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Identification of Antibiotic in Ethyl Acetate Fraction Produced by A Local Isolate PLS 80 Isolated from Shallow Sea Fumaroles

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Abstract. The need for the discovery of new compounds with antimicrobial activity is increasing as triggered by the resistance of pathogen microorganisms to the current drugs. Simultaneously, interests are growing in exploiting extremophiles to find new active organic compounds. A thermo-halophilic bacterium (dubbed PLS 80), previously isolated from underwater fumaroles, could produce antibiotic. Hence, the objectives of this study were to study the inhibition activity and identify the structure of the antibiotic partially. PLS 80 isolate was grown on TSB medium, and the supernatant was sequentially partitioned with n-hexane, ethyl acetate, and methanol. The extracts were tested for antimicrobial activity by the disc-diffusion antibiotic susceptibility test. The extract with the highest activity was identified for their antibiotic class by chemical reactions using ninhydrin, iodine vapor, and potassium iodine. It was then purified using column chromatography using silica G-60, and the pure substance was subjected to GC MS analysis. Ethyl acetate fraction showed the highest inhibition zone, even higher compared to the Gentamicin control. The qualitative identification by the chemical reactions showed that the antibiotic could be of the β-lactam group. The mass spectrum data indicated that the ion fragments could be derived from benzylpenicillin. The antibiotic has a potential to be studied further, particularly to elucidate the structure entirely. Eventually, structural modifications can be conducted to produce potent antibiotics to overcome antibiotic-resistant microorganisms.

Keywords: Antibiotic, pathogen, thermo-halophilic bacterium, inhibition activity, ethyl acetate

1. Introduction

Antibiotics are secondary metabolites produced by microorganisms, plants, and some animals to inhibit growth or kill pathogenic microorganisms [1]. New antibiotics discovery has become important, stimulated by the increasing resistance to the antibiotics currently available. The resistance occurs due to the adaptation of microorganisms caused by prolonged exposure and genes transfer between microorganisms from different taxa [2].

Some types of pathogen bacteria have been resistant to the existing antibiotics, such as methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Staphylococcus aureus (VRSA) and vancomycin-resistant Enterococci (VRE). Many studies are being conducted to find new natural antibiotics to overcome the problem, particularly those originating from microorganism [3-5]. The

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main antibiotics producers are Actinomycetes and fungi, although bacteria are also capable of producing them. About 75% of the antibiotic-producing Actinomycetes are Streptomyces sp. [6].

Recent studies have shifted the attention to the use of extremophilic microorganisms, which are known to produce primary and secondary metabolites with a better stability than that produced by the mesophiles [7]. The secondary metabolites, produced in response to the environmental stress, include antibacterial and antifungal compounds [8,9]. Amongst various extremophiles, thermophiles and halophiles have attracted more interests as the source of new natural antibiotics [10,11].

Our group has previously isolated several extremophilic microorganisms from underwater fumaroles. One of the isolates, PLS 80, has shown simultaneous thermophile and halophile characters, as it was isolated from an environment with high temperature and salt concentration. PLS 80 was able to produce antibiotics that inhibit the growth of human pathogens. In this study, the antibiotic from PLS 80 supernatant was fractionated with organic solvents and tested for its inhibition capability. The molecular structure was assessed, after purification, using Gas Chromatography–Mass Spectrometry method. Estimating the structure makes it possible to further structural modification in increasing the activity of the antibiotic.

2. Methods

2.1. Microorganism

Microorganism used to produce antibiotic was Pria Laot Sabang Isolate 80 (PLS 80), which was a culture stock of Biochemistry Laboratory of FMIPA Syiah Kuala University. PLS 80 was previously isolated from underwater fumarole in the area of Pria Laot Sabang of Weh Island, Aceh Province.

