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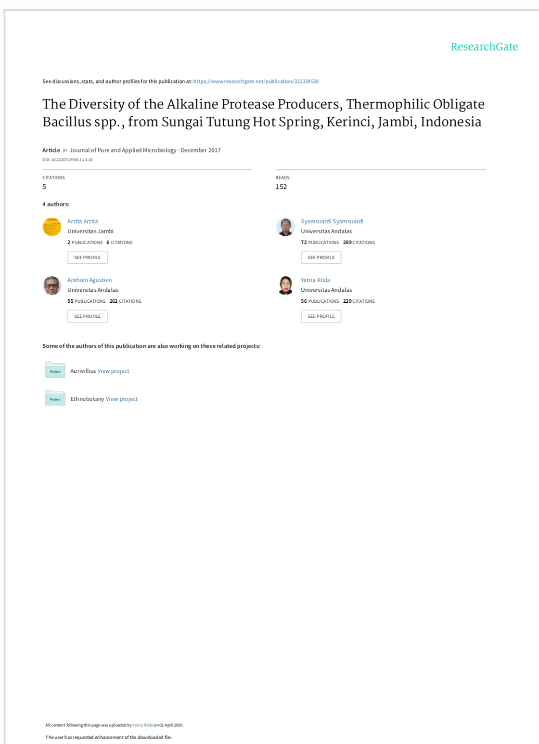

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Article *in* Journal of Pure and Applied Microbiology · December 2017
DOI: 10.22207/JPAM.11.4.18

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The Diversity of the Alkaline Protease Producers, Thermophilic Obligate *Bacillus* spp., from Sungai Tutung Hot Spring, Kerinci, Jambi, Indonesia

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http://dx.doi.org/10.22207/JPAM.11.4.18

(Received: 01 September 2017; accepted: 15 Ocotber 2017)

This study aimed to identify the diversity of the alkali protease producers, thermophilic obligate *Bacillus* spp., from Sungai Tutung Hot Spring in Kerinci, Jambi, Indonesia. The use of modified methods in exploring biodiversity from an extreme environment was useful as a tool for the discovery of more specific and thermostable enzymes from new sources. Based on the sequence analysis of the DNA isolate, there were six species with 14 strains identified from the hot spring. All strains grew well in thermophilic *Bacillus* medium at 60 ^{0}C with pH 8.0. They are also potentially able to produce alkali protease with the proteolytic index in the range of 0.25-6.15. Although characteristics of each strain were diverse, generally the color of the strains was from white to dark white and the shape was round with flat edges and raised elevations. Gram-positive bacteria with rod shape, central endospore, and purple color were observed under the microscope. Biochemical observation showed that all strains exhibited positive result from the catalase test, did not produce gas and sulfide, had positive mortality and to the sugar series test showed positive and negative variations. The identified species were: *Bacillus sonorensis, Bacillus lichenmiformis, Brevibacillus borstelensis, Paenobacillus, Bacillus aerius, dan Fictibacillus gelatini.*

Keywords: Diversity, *Bacillus*, thermophilic obligate, protease alkali, Sungai Tutung.

Protease is an important enzyme, 60% of the industrial production of enzymes in the world is a protease, and 25% of them are thermostable. The sale value for protease throughout 2013-2014 was equal to two-thirds of the world's enzyme market value. Demand for proteases is increasing, especially alkaline proteases from microbial species. In Indonesia, the need for a protease is also increasing and to fulfill it, this country still depends on imported products. The most appropriate strategies to anticipate the import of this enzyme are by increasing production of enzymes from microbes, exploring new bacterial sources from Indonesia's natural terrain, and increasing the isolation and screening of alkaline protease producing *Bacillus* in selective medium [1-3].

The thermophilic *Bacillus spp.* that produce alkaline protease are; *B. amyloliquefaciens, B. alcalophilus, B. brevis, B. clausii, B. cereus, B. halodurans B. licheniformis, B. lentus, B. subtilis* and *B. stearothermophillus.* This species is widely used in biotechnology, such as in food industry,

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medicine, chemical, silk and leather [3,4]. Special for detergent industries, they need thermostable protease, and it is usually produced by thermophilic obligate *Bacillus* [5]. Thermophilic obligate *Bacillus* spp. is widespread in extreme and high-temperature habitats, such as in hot springs, volcanic craters, geothermal, hot mud, mining, composting, lakes, and deep seas at temperatures between 60-80 ^{0}C, optimally at 70 ^{0}C [1,6].

