AN ABSTRACT OF THE THESIS OF

Younan Ma for the Master of Science

in Biology presented on July 11, 2016

Title: Characterization of the Antimicrobial Compound Produced by the Soil Bacterium *Brevibacillus laterosporus*

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Abstract approved: ______________________________

(Thesis Advisor Signature)

The resistance of pathogenic microbes to an increasing number of antimicrobial agents poses a serious threat to human health. Soil-dwelling bacteria have received great attention as a reservoir of new antibiotics because of large populations and great diversity. In this project, a new soil bacteria (named ZYD4) producing antimicrobial compound(s) was isolated from soil samples collected in the Rocky Mountain National Park. ZYD4 was identified as *Brevibacillus laterosporus* based on 16S rRNA gene sequence analysis. The antimicrobial compound(s) showed a broad spectrum of activity against 11 species of microorganism pathogens with clinical importance, including methicillin-resistance *Staphylococcus aureus* (MRSA). The specific substance was also proved to have strong inhibition on proliferation of melanoma and prostate cancer cells.

We explored the effects of using different media on the production of the antimicrobial agent(s), and found that the inhibitory activity of culture supernatant reached a peak after incubating ZYD4 in LB for 96 hours at room temperature. The addition of 20% (v/v) supernatant reduced the growth of *Escherichia coli* in log phase by 70%-80%. This project used the AlamaeBlue Assay, a method of measuring the viability and multiplication of cells, to examine the inhibitory effects of the antimicrobial compound produced by ZYD4. The antimicrobial agents showed stability to alkalinic pH and heat, and resistance to proteinase K. The purification

process involved ammonium sulphate precipitation/ethanol precipitation, Amberlite XAD-16 adsorption, n-butanol extraction, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) on a C18 reverse phase column. Three possible substances were isolated from the ZYD4 bacteria culture. The molecular weights were identified by a Mass Spectrometer (MS) as 1263, 1499 and 357 Da. The physical and chemical properties indicate that the antimicrobial agents are highly likely to be peptides, except the one with the smallest molecular weight. The antimicrobial agents identified here could be effective candidates for future clinical research.

Keywords: Antibiotics, *Brevibacillus laterosporus,* high performance liquid chromatography, Mass Spectrometer

Characterization of the Antimicrobial Compound produced by the Soil Bacterium *Brevibacillus laterosporus*

A Thesis

Submitted to

The Department of Biological Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

By

Younan Ma

July 2016

Approved by Major Advisor

Approved by Committee Member

Approved by Committee Member

Approved for Department of Biological Sciences

Approved for Dean of Graduate Studies and Research

ACKNOWLEDGEMENTS

I would like to extend my sincerest gratitude to my advisor, Dr. Yixin Yang, for his education, support, patience, encouragement and friendship throughout my graduate study. His intellectual guidance and keen insight in creative research provided me with a solid foundation on which I will build my future scientific career. His technical and editorial advice was essential for the completion of this thesis.

My thanks also go to the members of my committee: Dr. Scott S. Crupper and Dr. J. Richard Schrock for their kindness to serve on this committee and their comments to improve this thesis. I owe special thanks to Dr. Scott S. Crupper for providing instruments and reagents for this project and answering questions concerning technical details of research methods.

I would like to thank my lab mates, Yao Yan, Hui Qian, Guang Yang, Guangyi Gao Chunmiao Yu and Lin Wang, for their assistance in the lab and their understanding throughout my graduate study.

Last, but by no means least, I want my parents to receive my deepest gratitude for their education, dedication, encouragement and endless love in the whole of the past twenty years. Their excellence within their careers and their pursuit of perfection have been the driving forces of my motivation to be better. Their pride and happiness make this thesis meaningful.

PREFACE

This thesis was prepared following the publication style of *Microbiological Research*.