2.2. Cultivation of PLS 80 isolate

PLS 80 cells from glycerol stock were inoculated on a modified 1/2 Thermus solid medium (0.4% bacto peptone, 0.2% yeast extract, 1% NaCl, 0.25% glucose, 3% bacto agar). The medium was incubated at 70 °C for 24 hours. A single colony on the medium was transferred to 2.5% Tripton Soy Broth (TSB) liquid medium and incubated further at 70 °C, 150 rpm for 24 hours.

2.3. Antibiotic production

Bacterial culture from the cultivation step was further transferred into a fresh 2.5% TSB liquid medium and incubated at 70 °C, 150 rpm for 112 hours. The fermentation broth was centrifuged at 10000×*g* for 10 minutes, and the supernatant was then fractionated.

2.4. Fractionation of the supernatant

The supernatant after centrifugation was filtered using a 0.02 μm filter and partitioned with n-hexane (1:1). The water layer was then partitioned with ethyl acetate (1:1). Afterward, the water layer was further partitioned with methanol (1:1), and the methanol layer was separated.

2.5. Inhibition zone test

The antibiotic activity in the three fractions was tested by disc-diffusion antibiotic susceptibility test [12]. 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 *Escherichia coli* or *Staphylococcus aureus*. The media were then incubated at 37 °C for 24 hours. The diameter of the clear zone after incubation was measured. Gentamicin and sterile distilled water were used as the positive and negative controls, respectively.

2.6. Antibiotic class determination

The antibiotic class was checked by firstly separating the components in the extract with the largest inhibition zone (ethyl acetate) using thin layer chromatography (TLC) on three separate silica plates.

The solvent system used was a mixture of butanol, acetic acid and distilled water (3:1:1). Three staining procedures were used, i.e., ninhydrin, potassium iodine, and iodine vapor [13].

For staining with ninhydrin and potassium iodine, the TLC plate was initially sprayed with 1N NaOH to hydrolyze the lactam ring. The plates were then placed in a TLC chamber for 15 minutes. After separation, one of the plates was sprayed with 0.1% ninhydrin solution in ethanol and heated at 120 °C for 10 minutes. The other plate was sprayed with a solution containing 0.2 g of potassium iodine, 0.4 g of iodine dissolved in 20 ml of ethanol and 5 mL of 10% HCl. For identification using iodine vapor, several iodine crystals were placed at the bottom of the TLC chamber and closed tightly. After the vapor was evenly distributed, the TLC plate was placed into the chamber and left for 30 minutes. The plate was then sprayed with a 1% starch solution.

2.7. Purification and structure prediction of the antibiotic

The compounds in the extract with largest inhibition zone (ethyl acetate) were separated using silica gel G-60 column chromatography using a mixture of dichloromethane and methanol (70:30 v/v) as the eluent. The resulting fractions were identified using silica TLC with a mixture of butanol, acetic acid and distilled water (3:1:1) as the eluent. The results were visualized under UV light. Fractions having close Rf values were combined and further separated using silica TLC using the same eluent system. Visualization was carried out under UV light and by staining with iodine vapor. The combined fraction showing a single compound was further purified by column chromatography as described previously. The pure compound was analyzed using a Gas Chromatography-Mass Spectrophotometry technique.

3. Results and Discussion

3.1. Inhibition zone test

Fractionation of the supernatant of PLS 80 fermentation broth was successfully carried out using non-polar (n-hexane), semi-polar (ethyl acetate), and polar (methanol) solvents. All fractions were tested for their inhibitory capability on *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). The ethyl acetate fraction (EA) seemed to produce greater inhibitory zone than that of gentamicin control (Table 1, Figure 1). EA fraction showed a larger inhibitory zone against *E. coli* (23 mm) than *S. aureus* (14 mm). Meanwhile, the n-hexane and methanol factions did not show any inhibitory effect.

Table 1. Inhibition zone of PLS 80 supernatant fractionated using various organic solvents with different polarity.