Based on the optimum temperature, the thermophilic microbes are divided into three groups: thermophile with optimum temperatures between 50 ^{0}C and 64 ^{0}C and maximum at 70 ^{0}C, extremes with optimum temperatures between 65 ^{0}C and 80 ^{0}C, and hyperthermophile with optimum temperatures above 80 ^{0}C and maximum above 90 ^{0}C. *Bacillus* thermophilic obligate cannot survive at room temperature more than seven days, and most of the bacterial colonies from hot water survive at temperatures above 70 ^{0}C [7,8].

Bacillus gains popularity in biotechnology because it is relatively easy to isolate from various environments and can grow in a synthetic medium [7]. The fermentation ability of different types of *Bacillus* differs at various pH and temperature. The combination of alkaline pH and thermophilic properties in the *Bacillus* genus are indications that this genus is potential to be developed for producing stable enzymes at high temperatures [4]. Benefits of using thermophilic *Bacillus* spp. Are: the bacteria can be isolated from thermal environments with high cell growth rates, the contamination risk can be minimized, volatile compounds can be easily separated, the viscosity of the fermentation solutions is low, reaction rates are relatively fast, and the catalyzing reactions are more efficient and eco-friendly without byproducts [9].

Hot springs are widespread in Indonesia because this country had 22 active volcanoes [10]. This hydrothermal condition contains abundant and diverse thermophilic bacteria. However, up until now, there has been little attempt to isolate and characterize the thermophilic bacteria from hot springs [7]. The hot spring of Sungai Tutung is located in one of the volcanoes of Mount Kerinci, in Kecamatan Air Hangat, Kerinci District, Jambi Province, Indonesia (Fig 1). This hot water is unique because it has alkaline pH. The hot springs with alkaline water are rare, both in Indonesia and in the world. The alkaline hot springs usually contain a high amount of base mineral, which makes microbial populations are more diverse than in the acidic hot springs [11,12]. Therefore, this study aimed to determine the diversity of thermophilic obligate *Bacillus* spp. that could produce alkaline protease in Sungai Tutung.

Experimental section

Hot water sampling

The sampling of hot water at the studied location was done using a modified method of Agustien, Yetria [7] and Panda, Sahu [8]. The samples were taken 10 cm below the surface of the water with a 100 mL sterile bottle and 500 mL flask from ponds and streams of Sungai Tutung (Fig 2). There were four sampling points with temperatures of 70, 75, 80 and 85 ^{0}C. The temperature of the hot water samples was kept stable during the transportation to the laboratory by placing the sample containers in a box containing hot water. The samples were isolated from a medium of thermophilic *Bacillus* within 24 hours of the sample collection. Temperature, pH, and metal content of the water were analyzed. Microorganisms that presented at the edges and in the streams of the hot springs were also identified.

Isolasion of thermophillic obligate *Bacillus*

Thermophilic obligate *Bacillus* spp. isolation was carried out as follows: 1 mL of the hot water sample was transferred into a petri dish and 15 mL of TB medium (Thermophilic *Bacillus*) pH 8.0 was added [13]. The petri dish was wrapped and incubated at 60 ^{0}C for 24 hours. The *Bacillus* spp. colonies can be identified by the round shape and white color. The thermophilic *Bacillus* spp. colonies were purified on tilted TB medium and labeled as *Bacillus* spp. stock [7,14,15].

Screening of the thermophilic obligate *Bacillus* spp. that produced alkaline protease

Screening of the thermophilic obligate *Bacillus* spp. that produced alkaline protease was done following the method of Irfan, Safdar [16] with modification. Each pure thermophilic *Bacillus* spp stock was inoculated into a skim milk agar medium (SMA) pH 8.0 [13] and incubated at 60 ^{0}C for 24 hours. Bacterial colonies and clear zones formed around the colony were the indications of the proteolytic activity. The colonies were observed and the diameter was measured to determine its proteolytic index (PI) using the formula:

$$PI = \frac{\text{Ø clear zone} - \text{Øbacterial colony}}{\text{Øbacterial colony}}$$

Indentification of the thermophillic obligate *Bacillus* spp. that produced alkali protease