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I. INTRODUCTION

Infectious diseases were not expected to be such a big challenge after the discovery of penicillin which has been widely used. Over this last century, despite a diversity of chemotherapeutics and antibiotics, bacterial infections continue to be one of the leading causes of morbidity and mortality worldwide, attributed in part to the evolution of antibiotic resistance genes[1]. The resistance of pathogenic microbes to an increasing number of antimicrobial agents now poses a serious threat to human health. Especially in hospitals, massive use of antibiotics facilitates the selection and spread of antibiotic-resistant strains. According to the Infectious Disease Society of America, 70% of deaths resulting from nosocomial infections (infections acquired from hospitalization) have been attributed to infection with drug-resistant bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus*, and penicillin-resistant *Neisseria gonorrhoeae*[2]. On the other hand, the lack of basic medical knowledge among citizens, the weak prescription management system and the abusive use of antibiotics in developing countries, also threaten to spread antibiotic resistant strains to a wider community.

Despite the rapidly increasing rate of drug resistance in bacteria, researchers even predict that the development of new antimicrobial drugs might slow and cease in the future[3]. Because of high dollar investment, but a much shorter treatment course (7-10 days) of using antibiotics for treating bacterial infections compared to chronic disease medications that are often taken by patients for years, two-thirds of major pharmaceutical companies have greatly cut their effort in discovering and developing new antibiotics[4]. Thus, there has been a race between the development of antibiotic resistance in pathogenic microbes and the discovery of new antibiotics. Considering the downward trend of the development of new antibiotics for the

business market, researchers in the academic institutions must take more responsibility for pursuing antibiotic discovery.

The vast majority of microorganisms on earth flourish in soil and water. The high diversity and density of microorganisms in soil allow them to live in close proximity to each other. Bacteria in this interacting community compete for nutrients, availability of oxygen, and other abiotic components required for survival[5]. The production of antibiotics can inhibit the competing microorganisms, placing antibiotic-producing microorganisms in an advantageous position. Furthermore, soil bacteria have been shown to be a reservoir of new antibiotic-resistance genes, especially genes for resistance against aminoglycoside antibiotics and tetracycline[6]. There is good chance for other microorganisms in the same community to develop certain antibiotics that can counteract the resistance mechanism deriving from antibiotic-resistance genes. Therefore, the “gold-mining” of antibiotic-producing soil microorganisms is an effective and economical way to discover potent antibiotics.

In this project, by screening over 600 bacteria from soil samples collected in the Rocky Mountain National Park, a new soil bacteria (named ZYD4) producing antimicrobial compound(s) was successfully isolated. In these preliminary studies, ZYD4 was identified as *Brevibacillus laterosporus* based on 16S rRNA gene sequence analysis. The antimicrobial compound(s) showed a broad spectrum of activity against 11 species of microorganismic pathogens of clinical importance, including methicillin-resistance *Staphylococcus aureus* (MRSA). The specific substance was also proven to provide a strong inhibition on proliferation of melanoma and prostate cancer cells.

Brevibacillus was established as a new genus from reclassification of the *Bacillus brevis* group of species[7]. The pathogenic potential of *B. laterosporus* against insects has already been

demonstrated[8]. It can produce two different virulence factors: extracellular protease[9] and parasporal crystalline[10]. Spergualin is a known compound produced by some strains of *B. laterosporus.* It has been demonstrated to have a wide spectrum of biologicals activities, including immunosuppressive activity and anti-tumor activity against leukemia[11]. Tauramamide, a new lipopeptide, produced by the marine bacterial isolate *B. laterosporus* PNG276 was also reported to have a wide spectrum of antimicrobial activity[12]. Some antimicrobial peptides produced by soil *B. laterosporus* have also been reported[13]. In the present work, we investigate the purification, characterization and identification of novel agents secreted by *B. laterosporus* ZYD4.

II. MATERIALS AND METHODS

Chemicals and Reagents

AlamarBlue^{TM} was purchased from AbD Serotec (Raleigh, NC). Amberlite XAD16 was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Ammonium sulfate, ethanol, methanol, ethyl acetate, 1-butanol, Tris-EDTA and proteinase K were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ). Coomassie G-250 was purchased from Bio-Rad Laboratories, Inc. (Philadelphia, PA).

Bacterial Strains

Escherichia coli (ATCC 25922) was used as an indicator bacterial strain to test the antimicrobial activity of the supernatant produced by *Brevibacillus laterosporus* ZYD4.

