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Polypeptide Antibiotic Produced by a Thermo-Halophilic Bacterium from Pria Laot Sabang 76 Isolate

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Abstract

Extremophiles are known to produce antibiotics. This study aimed to isolate and purify polypeptide antibiotics from Pria Laot Sabang (PLS) 76, a bacterium isolated from an undersea fumarole. The antibiotic production was done by cultivating PLS 76 in a 2.5% TSB liquid medium and incubated at 70°C, 150 rpm for 112 hours. Ninhydrin assay on the crude supernatant after centrifugation indicated that the PLS 76 produced a polypeptide antibiotic. The supernatant was partitioned using methanol. The antibiotic activity was tested using the Kirby-Bauer method, and the inhibition zone was about 12 mm against *Escherichia coli* and *Staphylococcus aureus*. The methanol fraction was purified further by Preparative Thin Layer Chromatography and produced several fractions. Fractions with the same R_f were pooled and subjected to the ninhydrin assay. Fraction A, which gave a positive result after the ninhydrin assay, showed an inhibition zone of only 7 mm against *E. coli* and *S. aureus*. The Quadrupole Time-of-Flight (QTOF) Mass Spectrometry data indicated that the antibiotic's structure was similar to Polymyxin B2, an antibiotic of a polypeptide group.

Keywords: *Thermo-halophilic bacteria, polypeptide antibiotic, Pria Laot Sabang, Polymyxin*

Introduction

The discovery of antibiotics is one of the most significant medical improvements in infectious disease treatment in the last century. The annual production of antibiotics for health, food, and agriculture exceeds 100 thousand tons annually[1]. Various antibiotics have been produced commercially from different sources[2]. Microorganisms, including bacteria, algae, fungi, and actinomycetes, are common producers of antibiotics. Actinomycetes produce about 70% of commercial antibiotics, followed by fungi (20%), Bacillus (7%) and Pseudomonas (1-2%)[3]. Microorganisms have been used on an industrial scale as the primary source for antibiotics production because the production is easily controlled and has a minimal environmental impact.

Multi-antibiotic-resistant bacteria have become serious health problems. The prevalence of multiple drug-resistant microorganisms has been increasing, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), and vancomycin-resistant *Enterococci* (VRE). Furthermore, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* become

resistant to almost all antibiotics. Therefore, there is an urgent need to find novel antibiotics effective against multidrug-resistant bacteria[2].

Polypeptide antibiotics are short peptides with low molecular weights that inhibit or kill microorganisms[4],[5]. This type of antibiotic is known to be thermally stable, have good water solubility, have a broad spectrum, and kill fungi and protozoa[4], thus adequate to overcome antibiotic resistance[6],[7].

Indonesia has many areas of extreme conditions, such as hydrothermal vents or active volcanoes, which are habitats for extremophilic microorganisms[8]. Extremophilic microorganisms potentially produce metabolites, including antibiotics, that may possess unique characteristics due to their capability to adapt to extreme conditions. However, the potential of extremophilic microorganisms to produce such metabolites still needs to be utilized[9]. One of Indonesia's marine areas that has underwater hot springs from Shallow Sea Fumaroles in the area of Pria Laot Sabang of Weh Island, Aceh Province[10]. This hot spring is an isolation area of a thermo-halophilic bacterium of PLS 76[10].

Some studies report antibiotics productions from microorganisms living in extreme conditions. G*eobacillus toebii* isolated from a 60°C environment produces antibiotics of bacteriocin[11]. Antibiotics produced by *Bacillus atrophaeus* are stable at temperatures of 4° to 100°C and inhibit the growth of *Bacillus subtilis*[12]. *Serratia maculans*, which grows optimum at 80°C, can produce andrimid, an antibiotic used to preserve frozen food and pharmaceutical products[13]. *Bacillus pumilus* has the potential to produce bacitracin at 2.5% salt condition and can be used in the treatment of skin infections[14]. *Bacillus subtillis* have been reported to produce Bacitracin A and Polymyxin B which can inhibit pathagonic bacteria *Staphylococcus aureus*[15]. Thermo-halophilic bacterium of PLS 80 are known to produce β-Lactam antibiotics in ethyl acetate fraction[10].

