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PHYLOGENETIC ANALYSIS OF 16S rRNA GENE FRAGMENT OF ANTIBIOTIC-PRODUCING PLS 76 ISOLATE

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Abstract. Exploitation of extremophiles as novel bioactive compounds sources has been increasing. The aim of this study was to evaluate the activity and the class of antibiotic produced by a thermo-halophilic isolate PLS 76, as well as to identify the genotype of the isolate. The activity was determined by a disc diffusion method, while the antibiotic class was determined qualitatively by chemical reactions using ninhydrin, iodine vapour and potassium iodine. The genotype was determined by sequencing the 16S rRNA gene fragment and the phylogenetic tree from the sequence data. The results showed that PLS 76 was a Gram-negative bacterium and able to produce polypeptide antibiotic, which showed a slight activity on *E. coli* and *S. aureus*. Sequence alignment of the 16S rRNA gene fragment showed that PLS 76 was most related to *Geobacillus kaustophilus*. These results may be used to utilise the isolated for further antibiotic study.

Keywords: PLS 76, polypeptide, antibiotic, 16S rRNA and thermophilic, halophilic

I INTRODUCTION

Nowadays, extremophiles have been extensively studied to produce metabolites of biotechnological important for food, pharmaceutical, household products, alternative bioenergy and bioremediation [1,2]. Extremophiles are defined as microorganisms, inhibiting and thriving in environment with extreme conditions, such as high temperature (thermophile and hyperthermophile), high acidity (acidophile), high alkalinity (alkaliphile), high pressure (piezophile), high radiation (radiophile) and high metal content (metalophile). These microorganisms may be found in hydrothermal and geothermal active region, deep vent, deserts, alkaline or acidic lakes, saline lake and compost [1,2,3]. Indonesia is a maritime country and surrounding by the so-called the ring of fire [4]. The area is a fit habitat for polyextremophiles, especially thermophiles and halophiles [5]. Microorganisms isolated from these extreme environments are known to have unique characters because they are able to adapt to extreme environmental conditions [1] [2]. Therefore, Indonesia has been known as an excellent source for having polyextremophiles biodiversity. New isolated microorganisms are normally identified by two methods, i.e. phenotype and genotype. The phenotype identification includes microscopic observation, physiological and biochemical tests [6]. Genotype identification involves ribotyping analysis based on 16S rRNA gene sequence analysis using the well-established Polymerase Chain Reaction (PCR) methods. Although both methods can identify bacterial up to species level, ribotyping analysis is known to be more accurate, effective and the results are widely acknowledged [6,7]. Isolation of microorganisms from different habitats is able to discover new extremozymes and bioactive compounds with antibacterial and antifungal activities [8,9]. For example, microlatin type A and B of antibiotics have been produced by *Bacillus amyloliquefaciens* isolated from marine isolates in China [10]. Two antibiotics of depsipeptides (unnarmisin A and C) have been also isolated from *Photobacterium sp*. marine bacteria MBIC06485 strains [11]. Several thermo-halophilic bacteria have been isolated from underwater hot spring in Pria Laot Sabang in Aceh Province. These bacterial isolates are known to produce extremozymes and bioactive compounds [12,13]. In this study we reported the results of genotypic identification of the 16S rRNA gene fragment and evaluate the activity

and the class of antibiotic produced by a thermo-halophilic isolate. The results of the study may be used to utilise the isolate for further study of find novel bioactive compounds.

II METHODOLOGY

Microorganisms
Pria Laot Sabang Isolate 76 (hereinafter referred to as PLS 76) was used to produce an antibiotic. PLS 76 was a new isolate previously isolated from under sea fumarole in the area of Pria Laot Sabang of Weh Island, Aceh Province using ½ Termus (½T) medium. Microorganisms used for the antibiotic susceptibility test were *Escheria coli* and *Staphylococcus aureus*. All microorganisms were culture stocks of Biochemistry Laboratory of FMIPA Syiah Kuala University.

Gram-staining
Morphology of PLS 76 was evaluated using the standard Gram-staining method, and the result was observed under a microscope.