Sample	Average inhibition diameter (mm)	
	E. coli	***S. aureus***
n-hexane fraction	-	-
Ethyl acetate fraction	23	14
Methanol fraction	-	-
Positive control (gentamicin)	12.5	13.5
Negative control (distilled water)	-	-
Negative control (ethyl acetate)	-	-

The susceptibility or resistivity of *E. coli* and *S. aureus* to the antibiotics produced in this study was not determined. This can be studied by performing the minimum inhibitory concentration tests, by applying broth dilution, agar dilution or gradient diffusion techniques. Bacteria are classified as sensitive, intermediate or resistant based on breakpoint Minimum Inhibitory Concentration (MIC) values, which must be done on standard growth media, at optimum growth conditions and using the right inoculum size [14]. Although solvents may contribute to the MIC values, it can be easily monitored by performing the MIC test using the solvents as the control.

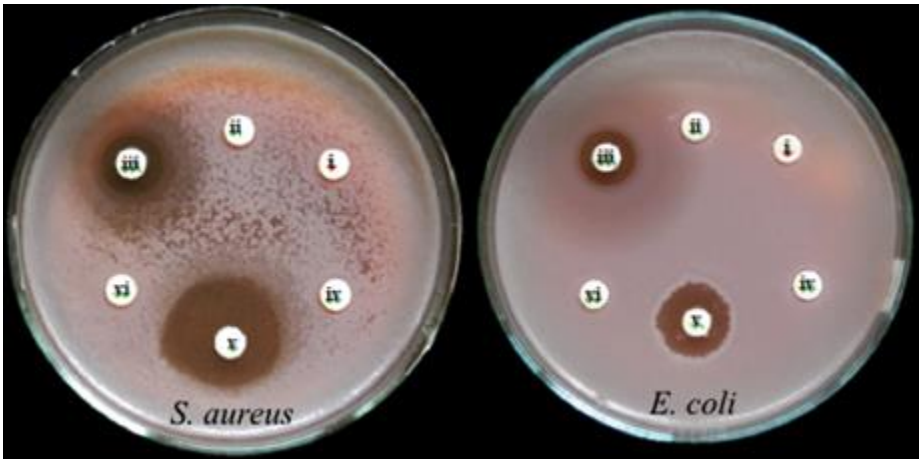

Figure 1. Inhibitory zone of organic solvent fractions from PLS 80 supernatant after 112 h incubation. (i) & (ii) Sterile distilled water; (iii) Gentamicin; (iv) n-hexane fraction; (v) Ethyl acetate fraction; (vi) Methanol fraction; (vii) Ethyl acetate solvent. The tested bacteria were grown on MHA media at 70 °C, 150 rpm for 24 h.

3.2. Antibiotic class determination

The EA fraction was tested further for its general antibiotic class as it showed the largest inhibitory zone. After separation using TLC, identification was carried out by chemical reaction using ninhydrin, potassium iodine and iodine vapor. The ninhydrin test produced a pale red color (Figure 2A), indicating that the EA fraction contained β-lactam or polypeptide antibiotics. The red color was formed due to the reaction between the antibiotic amine groups and the ninhydrin hydroxyl groups. A further test using iodine vapor produced a pale-yellow color, indicating that the EA fraction contained a β-lactam antibiotic (Figure 2B). This result was confirmed by potassium iodine test that produced a pale brown color (Figure 2C). These tests can be used as a preliminary identification of β-lactam antibiotics [13].

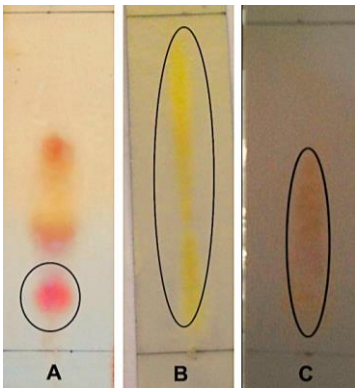

Figure 2. Results of (a) ninhydrin, (b) iodine vapor, and (c) potassium iodine tests after TLC separation using a solvent system of butanol: acetic acid: distilled water (3:1:1).