In this research, we studied the ability of a thermo-halophilic bacterium of PLS 76 to produce polypeptide antibiotics. The antibiotics were purified and tested for their ability to inhibit the growth of microorganisms. The results may be explored further to find new potent antibiotics.

Experimental

Equipment

The equipment used in this research were autoclave, shaker water bath (BS-II), , freezer, incubator (memmert), incubator (ultra clean "100"), analytical balance, oven (memmert), pH meter, Spectrophotometer Shimadzu GC-MS QP 2000 A, QTOF (LC-MS/MS) Q-Exactive-Thermo Ultimete RSLC nano 3000.

Material

The microorganisms used as antibiotic producers is a thermo-halophilic bacterium of PLS 76. The microorganisms used for the inhibition zone assay were *E. coli* and *S. aureus*. All chemicals used have proabalytical qualities (p.a).

Methods

Cultivation of Microorganism

Pria Laot Sabang (PLS) 76 isolate, a culture collection of the Biochemistry Laboratory FMIPA Universitas Syiah Kuala, was used to produce antibiotics. It was previously isolated from an undersea fumarole in Pria Laot Sabang of Weh Island, Aceh Province.

Cells of PLS 76 in glycerol stock were inoculated on a modified ½ 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 into a 2.5% Tripton Soy Broth (TSB) liquid medium and incubated at 70°C, 150 rpm for 24 hours [16]. The culture was later transferred to a fresh 2.5% TSB liquid medium and incubated at 70°C, 150 rpm for 112 hours.

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Partition of Supernatant

The fermentation broth from the procedure above was centrifuged at 10000×*g* for 10 minutes. The supernatant was filtered using a 0.2 μm filter and partitioned with n-hexane (1:1 v/v). The water layer was then partitioned with ethyl acetate (1:1 v/v). After ethyl acetate partition, the water layer was further partitioned with methanol (1:1 v/v). The methanol layer was finally collected and concentrated using a rotary evaporator for subsequent use.

Qualitative Determination of Polypeptide Antibiotic

The antibiotic in the methanol fraction was separated by thin-layer chromatography (TLC) on three separate silica plates. The separation used an eluent mixture of n-butanol: acetic acid: distilled water (3:1:1 v/v/v). After separation, the TLC plate was sprayed with 3% ninhydrin for the polypeptide antibiotic identification [17].

Inhibition Test

A disc-diffusion antibiotic susceptibility test was used to check the antibiotic activity of the methanol fraction. About 20 μL of the concentrated fraction was dropped onto a disc paper and placed in a 3% Mueller Hinton Agar (MHA) solid medium in a Petri dish, inoculated with *E. coli* or *S. aureus* [18]. The medium was incubated at 37°C for 24 hours. The diameter of the clear zone after incubation was measured. Sephazolin and sterile distilled water were used as the positive and negative controls, respectively.

Purification of Antibiotic

The methanol fraction was purified further by the preparative thin layer chromatography (TLC-P) using a mixture of *n*-butanol: acetic acid: distilled water (3:1:1 v/v/v). After separation, the TLC plate was subjected to a UV light. Significant spots were marked, scraped off the plate, pooled in test tubes, and dissolved in the same eluent mixture. The fractions were then filtered using a 0.2 μm filter. The pooled fractions were separated individually using the TLC using the above eluent mixture to check the purity level. The fraction with the highest purity was tested for inhibition activity.

SDS-PAGE Analysis

Sample of the combined fractions were analyzed on Sodium Dedocy Sulfate Polyacrylamide gel Electrophoresis based on Laemli to determine Molecular Weight from Polypeptide Antibiotic.

Antibiotic Structure Determination

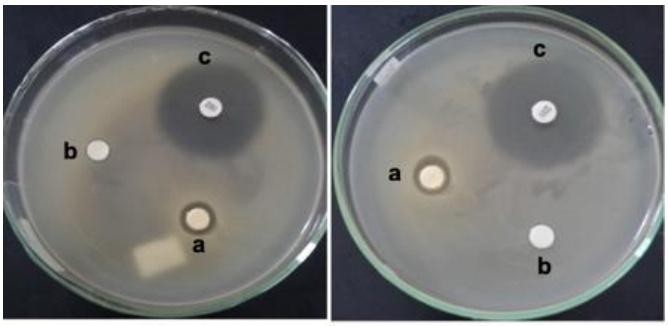
The antibiotic structure in the fraction with the highest purity was characterized using QTOF (LC-MS/MS). The sample was first fragmented into shorter peptides to facilitate a simple analysis. Separation by LC was carried out with Easy Spray Colum PepMap RSLC C18 for 57 minutes. MS analysis was performed in a positive mode. The ionization used nanoelectrospray with a resolution of 70,000 (full MS) and 17,500 (MS/MS) with a scan range of 200-2000 m/z and fragmentation energy of 30 eV.