Regeneration and cultivation of PLS 76
The glycerol stock of PLS 76 (100 µL) was grown aseptically on modified ½T solid medium (0.4% bacto peptone, 0.2% yeast extract, 1% NaCl, 0.25% glucose, 3% bacto agar) at 70°C for 24 h. A single colony was transferred into ½T liquid medium and incubated at 70°C, 150 rpm for 24 h. To study the microbial growth, the PLS 76 culture in ½T medium was transferred into 2.5% TSB medium and incubated at 70°C, 150 rpm for 120 h. Samplings were done in time intervals and the samples were centrifuged at 10000×*g* for 10 min. The pellet was used for dry cell weight determination by gravimetric method, while the supernatant was used for the disc-diffusion antibiotic susceptibility test.

Production and fractionation of antibiotic
Antibiotic was produced in 2.5% TSB medium, which was incubated at 70°C, 150 rpm for 116 h. The fermentation broth was centrifuged at 10000×*g* for 10 min. The supernatant was successively fractionated using n-hexane, ethyl acetate and methanol with 1:1 ratio. All extracts were tested for their inhibition zone activity.

Bacterial susceptibility test
The antibiotic activity of the three fractions was tested by disc-diffusion antibiotic susceptibility test [14]. Each extract (20 µL) was dripped onto separate disc papers and placed in a 3% Mueller Hinton Agar (MHA) solid medium that had been inoculated with E. coli or S. aureus. The media were then incubated at 37°C for 24 h. The diameter of the inhibition zone was measured. Cephazolin and the solvents were used as the positive and negative controls, respectively.

Antibiotic class identification
Extract showing the best inhibition zone was identified for its antibiotic class by initially separating its components using thin layer chromatography (TLC) on three separate silica plates. A mixture of butanol, acetic acid and distilled water (3:1:1) was used as the eluent. Ninhydrin, potassium iodine and iodine vapour tests were employed to check the possibility of lactam class antibiotic [15]. Staining with ninhydrin and potassium iodine required the TLC plates to be sprayed with 1N NaOH to hydrolyze the lactam ring. The plates were placed in a TLC chamber for 15 min. Lactam and polypeptide antibiotics were checked by spraying the plate with 0.1% ninhydrin solution in ethanol, and heated at 120°C for 10 min. The other plate was sprayed with a solution containing 0.2 g of potassium iodine (0.4 g of iodine in 20 ml ethanol and 5 ml 10% HCl). For identification using iodine vapour, several iodine crystals were allowed to vaporize in a closed TLC chamber. A TLC plate then was inserted into the chamber. After separation, the plate was then sprayed with 1% starch solution.

Genotypic identification of PLS A
Chromosomal DNA was isolated using *Rapidwater™ DNA isolation kit*. The 16S rRNA gene fragment was amplified using a set of primers, i.e. Com_1F (5'-CAGCAGCCGCGGTAATAC-3') and Com_2R (5'-CCGTCAATTCCTTTGAGTTT-3') [16]. The PCR conditions are shown in Table 1.

Table 1 The PCR conditions for the amplification of PLS 76 16S rRNA gene fragment.

PCR step	Temp. (°C)	Time (min)	Cycle
Denaturation	95	5	2
Annealing	50	1	30
Elongation	72	2	30
Final elongation	72	10	1
Cooling	4	15	1

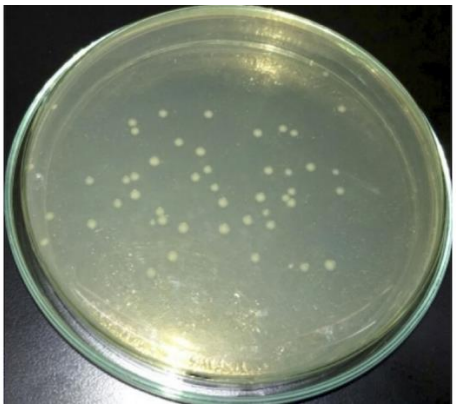
The chromosomal DNA and the amplification result were checked on 1% agarose gel electrophoresis, conducted at 85V, 400 mA for 100 min. The DNA fragments on the gel were visualized on agarose gel using UV Trans-illuminator. The 16S rRNA gene fragment was then sequenced by Dye Terminator method using Com 1F as the sole primer. Phylogenetic

analysis was performed by sequence alignment using data in the gene bank (NCBI, www.ncbi.nlm.nih.gov/blast) using Basic Local Alignment Search Tool (BLAST) program. Some sequences with high homology were selected to construct the phylogenetic tree using Mega 6.06 program.