3.3. Purification of antibiotic

As it showed the best inhibition zone than the other fractions, the ethyl acetate fraction was concentrated and further purified using column chromatography with silica gel G-60. The best solvent system for separation was a mixture of dichloromethane: methanol 70:30 (v/v). The chromatography yielded 50 fractions, which were combined with nine fractions based on their Rf values (Figure 3).

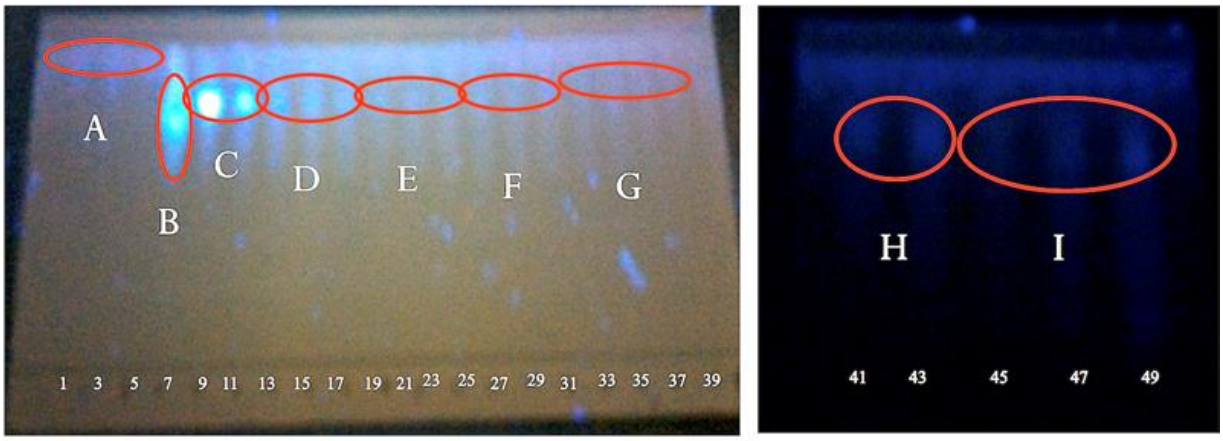

Figure 3. Chromatogram of EA fraction after column chromatography silica gel G60 using DCM: MeOH 70:30 (v/v) as the eluent. Combined fractions were A (1-5); B (6-8); C (9-11); D (12-18); E (19-25); F (26-30); G (31-38); H (39-43); I (44-50)

To determine the fraction containing β-lactam, the combined fractions were further separated using TLC and identified using UV and potassium iodine reagent. The results showed that fraction H positively contained β-lactams (Figure 4). The stain was formed due to the reaction between iodine and penicilloic acid, which was an intermediate of alkaline hydrolysis of the lactam ring. The H fraction was used for structural identification using GC-MS.

