**4.63**	**White, Circular, Entire, Convex, 3.86**	**Diplobasil, G+, Terminal**	+	+	-	-	M/K	-	-	-	-	-	-	+	-	+	+	-	+	-	+	+
SA-17	0.19	Pale white, Circular, Filament, Raised, 3.71	Diplobasil, G+, central	+	-	-	-	K/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-18	0.30	White, Circular, Filament, Convex, 2.70	Basil, G+, Central	+	+	-	-	K/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-19	0.26	Pale white, Circular Undulate, Raised, 2.22	Diplobasil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-20	0.59	Dark white, Circular, Entire, Flat, 2.79	Diplobasil, G+ Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-21	0.66	White, Circular, Entire, Raised, 2.88	Streptobasil, G+, Subterminal	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-22	0.57	Pale white, Irregular, Entire, Convex, 3.30	Basil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	+	-	+	+
SA-23	1.10	Pale white, Circular, Entire, Raised, 1.54	Diplobasil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-24	0.54	White, Irregular, Filament, Raised, 2.35	Diplobasil, G+ , Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-25	0.40	Pale white, Irregular, Erose, Raised, 2.24	Basil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-26	0.44	Pale white, Circular, Erose, Raised, 1.87	Streptobasil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+
SA-27	0.44	Pale white, Irregular, Filament, Convex, 5.15	Streptobasil, G+, Central	+	-	-	-	M/K	+	-	-	-	-	+	+	+	+	+	-	-	-	+	+
SA-28	0.58	Pale white, Circular, , Entire, Raised, 2.63	Basil, G+, Terminal	+	-	-	-	M/K	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+

verse. This may be influenced by the alkalinity of the water (pH 8.6), water temperature (67-92 oC), and minerals content: NO3 3.2 mg/l, Fe 0.45 ppm, and Ca 3.77 ppm. Moreover, various plants were grown around the pool of the hot springs, which may support the growth of *Bacillus* spp. thermophilic in extreme environment. Thermophilic bacteria in hot spring can survive at temperatures of 70 to 90 °C. The ability to adapt at high temperatures is due to their molecular modification at cellular and subcellular levels. For example, cell membranes are rich in saturated fatty acids with long methylated carbon chain that could form strong hydrophobic bonds to maintain fluidity and permeability of cell membranes at extreme temperatures. Other than temperature, the wide range of pH at alkali condi-

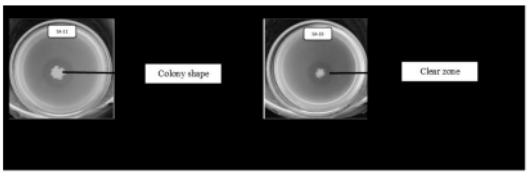

Fig. 3. Morphology of *Bacillus* spp.

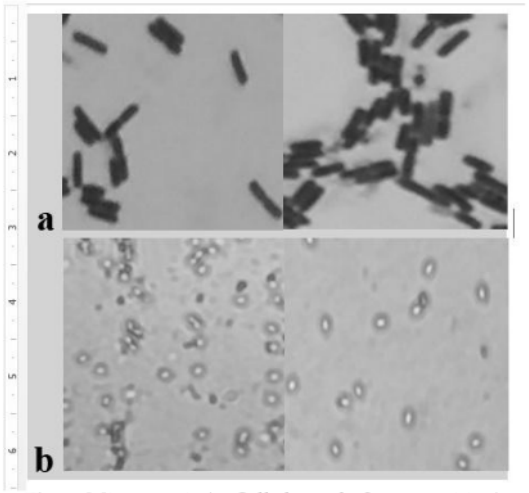

Fig. 4. Microscopic (a. Cell shape, b. Spore position)