Supernatant Preparation

100 µl of overnight culture (OD_{450}=0.5) of ZYD4 was inoculated in 100 ml of Luria Broth (LB) and was grown on the shaking platform at 25℃. The supernatant was collected at 96h after inoculation, and then filtered through a sterile Nylon syringe filter (0.22µm, Fisherbrand). The sterile filters were always washed with at least 30 ml ddH_2O immediately prior to using them to filter supernatant or any other solutions to reduce the possibility of substances being released from the filter cartridges into the filtrates. The filtered supernatant was stored at 4 ^{o}C for subsequent assays.

Determination of Antimicrobial Activity

The overnight culture of *E. coli* was inoculated in Tryptic Soy broth (TSB) and grown into log phase (OD_{450}=0.5). The log phase culture was then diluted 10 times with TSB. 20 µl of supernatant, supernatant extracts, or LB (positive growth control) was added to 70 µl of diluted bacterial culture in a 96-well microplate. Then 10 µl AlamarBlue reagent was added to the mixture and incubated at 37°C for 2.5-8h. Absorbance was measured at wavelengths of 570 nm and 600 nm after incubation.

The percent difference in reduction $= \frac{(\varepsilon OX)\lambda 2A\lambda 1 - (\varepsilon OX)\lambda 1A\lambda 2}{(\varepsilon OX)\lambda 2A°\lambda 1 - (\varepsilon OX)\lambda 1A°\lambda 2} \times 100\%$

(ε_{OX} = molar extinction coefficient of AlamarBlue oxidized form, A = absorbance of test agent well, A° = absorbance of positive growth control well, λ_1 = 570nm, λ_2 = 600nm, $(\varepsilon_{OX})\ \lambda_2$ = 117216, $(\varepsilon_{OX})\ \lambda_1$ = 80586).

The growth inhibition percent = (1 – percent difference in reduction) × 100%.

Effect of Extreme pH, Temperature and Proteinase K on Microbial Activity of Supernatant

Supernatant was treated with acidic pH (pH = 4.6) or alkalinic pH (pH = 12.1) solutions for 45 min. at 25°C, and was adjusted back to the original pH (pH = 7.8); alternatively, it was heated in boiling water for 30 min.; or, it was treated with proteinase K (10 mg/ml) at 37°C for 60 min. The supernatant subjected to above treatments was tested by AlamarBlue assay for the stability of antimicrobial activity.

Determination of Molecular Size of Antimicrobial Substances

Dialysis was performed against 50mM Tris-EDTA buffer for 10h at 4°C using 0.1-0.5 KD cut-off (Spectra/Por Biotech-Grade CE Membrane) and 10-12 KD cut-off (Fisher Scientific) dialysis tubing. The supernatant after dialysis was tested for antimicrobial activity by using the

AlamarBlue assay. The positive control used 50mM Tris-EDTA buffer.

Precipitation of Antimicrobial Substance from ZYD4 Supernatant with Ammonium Sulfate and Ethanol

Supernatant was saturated with ammonium sulfate to 55% by adding 1.78g of ammonium sulfate to 5 ml of supernatant. After centrifuging at 10,000g for 10 min, the precipitation fraction was removed and dissolved in 2 ml of 50 mM Tri-EDTA (pH = 7.8) buffer. The supernatant fraction was added with 1.02 g ammonium sulfate to get a 85% saturated solution, and centrifuged at 10,000g for 10 min. The pellet was again dissolved with 50 mM Tris-EDTA (pH = 7.8) buffer. Both the 55% and 85% ammonium sulfate pellet re-dissolved solutions were put into dialysis tubing (100-500 Dalton cut-off) and incubated in 50mM Tris-EDTA buffer for desalting overnight. The precipitation fractions were evaluated for antimicrobial activity by the AlamarBlue assay.

Ethanol of 3 ml, 6 ml or 12 ml was slowly added to three supernatant samples each with the volume of 3 ml, reaching final 50%, 67% and 80% (v/v) ethanol solution. The mixture was stirred and the samples were kept in an ice water bath for 15 min. After centrifuging at 10,000g for 10 min, each pellet was dissolved in 3 ml LB. Precipitated fractions were tested for antimicrobial activity.