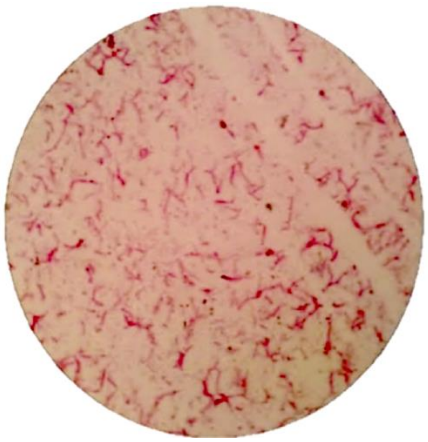
III RESULTS AND DISCUSSION

Morphology identification

Bacterial growth of PLS 76 isolate was characterized by yellowish white colonies. Gram staining gave a pink colour, indicating that PLS 76 was a Gram-negative bacterium with a rod shape (Figure 1). It could belong to basil group, which has size of 0.3-1µm wide and 1.5-4 µm long [13].



(A)



(B)

Figure 1 (A) Colonies of PLS 76 on ½ T solid medium incubated at 70°C for 24 h. (B) Gram-staining of PLS 76 (with 500x magnifications)

Nazina et al. reported that Geobacillus genus was Gram-positive (to Gram variable), rod-shaped, spore forming, for example *Geobacillus thermoleovorans*, *Geobacillus kaustophilus*, *Geobacillus uzenensis*, *Geobacillus stearothermophilus*, *Geobacillus thermocatenulatus*, *Geobacillus thermoglucosidasius*, *Geobacillus thermodenitrificans* [17]. The result suggested that most of Geobacillus genus were Gram-positive and rare to be found Gram-negative [17].

Production of antibiotic

The PLS 76 isolate was propagated on TSB medium before transferred into similar medium to determine the stationary phase, during which secondary metabolites are normally produced as a response to the environmental stress [13]. In the stationary phase, the growth rate is comparable with the death rate so the number of viable cells remains the same. Although a complete microbial growth curve was available, only truncated data is shown (Figure 2). This was done to determine the incubation period for antibiotic production, during nutrient limitation. The stationary phase of PLS 76 in TSB medium was observed between 96-120 h. Each sample in the stationary phase was checked for their antibiotic activity by disc diffusion method. Although having considerably less inhibition activity than the positive control (Cephazolin), the antibiotic was nevertheless produced in the late stationary phase. It was more active against *E. coli* than *S. aureus*. The 104 h and 120 h samples gave a slight inhibition against *E. coli* but not against *S. aureus*. Samples from 108 to 116 h showed inhibition activity against both tested bacteria. It seemed that the optimum time for antibiotic production was at 116 h (Table 2).

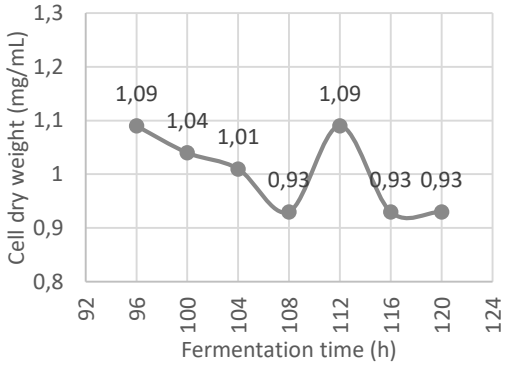


Figure 2 The stationary phase of the PLS 76 growth curve on TSB medium incubated at 70°C at 150 rpm.