Results and Disscusion

Antibiotic Identification from PLS 76

The antibiotic from PLS 76 was produced on a TSB liquid medium at 37°C for 116 hours [16]. Prolonged incubation time was used because secondary metabolite, such as antibiotics, is typically produced during the late stationary phase [18]. The TSB medium seemed to provide PLS 76 with nutrition for gradual metabolism [19]. Several studies show that some bacteria produce antibiotics in TSB with an extended stationary phase [20].[19].

The supernatant of the production broth was partitioned consecutively using non-polar (*n*-hexane), semi-polar (ethyl acetate), and polar (methanol). The consecutive strategy was done to isolate small polar peptide molecules which is water-soluble. The A disc-diffusion antibiotic susceptibility test of the methanol fraction showed an inhibitory zone against *E. coli* (12 mm) and *S. aureus* (12 mm) (Fig. 1).



(a) (b)

Sample	Inhibition zone (mm)	
	E. coli (left panel)	*S. aureus* (right panel)
Methanol extract, concentrated (a)	12	12
Negative control (metanol) (b)	6	6
Positive control (sephazolin) (c)	29	29

Figure 1. The inhibitory activity of the methanol fraction of PLS 76 supernatant on a TSB medium. The tested bacteria were grown on an MHA medium at 37°C for 18 hours. The left panel (a) MHA inoculated with *E. coli,* and the right panel (b) MHA inoculated with *S. aureus.*

Figure 2. Polypeptide antibiotics determination in the methanol fraction after staining with 3% ninhydrin. The eluent system for TLC separation was a mixture of n-butanol: acetic acid: distilled water (3:1:1 v/v/v).

As the target compound was polypeptide antibiotics, the presence in the methanol fraction was checked qualitatively using ninhydrin reagents. Initially, thin-layer chromatography (TLC) separation was performed to separate the components in the mixture. The ninhydrin test gave a positive result, indicating that PLS 76 produced antibiotics of the polypeptide group (Fig. 2).

The methanol fraction was purified further using preparative TLC. The separation results were viewed under UV light. Spots were marked, scraped off the plate, pooled in test tubes, and dissolved in n-butanol: acetic acid: distilled water (3:1:1 v/v/v). Seven fractions were collected after this step based on the similarity of their R_f (f1 – f7). To check the purity level, all fractions were subjected to TLC separation again using an eluent system of n-butanol: acetic acid: distilled water (3:1:1 v/v/v). After separation, the TLC plate was stained with ninhydrin. The pattern in fractions 1-4 indicated that the fraction was not pure (Fig.

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3a). Meanwhile, fractions 5, 6 and 7 showed a single spot with similar R_f. Therefore, the three fractions were combined and separated again using TLC. After testing with ninhydrin, the separation results showed a single spot (Fig. 3b).

The inhibitory activity of the combined fractions 5, 6, and 7 was tested against *E. coli* and *S. aureus*. The combined fraction gave an inhibitory zone of only about 7 mm against both bacteria (Fig. 4). The activity was lower than that of crude methanol extract (see also Fig. 1) and equivalent to that of the negative control. The low activity of the combined fraction may be due to the low concentration of antibiotics in the sample. In addition, it may be due to the loss of synergistic properties, as several other compounds that may increase activity were removed [21].