De-proteinization of Supernatant with Sevag Reagent

One volume of Sevag reagent (n-butanol : chloroform = 1 : 4) was added to five volumes of supernatant, shaken for 20 min, then centrifuged at 10,000 rpm for 10 min to remove denatured protein (pellet between aqueous and organic layer) and organic reagent (lower layer). This process was repeated three times. Each time the protein concentration of the aqueous layer

was determined by the Bradford assay, and the antimicrobial activity was tested by the AlamarBlue assay.

Extraction of Supernatant with Amberlite XAD-16 Resin

1 g Amberlite XAD-16 was added into 50 ml supernatant and incubated at 25 ^{o}C overnight. After the supernatant was removed, the resins were washed with 10 ml methanol, 10 ml acetone, and 10 ml ethyl acetate sequentially. The eluent solutions were concentrated by a rotary evaporator, and the dried content was dissolved in ddH_2O. The methanol, acetone, ethyl acetate eluent contents and the remaining supernatants were assayed for antimicrobial activity by the AlamarBlue assay.

Extraction of Supernatant with Ethyl Acetate and n-Butanol

Supernatant was extracted by equal volume of HPLC-grade ethyl acetate for three times, and the organic layer was concentrated by 10 times on an evaporator (re-dissolved with ddH_2O). The aqueous layer was then extracted with equal volumes of HPLC-grade n-butanol for three times and then concentrated 10 times on a rotary evaporator (re-dissolved with ddH_2O). The antimicrobial activity of concentrates was evaluated by the AlamarBlue assay.

Purification of Antimicrobial Reagents

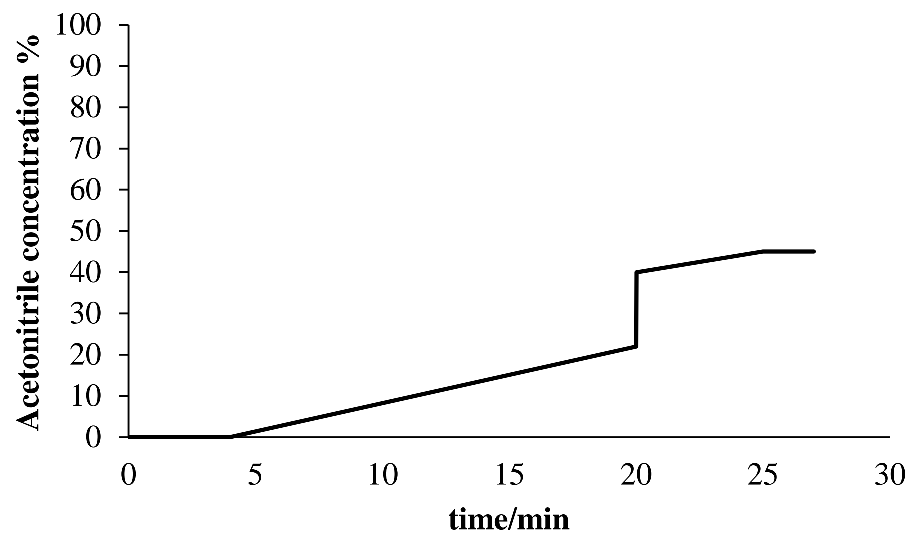
Thin-layer Chromatography Assay: The thin-layer chromatography (TLC) was carried out with silica gel 60 F_{254} (Teledyne Isco) and Basic Alumina F_{254} (EMD Millipore) pre-coated plates using the BAW system (butanol : acetate acid : water = 4 : 6 : 1, mixed and used the upper layer).

High Performance Liquid Chromatography (HPLC) Assay: The butanol extract was

separated by an HPLC system (Shimadzu LC-20AB) employing a reverse phase column (Restek, Viva C-18, 5µm, 200 × 4.6 mm). This system was operated at 25 ^{o}C with a 0% isocratic gradient (0-4 min), 0% to 22% linear gradient (4-20 min), 40% to 45% linear gradient (20-25 min) and 45% isocratic gradient (25-27 min) of acetonitrile as mobile phase with a flow rate of 0.8 ml /min (Figure 1). Alternatively, 0.05% trimethylamine (TEA) was added into water and acetonitrile, and adjusted for a pH = 7 by acetic acid. The protocol for the mobile phase gradient was the same. UV detector absorbance was set to 235nm and 255nm. The peaked fractions were dried with nitrogen flow, re-dissolved with ddH_2O to an appropriate concentration, and tested for antimicrobial activity by the AlamarBlue assay.