Bacillus sp. isolates living in alkaline and high-temperature conditions were identified macroscopically, microscopically, biochemically, and molecularly using a 16S r RNA method. The steps are as follows: 1) the bacteria colony morphology was macroscopically observed from the agar plate under the age of 24 hours; 2) microscopic observation was done by coloring the pure cell and spore isolates; 3) biochemical observations were carried out by testing the catalase oxidase, gelatin, amylase hydrolysis, gas formation, and motility [11,14]; 4) the bacteria molecular was analyzed following the method from [8] with modification. The molecular analysis was initiated by isolating isolate DNA from TB medium grown at 60 ^{0}C, pH 8.0, for 8 hours using lysozyme. The DNA from polymerase chain reaction (PCR) product was electrophoresed on 1% agarose gel and purified with QIA quick PCR (Qiagen). The measurement was done with A260 / A280 spectrophotometry, 16S rDNA isolate sequence amplification with Big Dye ABI PRISM

Tabel 1. Proteolytic index of thermophilic obligate *Bacillus* spp. from hot spring in the Sungai Tutung

Code Isolates	Proteolytic Index
ST-1	0.78
ST-2	0.64
ST-3	0.25
ST-4	0.76
ST-5	1.91
ST-6	0.68
ST-7	0.92
ST-8	6.15
ST-9	0.59
ST-10	0.90
ST-11	0.32
ST-12	0.46
ST-13	1.48
ST-14	2.06

Chemistry, Biosystems, USA, with 1492R primers (5'-TACTACGGYCTTGTTACGACT-3 ') and 27F (5'-AGAGTTGAT CMTGGCTCAG-3') on a PCR machine. The PCR protocol was performed with one denaturation cycle at 94 ^{0}C for 1 minute, annealing at 48 ^{0}C for 1 minute, 30 cycles of elongation at 72 ^{0}C for 2 minutes per cycle, and an additional elongation for 10 minutes after the amplification process [17]. The pure mixture of sequence reaction was electrophoresed with automatic DNA sequencer Biosystems Model 310 (Perkin Elmer, USA). PCR fragments were analyzed by a Macrogen sequencer machine and the sequence results were then compared with Gen Bank database using Blast N program. The analysis of phylogenetic isolates was done with the Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 [18]. The phylogenetic tree was constructed based on genetic kinship distance by neighbor-joining (NJ) method [19]. The strength of phylogenetic trees was tested using bootstrap with 1000 repetitions [20].

RESULTS AND DISCUSSION

The hot spring of Sungai Tutung is part of the Kerinci Seblat National Park area under the Mount Kerinci (Jambi Local Government Data). This river is located on 20 'S 02 02' 19.4 "and E 101 26 '37.9", 812 M above sea level. The temperature of the river is in the range of 70-85 ^{o}C with alkaline pH (8.4). The hot spring is alkaline (pH 7,5 - 14), rich in minerals, and contains sodium carbonate, which causes a diversity of its biotas. Wang, Xu [3] and Logares, Lindström [21] stated that the environment affects community development in extreme habitats and special habitat is more responsive to the environmental selection. The water pH increases as it flows to the surface because it dissolves minerals and CO_2. Even though H_2S sometimes present in the water, sulfur oxidation on the surface does not affect the pH, as it stays constant in the range of 9-10 due to water abundance. Mothe and Sultanpuram [15] reported that the optimum pH and temperature for protease activity were at 8.0 and 60 ^{o}C, respectively.

The composition of some minerals in the hot springs of Sungai Tutung was: 2.8 mg/L NO_3, 0.37 ppm Fe, and 0.54 ppm Ca. Trashes that were

Table 2. The identification of Bacillus spp. Thermophilic obligate that produced alkaline protease isolates from hot springs Sungai Tutung Kerinci Jambi Indonesia