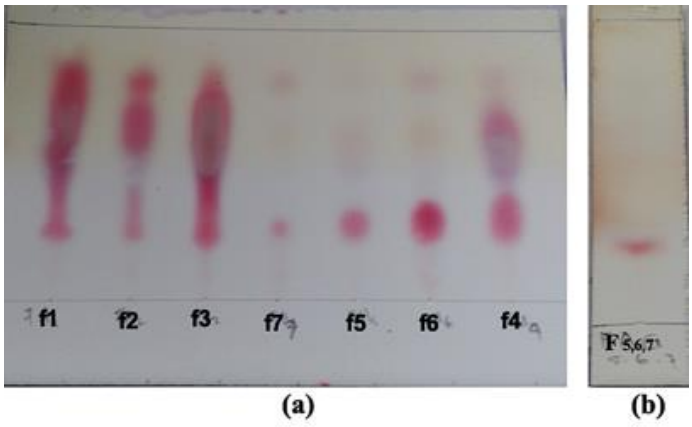
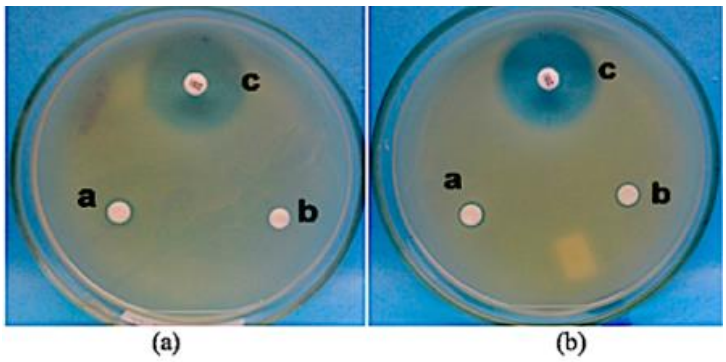


Figure 3. Chromatogram of methanol extracts sub-fractions separated using an eluent of n-butanol: acetic acid: distilled water (3:1:1 v/v/v) (a) Fractions from preparative TLC separation, (b) A combined fraction of 5, 6 and 7. Spots coloured were developed using ninhydrin.



Sample	Inhibition zone (mm)	
	E. coli (left panel)	***S. aureus*** **(**right panel)
Combined fractions 5, 6, 7 (a)	7	7
Negative control (distilled water) (b)	6	7
Positive control (Sephazolin) (c)	29	29

Figure 4. The inhibitory activity of the combined fractions 5, 6 and 7. The tested bacteria were grown on an MHA medium at 37°C for 18 hours. The left panel (a) MHA inoculated with *E. coli,* and the right panel (b) MHA inoculated with *S. aureus.*

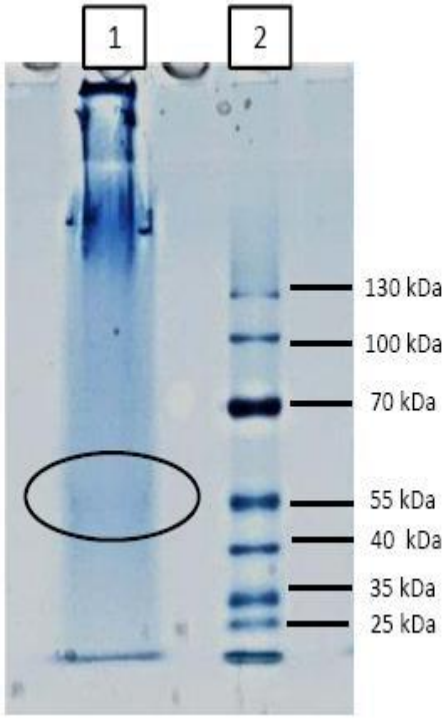


Figure 5. SDS-PAGE result of the antibiotic fraction (1) The antibiotic from PLS 76 of the combined fractions (2) Protein Marker (PageRuler Prestained Protein Ladder).

Determination of Molecular Weight from Polypeptide Antibiotic

SDS-PAGE electrophoresis was carried out on the combined fractions to show the protein molecular weight and purity level if the separation results by preparative TLC separation. Figure 5 was showed the results of gel electrophoresis staining using Coomassie Brilliant Blue. The results obtained only one protein band with a molecular weight of 55 kDa which was thought to be a polypeptide antibiotic compound.

The antibiotic by isolate PLS 76 can be categorized as polypeptide which has molecular weight 55 kDa. Pilonieta *et al*. (2009) reported a polymixin antibiotic properties with a molecular weight of 40 kDa [22] . The polypeptide grup of antibioticts from P.javanica has molecular weight af around 7-55 kDa [23]. (Waluyo 2007). The results obtained in this study and comparions with previous studies show that the polypeptide group antibiotics produced by PLS 76 isolater was molecular weight of around 55 kDa.