Figure 1. Mobile phase protocol of HPLC system

The mobile phase pumped through HPLC system started from 0% isocratic gradient (0-4 min), 0% to 22% linear gradient (4-20 min), 40% to 45% linear gradient (20-25 min) and 45% isocratic gradient (25-27 min) of acetonitrile, with a flow rate of 0.8 ml /min.


Acetonitrile concentration %
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
time/min

Identification of the Antimicrobial Substance by Liquid Chromatography-Mass Spectrometry (LC-MS)

An electrospray ionization (ESI) mass spectra was operated by LCMS-2020 (Shimadzu), connected to the interface of the HPLC system. The instrument was operated in the positive mode with a scan speed of 2143 u/s. For the MS analysis, the data were acquired in the MS scanning mode using a scan range of 20–2000 (m/z).

Statistical Analysis

Statistical analysis was performed with Excel. The percentage of growth inhibition of melanoma cells was expressed as mean ± SD. A *t*-test was applied to test for differences in activity between a differently-treated supernatant and a non-treated supernatant in the assay of stability of ZYD4 supernatant, while a one-way ANOVA was used to test for differences in activities among the multiple groups of different treatments. In all comparisons, statistical significance was set at $p \leq 0.05$.

III. RESULTS

Optimization of the Peak Time for the Antimicrobial Substance Produced by ZYD4

As shown in Figure 2, the antimicrobial activity of supernatant against *E. coli* was stronger when ZYD4 was grown in LB broth than in TSB or NB broth. The antimicrobial activity reached a peak at 96 hours (the fourth day) in LB broth in a 5-day growth course.

Physical and Chemical Characterization of the Antimicrobial Agents in ZYD4 Supernatant

The antimicrobial activity was found to be acidic-stable: exposure to pH = 4.6 for 45 min did not reduce the activity. Further, the activity was preserved after incubating in 100°C water. Also, the treatment with proteinase K had no effect on the antimicrobial activity. However, the antimicrobial activity was significantly decreased in the solution of extreme alkalinity (Figure 3). Thus, the antimicrobial substances were heat-, acid-stable, proteinase-resistant, and alkali-unstable.

Determination of the Range of Molecular Size of Antimicrobial Substance

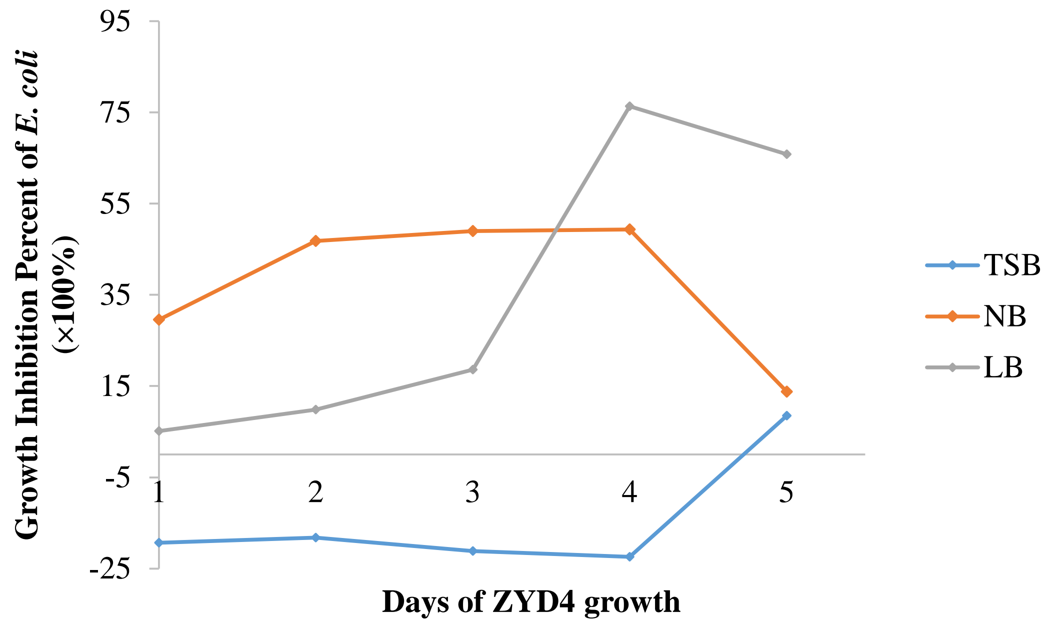
The antimicrobial activity remained after dialysis with a 100-500 Dalton cut-off membrane, but was lost after dialysis with a 10,000-12,000 Dalton cut-off membrane (Figure 4). Thus we conclude that the molecular weight of the antimicrobial substance(s) present in ZYD4 supernatant could be between 500-10,000 Dalton.