Isolate Code	Colony morphology (color, shape, edge, surface)	Microscopic (cell shape, Gram, spore location)	Biochemistry																			
			Catalase	Oxydase Gas	Sulfide	TSIA	Mortility	Indol		Urea	Citrate	Lactose	Glucose	Sucrose	Mannitol	MR	VP	OF	Arabinose	Xylose	Nitrate	Gelatin
ST-1	White, circular, entire, flat	Diplobacillus, G+, subterminal	+	-	-	-	M/K	+	-	-	-	-	-	+	-	+	+	-	+	-	+	+
ST-2	Dark white, circular, entire, convex	Bacillus , G+ , subterminal	+	-	-	-	M/K	+	-	-	-	-	+	+	+	-	+	-	-	-	+	+
ST-3	Dark white, circular filament, flat	Polimorpis , G+ , terminal	+	+	-	-	M/K	+	-	-	-	-	+	+	+	-	+	-	-	-	-	+
ST-4	White, circular entire, flat	Diplobacillus , G+ , central	+	-	-	-	M/K	-	-	-	-	-	-	+	-	+	+	-	-	-	+	+
ST-5	Dark white, circular, undulate, raised	Diplobacillus , G+ , central	+	-	-	-	M/K	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+
ST-6	White, circular, undulate, raised	Bacillus , G+ , subterminal	+	-	-	-	M/K	+	-	-	-	-	-	+	+	-	+	-	+	-	-	+
ST-7	Dark white, circular, entire, convex	Bacillus , G+ , central	+	-	-	-	M/K	+	-	-	-	-	-	+	+	-	+	-	+	-	+	+
ST-8	Dark white, irregular, lobate, raised	Bacillus , G+ , central	+	-	-	-	K/K	+	-	-	-	-	-	+	+	-	+	-	-	-	+	+
ST-9	Dark white, circular, undulate, raised	Diplobacillus , G+ , central	+	-	-	-	M/K	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
ST-10	Dark white, circular, undulate, raised	Diplobacillus , G+ , central	+	-	-	-	M/K	+	-	-	-	-	+	+	+	+	+	-	+	-	-	+
ST-11	White, circular, lobate, raised	Bacillus , G+ , central	+	-	-	-	M/K	+	-	-	-	-	-	+	-	-	+	-	+	-	+	+
ST-12	Dark white, circular, entire, convex	Bacillus , G+ , subterminal	+	-	-	-	M/K	+	-	-	-	-	-	+	+	-	+	-	+	-	+	+
ST-13	White, circular, entire, convex	Bacillus , G+ , central	+	-	-	-	M/K	+	-	+	-	-	+	+	+	-	+	-	+	-	-	+
ST-14	White, circular, filament, undulate	Diplobacillus , G+ , central	+	-	-	-	M/M	+	-	-	-	-	-	+	+	-	+	-	-	-	+	+

found at around the ponds and streams (Fig.1) contained various biotic compounds: 23 plant species were identified from 15 families. This environmental condition was favorable for the development of different types of thermophilic and alkaliphilic *Bacillus*. All isolated bacterial strains produced alkaline proteases with their proteolytic modulations ranging from 0.25 to 6.15 (Table 1 and Fig.3).

Biological and physicochemical interactions play a role in shaping the conditions of microbial compositions in hot springs. In addition to the temperature, pH, geochemical composition and geographic distance determine the colony structure and bacterial populations. The presence of particular strains contributes significantly to habitat composition and is correlated with the geochemical properties of hot springs [12].