Structure Determination

The antibiotic structure determination was conducted by QTOF (LC-MS/MS). The results showed that the antibiotic was of a polypeptide that had similarities to polymyxin B2 based on the precursor mass and ion products compatibility with the reference database [24],[25],[26]. LC-MS/MS analysis was carried out by matching the experimental mass with the mass of the reference compounds of polymyxin B1, polymyxin B2, polymyxin E1, polymyxin E2, mycocystin, bacitracin, fenticonazole and mifepristone. In the matching process, two types of mass were used, i.e. the mass of ionic precursors and ionic products. The mass of the ionic precursor was obtained from the complete MS analysis and was the molecular mass of the sample. The precursor mass and mass of ionic products did not match the references, except polymyxin B2 (Fig. 6).

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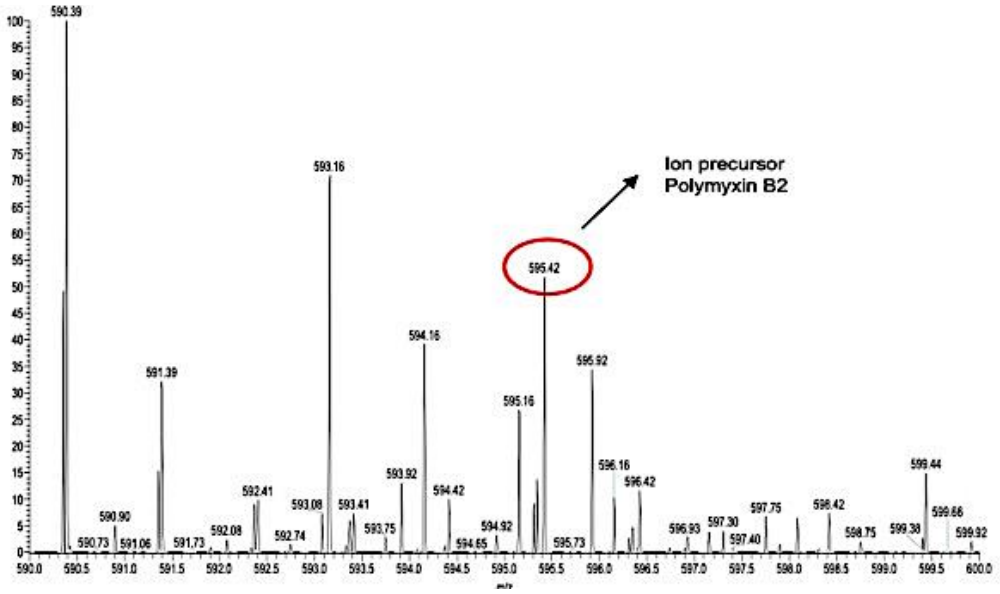


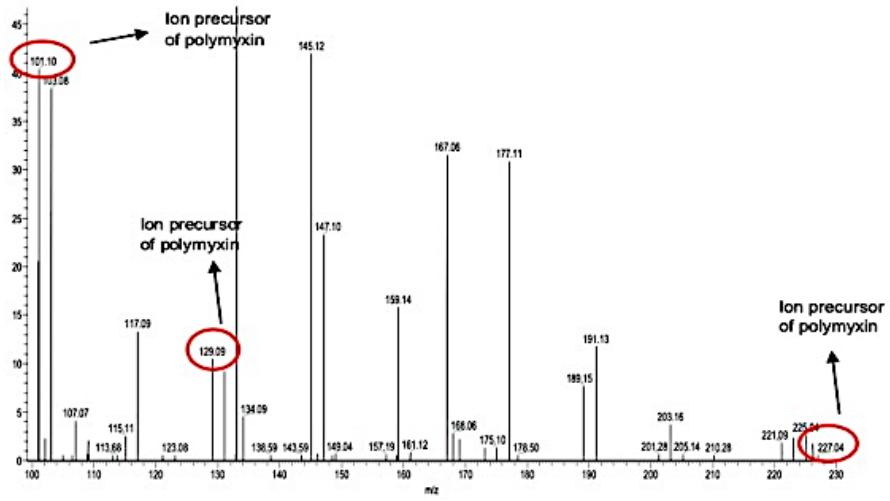
Figure 6. The mass spectra of the polypeptide fragmentation using QTOF (LC-MS/MS)

The mass-to-charge ratio (m/z) of the polypeptide was 595.42. Meanwhile, the mass of the precursor ion was half that of polymyxin B2 (m/z 1010). The fragmentation pattern from Fig. 6a was further confirmed using the mass of the polymyxin B2 ion products (Fig. b). Thomas et al. 2012 [25] reported that the ion product of $[M+2H]^{2+}$ of m/z 595.5 and 602.6 were polymyxin B2 and polymyxin B1, respectively.