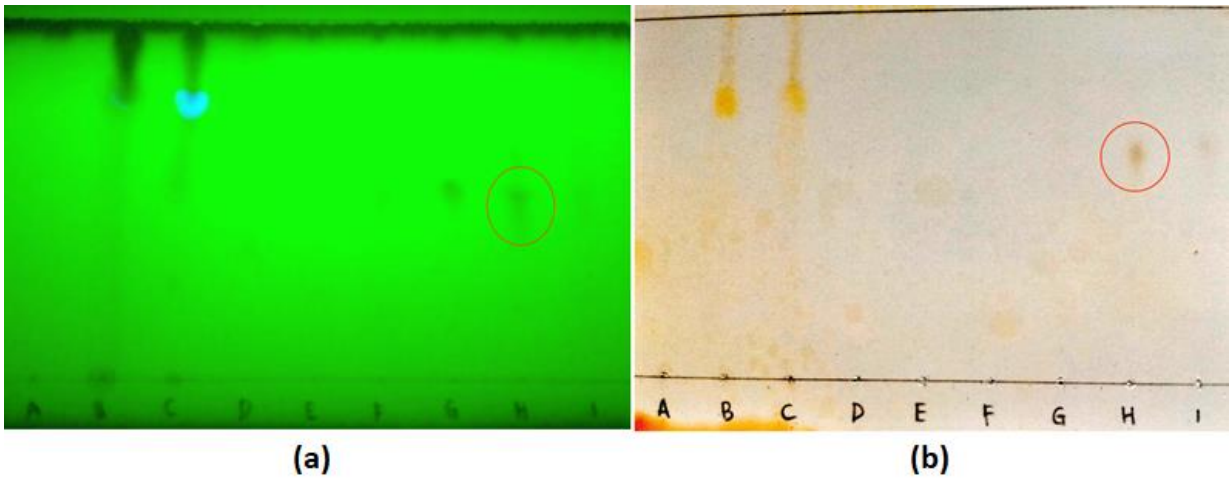

Figure 4. Thin layer chromatography results of the combined fractions using butanol: acetic acid: distilled water (4: 1.5: 1.5 v/v/v) as the eluent (a) detected by UV; (b) reacted with iodine vapor.

3.4. Identification of the antibiotic structure

The mass spectrum data were used to predict the structure of antibiotic from PLS 80 (Figure 5). Based on the data, the antibiotic might be benzylpenicillin with m/z = 334. The fragment of m/z = 292 $[M - H]^+$ was estimated as C_3H_6 in the form of ion-molecule. The fragment of m/z = 204 $[M + H]^+$ indicates the presence of a compound with a molecular formula of $C_2H_2O_2S$. Meanwhile, the fragment of m/z = 121 was likely to be $C_3H_5N_2O$. The base peak at m/z 91 was formed by the loss of the –COH group in the structure (Figure 6).