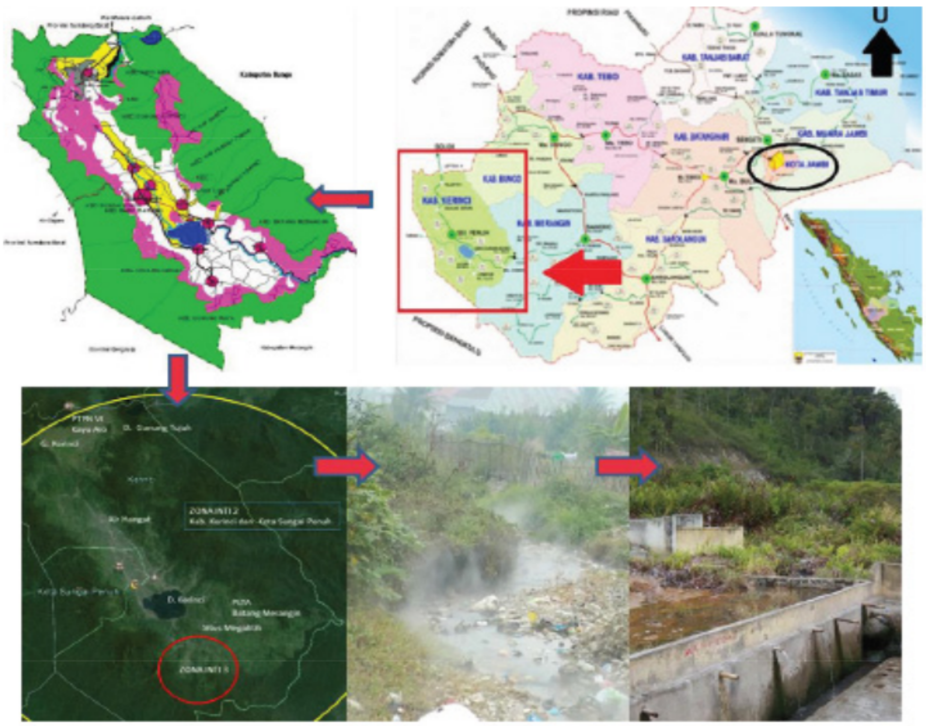

Fig. 1. Sungai Tutung in Kecamatan Air Hangat, Kerinci, Jambi, Indonesia

Fig. 2. Hot spring water sampling

Identified isolates from the hot springs of Sungai Tutung are shown in phylogenetic trees in Fig.4. Six species of thermophilic *Bacillus* with 14 strains were successfully identified. They were: *Bacillus sonorensis* (1 strain), *Bacillus lichenmiformis* (6 strain), *Paenobacillus residui* (1 strain), *Brevibacillus borstelensis* (2 strain), *Bacillus aerius* (3 strain), *and Fictibacillus gelatini* (1 strain). Some of these species have also been discovered by Habibie, Wardani [22] in hot mud Lapindo (temperature 45-70 ^{0}C, pH 7, 14 isolates), by Aanniz, Ouadghiri [23] in hot springs in Morocco (temperature 39-57 ^{0}C, pH 7-7.4), by Panda, Sahu [8] in the Tarabalo hot spring in India (temperature 50-90 ^{0}C, 22 isolates). Sungai Medang hot springs; 39 isolates [24]. One Genus of *Fictibacillus* has also been found in the Chorao Island marine sediments, Goa Provence, India [25].

Only *Fictibacillus gelatini* had not been found in any hot springs, but according to Glaeser, Dott [26] this species has a growth high temperature. The discovery of a new strain of the thermophilic *Bacillus* at this hot spring water of Sungai Tutung was most likely because of the uniqueness of this hot water. As explained before, this hot spring had not only high-temperature water but also had

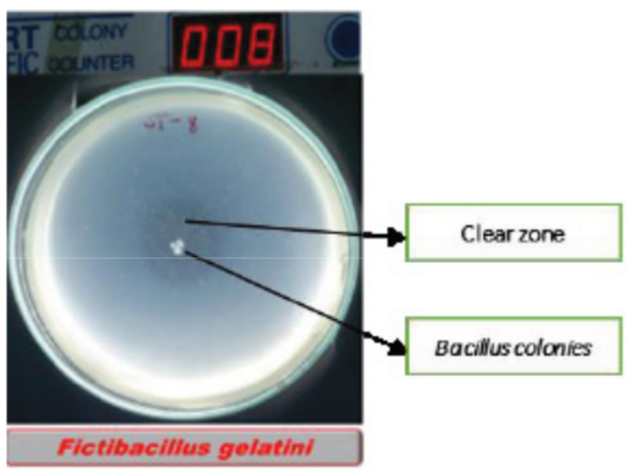

Fig. 3. Screening of the thermophilic obligate *Bacillus* spp

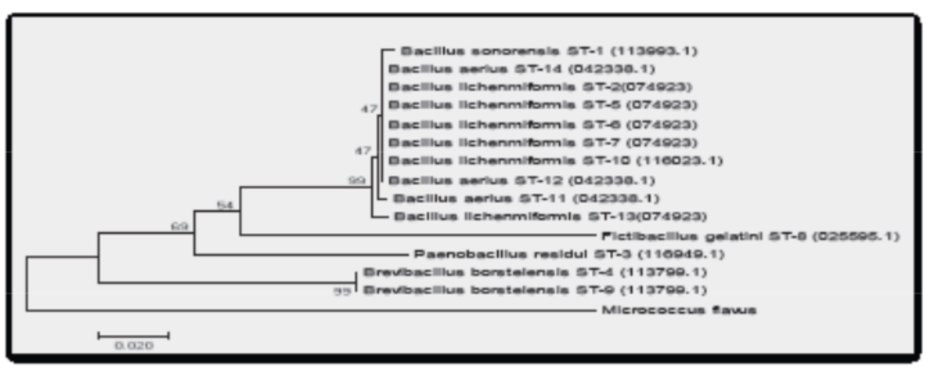

Fig. 4. The phylogenic tree of *Bacillus* thermophilic obligate that produce protease alkali isolate from SAP. Sungai Tutung

alkaline pH, contained various minerals, and the origin of the source sluice is also different.