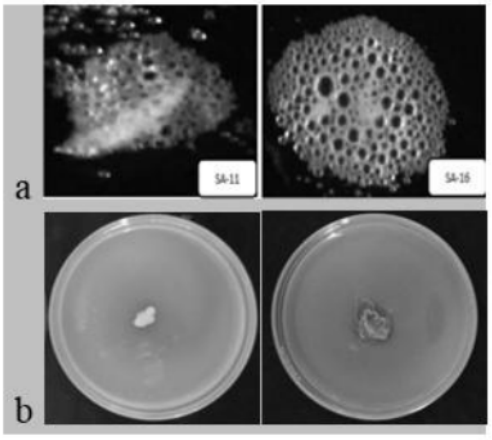

Fig. 5. Biochemical observation (a. Catalase test, b. Gelatin test)

tion (7.5-12) also greatly affects the diversity of the *Bacillus* species (Agustien *et al.*, 2015; Canganella and Wiegel, 2014; Lu *et al.*, 2013; Panda *et al.*, 2013). From the 28 identified strains of *Bacillus* spp. thermophilic obligate, there were two strains that have a potential to produce protease: the SA-11 and SA-16 with the proteolytic index of 2.68 and 4.63, respectively (Table 1 and Fig. 6). Based on molecular properties, these two isolates were identified as *Bacillus sonorensis* and *Bacillus aerius*. The phylogenetic tree is shown in Fig. 7. Both species have previously

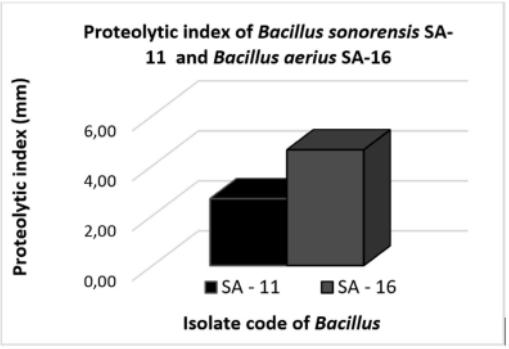

Fig. 6. Proteolytic index of *B. sonorensis* and *B. aerius*

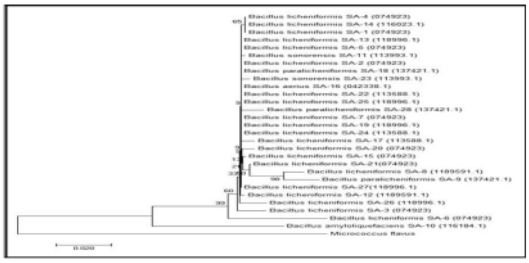

Fig. 7. Phylogenetic tree of *Bacillus spp.* thermophilic obligate producing serine alkaline protease isolates from hot springs in Sungai Abu, Kerinci, Jambi, Indonesia using *Micrococcus flavus* as an outgroup

been found in several hot springs such as in Morocco (Aanniz *et al.*, 2015), in Tarabalo India (Panda *et al.*, 2013), in Indonesia in Changhar Malang (Ibrahim *et al.*, 2013), and in the hot mudflow Lapindo (Habibie, 2014). The protease activity of *B. Sonorensis* and *B. aerius* in basal media was 0.137 and 0.186 U/ml, respectively (Fig. 8). The PMSF 0.1 mM test showed 80-86% inhibition, indicating that the protease from the *Bacillus* from Sungai Abu hot spring could be classified as serine protease and was suitable for application in the detergent industry (Ghareib *et al.*, 2014; Niyonzima *et al.*, 2013; Mothe and Sultanpuran, 2016).

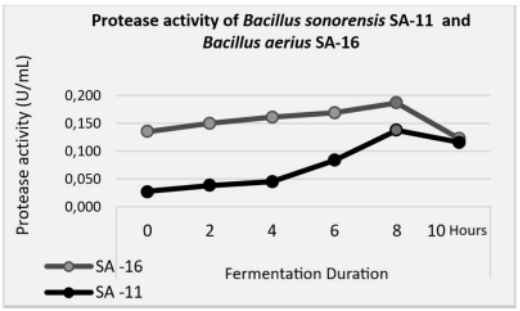

Fig. 8. Protease activity of *B. sonorensis* and *B. aerius*

Conclusion

There were 28 strains of *Bacillus spp.* thermophilic obligate producing alkaline proteases identified from the hot springs of the Sungai Abu, Kerinci, Jambi, Indonesia. Two strains were identified as *B. sonorensis* and *B. aerius* generating potential serine alkaline protease with a proteolytic index of 2.68 and 4.63 and protease activity on basal media 0.137 and 0.186 U/ml, respectively.

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