The antibiotics production for *Streptomyses griseus* and *Bacillus subtilis* were detected in the stationary phase [18]. Several other studies have reported that the antibiotics production corresponded to stationary growth phase of bacteria from 48-72 h [19,20]. While Kittikun

et al 2015 and Zhu et al 2014 reported that bacteriosin and plantarisin ZJ008 can be produced antibiotics to log growth phase (24 and 16 h respectively) [21,22]. The result suggested that antibiotics can be produced at different growth phase of bacteria. TSB medium has been known as the best medium to produce antibiotics, although some other media may be used. For examples, some bacteria produce antibiotics better in TSB than in Luria Broth (LB) and Nutrien Broth (NB) media [23]. Also, *Bacillus subtilis* produces antifungal well in TSB medium than in NB, Lactosa Broth (LB), Trypticase Soya Broth (TSB) or Brain Heart Infusion Broth (BHI) media [24].

Table 2 Inhibition test of PLS 76 supernatant at various fermentation time in the stationary phase. The tested bacteria were grown on MHA medium at 37°C for 18 h.

Fermentation time (h)	Inhibition diameter (mm)	
	E. coli	*S. aureus*
96	-	-
100	-	-
104	7	-
108	7	6.5
112	7.5	7
116	8	7
120	7.5	-
Cephazolin (control +)	35	33
Distilled H_2O (control -)	-	-

Data analysis was carried out with three replication test

Fractionation of antibiotic

The supernatant of the PLS 76 fermentation broth was fractionated sequentially with solvents of different polarity, i.e. n-hexane (non-polar), ethyl acetate (semi polar) and methanol (polar). All fractions were tested for their antibiotic activity against *E. coli* and *S. aureus*. The results were presented in Table 3. The n-hexane and ethyl acetate extracts showed a slight inhibitory activity only against *E. coli*. The methanol extract showed a comparable inhibition power and it worked for both tested bacteria. Although the inhibition zone by the methanol extract was small, it could still contain antibiotics with considerable activity. Problems could arise due to the impurity in the extract that may inhibit the activity [25].

Antibiotic class test

The antibiotic class of the methanol extract was determined by staining using ninhydrin, iodine vapour and potassium iodine after separation with thin layer chromatography (TLC). The tests are originally designed for testing class antibiotics family [15]. The results in Figure 3 showed that ninhydrin gave a positive results indicating by the red colour, as a reaction result between the antibiotic amine groups and the ninhydrin hydroxyl groups. Meanwhile, iodine vapour and potassium iodine tests for lactam antibiotic identification gave negative results. This result was confirmed by ninhydrin test that produced red colour as an identification of peptide antibiotics (Figure 3A).

Table 3 Inhibition test of extracts of PLS 76 supernatant. The tested bacteria were grown in MHA medium at 37°C for 18 h.

Sample	Inhibition diameter (mm)	
	E. coli	*S. aureus*
n-hexane extract	8	-
Ethyl acetate extract	6,5	-
Methanol extract	8	8
Control-(ethylacetate)	-	-
Control - (n-hexane)	-	-
Control - (methanol)	-	-
Control+(cephazolin)	32	29

Data analysis was carried out with three replication test.

Genotypic identification of PLS 76

As it was isolated from under sea fumaroles with high temperature and salt concentration, PLS 76 could be a fascinating bacterial strain. The identification was done by partial 16S rRNA gene sequencing.

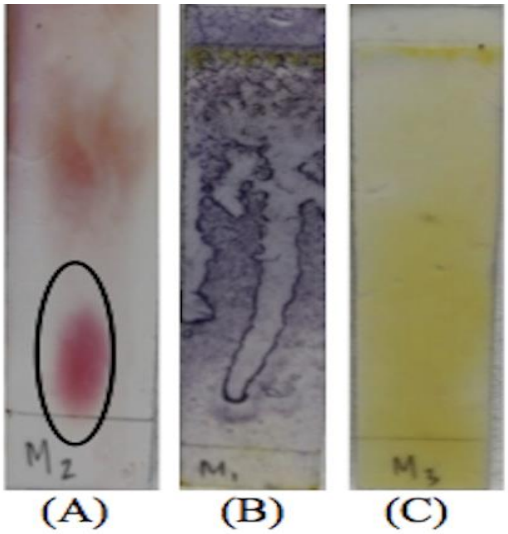


Figure 3 Results of antibiotic class tests using (A) ninhydrin, (B) iodine vapour, and (C) potassium iodine after TLC separation using a solvent system of butanol:acetic acid:distilled water (3:1:1)