Precipitation of Antimicrobial Substance from ZYD4 Supernatant with Ammonium Sulfate and Ethanol

The antimicrobial activity can be precipitated by only 85% ammonium

Figure 2. Antimicrobial activity of supernatant when grown in different media on a 5-day growth course. Data represent the average of three experiments, and results are means ± SD.

ZYD4 was grown in TSB, NB or LB broth to examine the effect of different media on the antimicrobial activity, and the time to reach the peak antimicrobial activity. The antimicrobial activity were tested by AlamarBlue assay. The supernatant produced by ZYD4 growing in LB broth showed a stronger antimicrobial activity than in TSB and NB broth. The antimicrobial activity peak was reached after 96h growing in LB broth.



Growth Inhibition Percent of E. coli (×100%)
95
75
55
35
15
-5
-25
1
2
3
4
5
Days of ZYD4 growth
TSB
NB
LB

Figure 3. The Stability of the Bioactive Substance Produced by ZYD4 to Physical or Chemical Treatment. Data represent the average of three experiments, and results are means ± SD.

The stability of the antimicrobial agents was tested by treating with heat, acid and alkaline solutions, proteinase K. The antimicrobial activity in the supernatant produced by ZYD4 only significantly decreased ($p<0.05$) with the exposure to alkaline solution (pH = 12.1). The antimicrobial activity was preserved with the treatment of heat, acid solution and proteinase K.