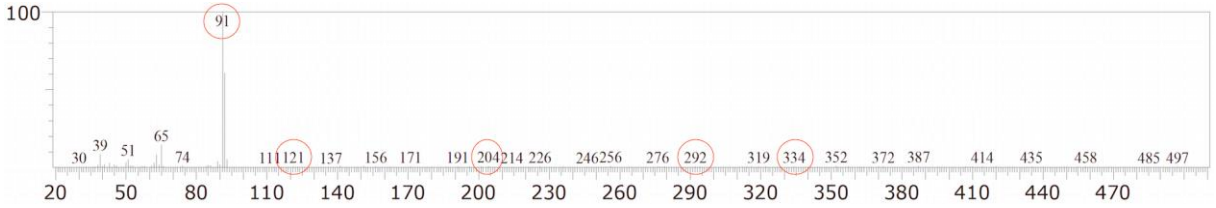

Figure 5. The m/z spectrum of antibiotic in Fraction H after purification

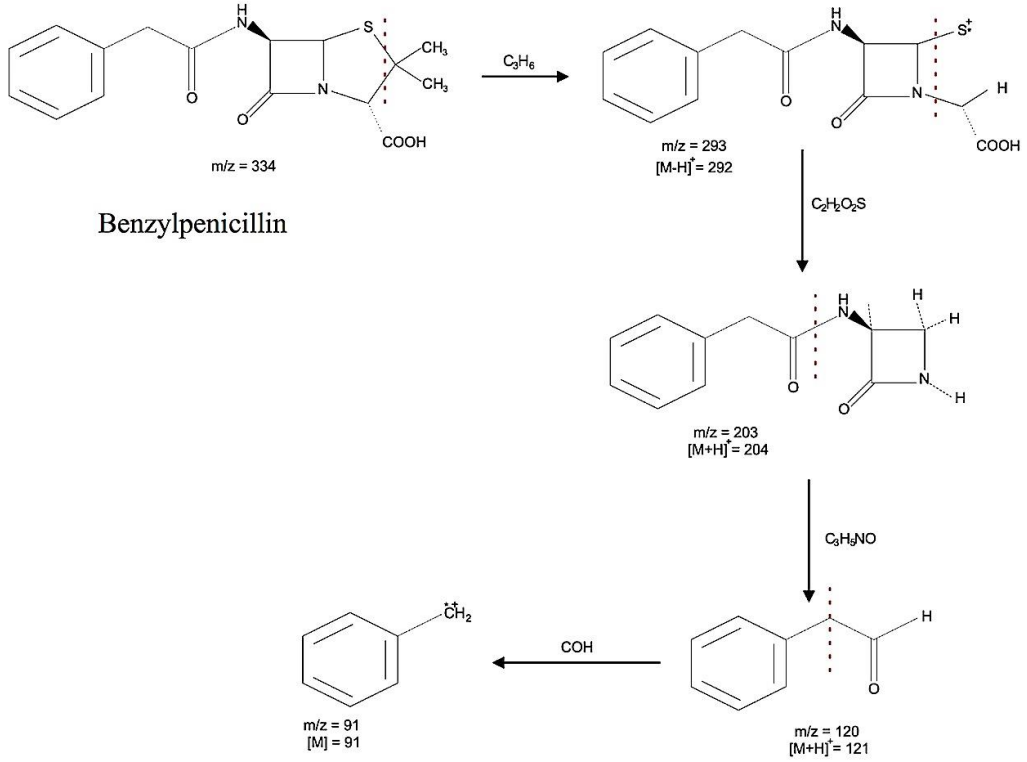

Figure 6. The proposed fragmentation pattern of the antibiotic from PLS 80

Benzylpenicillin is one of the naturally occurring lactam antibiotics that is characterized by four-membered β-lactam and thiazolidine rings and a variable side chain in the molecule. The side chains, which are either hydrophobic or hydrophilic, distinguish the type of penicillin. Benzylpenicillin is hydrophobic penicillin that prevents bacterial cell wall synthesis and causes cell lysis. It is known effective against most Gram-positive bacteria but less active against Gram-negative [15].

One of the factors affecting the type of penicillin produced by microorganisms is the kind of the side-chain precursors available in the fermentation medium. It has been recognized that corn steep liquor, phenylalanine, β-phenylamine favor the synthesis of benzylpenicillin [16]. In this study, tryptone in the TSB medium could provide phenylalanine for the synthesis of the side chain as tryptone, a trypsin-digested product of casein, contains peptides of various sizes.

PLS 80 was rod-shaped, spores forming and Gram-negative bacterium, and belonged to the genus of *Geobacillus* by partial 16s rRNA sequence analysis (data not shown). This is somewhat interesting as although benzylpenicillin is a broad-spectrum antibiotic, its activity is uncommon against Gram-negative bacteria. This implies that the structure of antibiotic from PLS 80 may be unique. The uniqueness could drive further structural modification for two reasons, firstly to produce compounds with more potent antimicrobial activity and secondly to broaden the activity beyond merely antimicrobial. A structural modification is done either by synthesis from precursors [17] or altering the

biosynthetic pathway and precursor feeding [18]. The new discovery of antibiotics or their derivatives become urgent as after its first discovery in the first half of the 20th century, the new class of antibiotics has been rarely introduced to the market. The analogs development is not able to compete with the emergence of microbial resistance, particularly the Gram-negative pathogens [19].

4. Conclusion

PLS 80 was able to produce antibiotic of the β-lactam group that showed inhibition activity both against Gram-positive and Gram-negative bacteria. Although it could be benzylpenicillin, the structure is not yet clear, and GC-MS alone cannot be used to elucidate the structure. The complete structure elucidation is essential, mainly using NMR, to assess the possibility for structural modification to produce more active compounds.

Acknowledgments

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