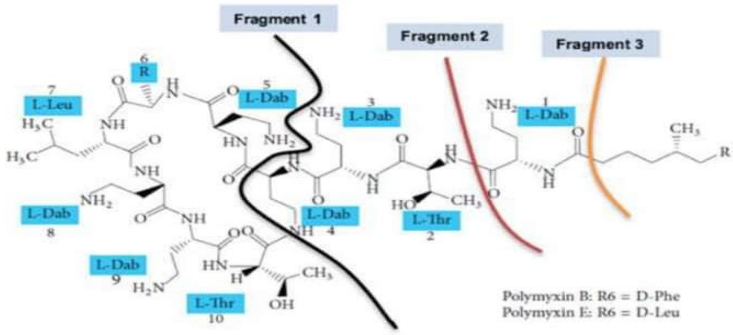
The mass of the ion product with an m/z of 227.04 already lost an m/z of 369. The loss was likely due to the separation of two methyl groups, L-Dab (1), L-Thr (2) and L-Dab (3). The m/z 227 was further fragmented to m/z 129.09. The structure then lost alkyl chains of amino acids to give an m/z 101.19 (Fig. 6b). The fragmentation patterns were in agreement with the reference compound of polymyxin B2 [24],[25],[26]. Polymixin are composed of a cyclic heptapeptide core linked to a fatty acid tail. Polymyxin has the characteristics of L-α, γ-diaminobutyric acid (Dab), threonine amino acid, and branched fatty acids in the stucture. Dab residues contribute to the overall positive charge of polymyxins, making them cationic. Each polymyxin was a different amino acid composition, where polymyxin B and C contain phenylalanine, polymyxin C contains leucine and polymyxin D contain serine [27].

Structure determination showed that the antibiotic from PLS 76 was a polypeptide antibiotic, with similarities with the fragmentation pattern of polymyxin B2. Polymyxin is a basic peptide and one of five polypeptide antibiotics derived from Bacillus species, active against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*[11].

Another test result of polypeptide antibiotic of PLS 76 was protein identification. The result suggested that the polypeptide antibiotic was similarity with protein from several bacteria in the same genus (Table 4.1).



(a)



(b)

Figure 6. a) Matching the ion mass products of polypeptide from PLS 76 with those of polymyxin B2. (b) Fragmentation pattern of polymyxin B2 based on LC-MS/MS result.[28]

Table 4.1 Protein characteristics of polypeptide antibiotic of PLS 76 was compared with protein from the closest similarity in the same genus.

No	Types of Protein	Uni prot Protein ID	Bacteria	Peptida of PLS 76
1	Elongation factor Tu	A0A2B4MB01	*Bacillus sp*	CDMVDDAELLELVEMEVR AVDGTFLMPVEDVFSISGR LDQGQAGDNVGILLR LLDQGQAGDNVGILLR
2	ATP Synthase beta subunit	A0A060M6R0	*Bacillus lehensis*	FTQAGSEVSALLGR VGLFGGAGVGK
3	*Aldehyde dehydrogenase*	A0A1B9B6N5	*Bacillus wudalianchiensis*	LIMQYASQNIIPVTLELGGK ETMAADIPLAIDHFR

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Based on Table 4.1, the result suggested that antibiotic produced from PLS 76 was polypeptide antibiotic. The results of LC-MS/MS analysis was found to be polymyxin B2 antibiotic.

Conclusions

PLS 76 isolate produced an antibiotic of the polypeptide group, which had a similar structure to polymyxin B2. The antibacterial activity in the pure form was not as high as that in the methanol fraction. Therefore, different purification methods may be necessary to isolate different pure fractions. In addition, the synergistic effect of the antibiotic needs to be investigated further.

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