Initially, the chromosomal DNA was isolated. The molecular weight of the DNA could not be determined precisely as the marker lacked molecular weight above 10 Kb. However, Figure 4 shows a band well above 10 Kb, indicating that the chromosomal DNA had been

successfully isolated. Having a circular shape, bacterial chromosomes have a difficulty to migrate through agarose gels, so it barely migrates from the gel well. The size of bacterial chromosomal DNA is generally about 22-23 Kb [26]. As the chromosomal DNA has been successfully isolated, it was used as a template for the amplification of the 16S rRNA gene fragment. The amplification was carried out using 1 set of primers, i.e. Com_1F and Com_2R. The Com_1F primer was a specific forward primer used to conserve region (519-536) in the overall genetic sequence of the 16s rRNA gene. The Com_2R primer was a reverse primer used to conserve region (907-926) [16,27]. The amplification using both primers successfully amplified a gene fragment to approximately 500 bp in length (Figure 5).

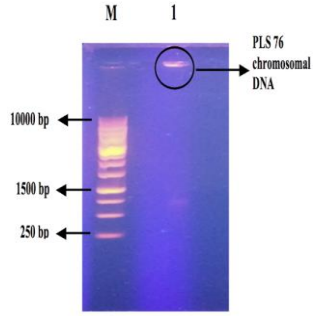


Figure 4 Electrophoregram of PLS 76 DNA chromosomal on agarose gel. Separation was done at 85V, 400 mA for 100 min. (M) Marker, (1) Chromosomal DNA.

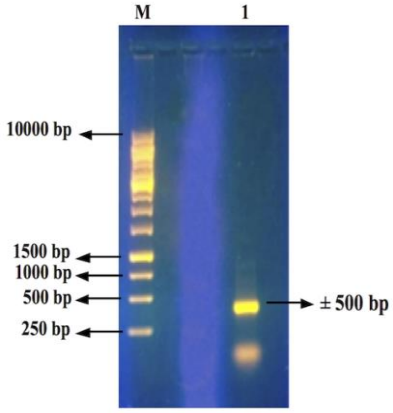


Figure 5 Electropherogram of the amplicon 16S rRNA gene from PLS 76. Separation was done at 85V, 400 mA for 100 min. (M) Marker, (1) 16S rRNA gene fragment amplified using Com_1F and Com_2R primers.

Phylogenetic analysis of the 16S rRNA gene fragment

The amplification result of the 16S rRNA gene was further sequenced and analyzed using BLAST direct sequence to compare the homology of the gene fragment with sequences in the NCBI GenBank. Data alignment was analyzed by Neighbour-Joining (NJ) and bootstrap methods to construct the phylogenetic tree. Bootstrap value used in this study was 1000. The higher the bootstrap value, the more reliable is the phylogenetic tree topology [28].

Table 4 Best 10 homologs with 16S rRNA gene fragment from PLS 76 isolate

Strain	Access no	Homology
Geobacillus thermoleovorans strain EC-5 16S ribosomal RNA gene, partial sequence	MH183214.1	97%
Geobacillus thermoleovorans strain EC-3 16S ribosomal RNA gene, partial sequence	*MH183212.1*	97%
Geobacillus thermoleovorans strain EC-2 16S ribosomal RNA gene, partial sequence	MH183211.1	97%
Geobacillus sp. strain PCH119 16S ribosomal RNA gene, partial sequence	KY628937.1	97%
Geobacillus sp. strain PCH112 16S ribosomal RNA gene, partial sequence	KY628930.1	97%
Geobacillus sp. strain PCH111 16S ribosomal RNA gene, partial sequence	KY628929.1	97%
Geobacillus sp. strain PCH103 16S ribosomal RNA gene, partial sequence	KY628921.1	97%
Geobacillus kaustophilus strain WSUCF-020C 16S ribosomal RNA gene, partial sequence	*MF965141.1*	97%
Geobacillus kaustophilus strain WSUCF-020B 16S ribosomal RNA gene, partial sequence	*MF965140.1*	97%
Geobacillus subterraneus subsp. aromaticivorans strain Manikaran-099 16S ribosomal RNA gene, partial sequence	*MF965134.1*	97%