The characteristics of the thermophilic obligate *Bacillus* spp. strains that produced alkali protease from Sungai Tutung are listed in Table 2. The color of the colonies of *Bacillus* spp. was white and dark white. The shape, edges, and elevation of colonies were varied. Most of the strains had rounded colonies with flat edges and showed raised elevation (Fig.5). According to Pandey, Dhakar [11], the thermophilic *Bacillus* spp colony is characterised by a round shape flat edge and white color. Based on this morphological observation, nine types of strains were identified. Five types of thermophilic and alkaliphilic *Bacillus* were identified from the microscope observation. All of these five types bacteria were gram-positive, which characterized with a purple color. The cell shape varied from polymorphic, diplobacillus, and half-shaped bacillus. The spores of the thermophilic obligate *Bacillus* spp. were mostly located in the center of the cell, partially located almost to the end of the cell, and spores of one strain located at the end of the cell (Fig.5). Based on the biochemical characters (Table 2), the whole strains showed the characteristics of thermophilic obligate *Bacillus* spp. that generated protease. The bacteria could be grouped into 13 strains based on the differences in biochemical characters, especially in sugar series reaction [8,17,27].

The cell shape of thermophilic *Bacillus* spp. consisted of 50% bacillus, 36% diplobacillus

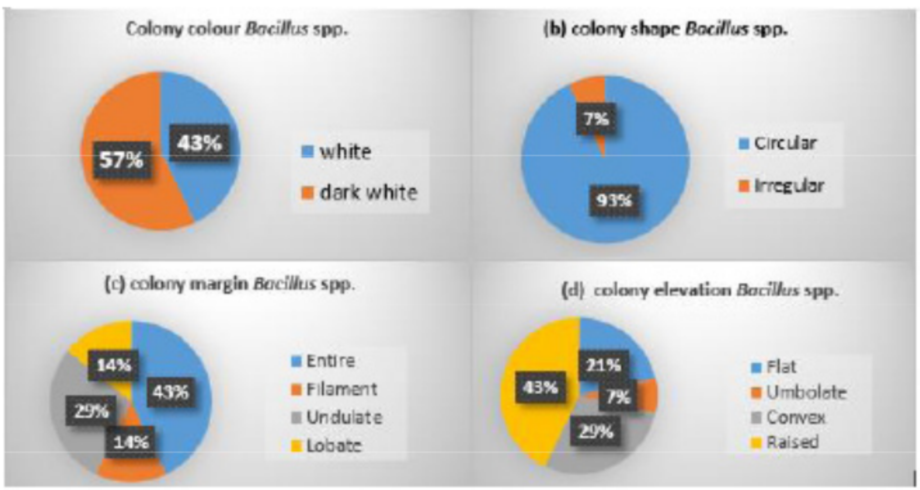

Fig. 5. Colony morphology *Bacillus* spp. obligate thermophillic (a. color; b. shape; c. margin; d. elevation)

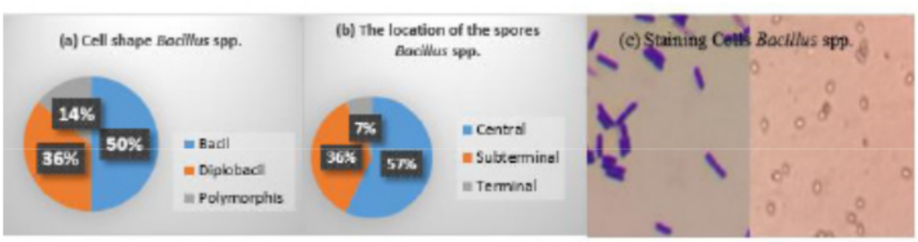

Fig. 6. Microscopic observations *Bacillus* spp. obligate thermophilic (a.cell shape, b.the location of the spores, c.staining cells)

and 14% polymorphism (Fig 6a). About 57% of *Bacillus* spp. had spore located in the middle of the cell, 36% located in the subterminal of the cell, and 7% located at the end of the cell (terminal) (Figure 6 b). Gram staining showed that the cells were purple with the shape of short bacillus, which indicated a Gram-positive bacteria that form endospores.

CONCLUSION

The hot spring of Sungai Tutung in Kerinci, Jambi, Indonesia contained varieties of the thermophilic obligate *Bacillus.* Six species with 14 different strains were identified from the analysis of the DNA sequences. All of these strains had the potential of producing alkali proteases with a proteolytic index ranging from 0.25 to 6.15. Each strain showed different morphological, microscopical, and biochemical properties.

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