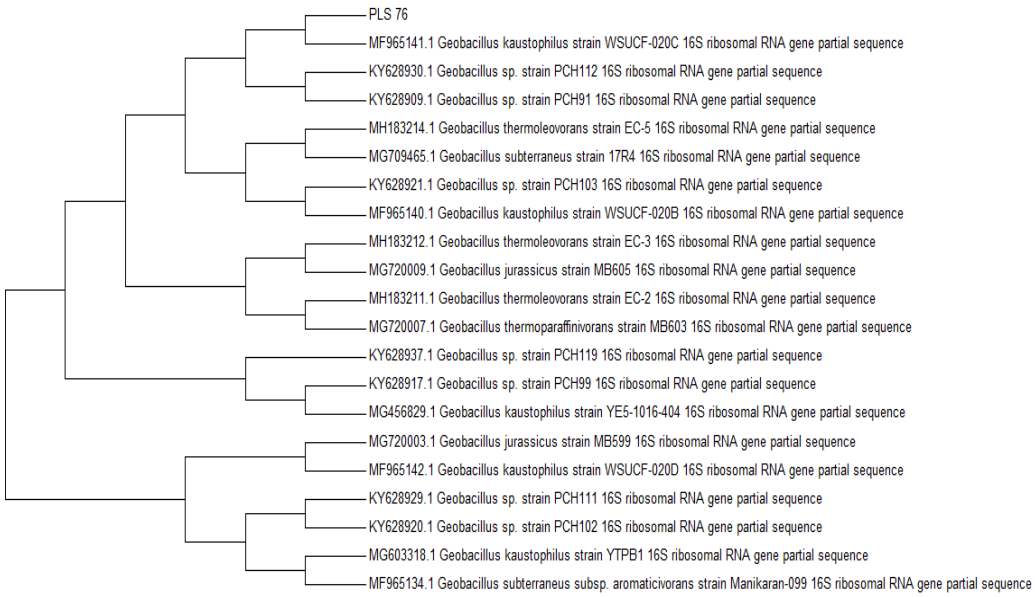


Figure 6 Phylogenetic tree of 16S rRNA gene fragment of PLS 76 in relation to 20 sequences using the neighbour-joining method in MEGA 6.06 with 1000 bootstrap replicates

The 16S rRNA gene fragment from PLS 76 has 97% homology to the gene fragments from four bacteria i.e. (1) *Geobacillus thermoleovorans*, (2) *Bacillus* sp., (3) *Geobacillus kaustophilus* and (4) *Geobacillus subterraneus* (Table 4). Microorganisms with 99% similarity could be of the same species, while 97-99% similarity suggests they could be in the same genera. Similarity below 97% suggests the microorganisms could be new species [29]. Based on phylogenetic tree analysis, the gene fragment of 16S rRNA from PLS 76 had a homology that was most closely related to *Geobacillus kaustophilus* as they were clustered in the same branch (Figure 6). Further alignment using an intact 16S rRNA gene is still needed to confirm this result.

CONCLUSION

PLS 76 isolate produced a polypeptide group antibiotic that show a slight inhibition against *E. coli* and *S. aureus.* The homology of an approximately 500 bp gene fragment of DNA 16S rRNA from PLS 76 isolate showed that the isolate was related to *Geobacillus kaustophilus*, with 97% sequence similarity. Further study is needed to test the antibiotic activity in its purified form. Full sequence alignment of the 16S rRNA intact gene is required to determine the species of PLS 76 isolate.

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