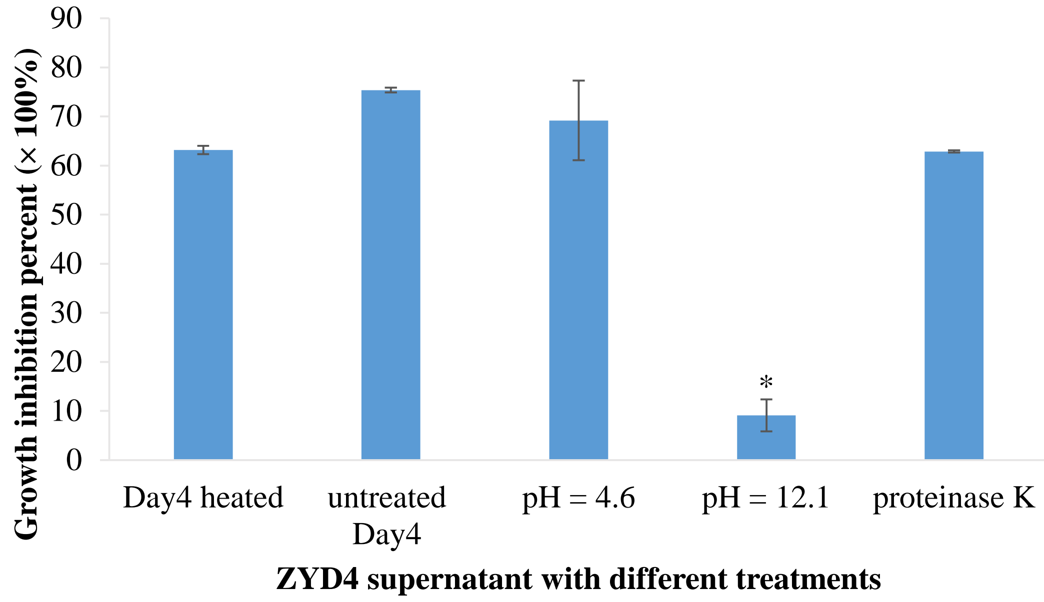
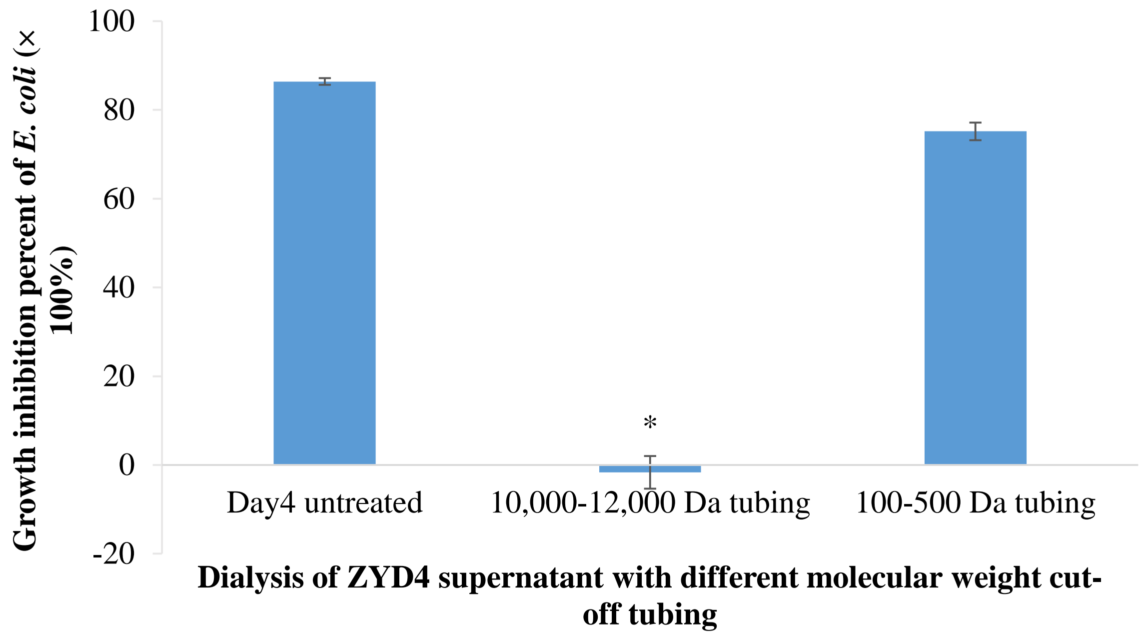

90
80
70
60
50
40
30
20
10
0
Growth inhibition percent (× 100%)
*
Day4 heated
untreated Day4
pH = 4.6
pH = 12.1
proteinase K
ZYD4 supernatant with different treatments

Figure 4. Determination of the Range of Molecular Size of Antimicrobial Substance by Using Dialysis Membrane Tubing. Data represent the average of three experiments, and results are means ± SD.

The ZYD4 supernatant was out into dialysis bag with a 100-500 Da or 10,000-12,000 Da molecular weight cut-off against 50mM Tris-EDTA overnight. The antimicrobial activity of supernatant dialyzed by 100-500 Da and 10,000-12,000 cut-off membrane were determined by AlamarBlue assay. The antimicrobial activity was significantly decreased ($p<0.05$) after incubating in 10,000-12,000 Da cut-off dialysis membrane, but retained after dialyzing with 100-500 Da cut-off membrane.


100
80
60
40
20
0
-20
Growth inhibition percent of *E. coli* (× 100%)
*
Day4 untreated
10,000-12,000 Da tubing
100-500 Da tubing
Dialysis of ZYD4 supernatant with different molecular weight cut-off tubing

sulfate but not 55% ammonium sulfate. Although precipitation fractions of 55% and 85% ammonium sulfate both showed strong antimicrobial activity, the high concentration of ammonium sulfate contained in the pellet can have a cytotoxic effect on the indicator bacteria. In order to remove ammonium sulfate from the pellet, a re-dissolved pellet was dialyzed with the 100-500 Dalton cut-off dialysis membrane in Tris-EDTA buffer. After overnight incubation, the activity was only recovered in the 85% saturation pellet (2.5-times concentrated re-dissolved solution). The activity of the 55% saturated pellet was significantly reduced, indicating that a high concentration of ammonium sulfate contributed the antimicrobial activity of the 55% saturated pellet (without desalting) (Figure 5A). For the one-fold re-dissolved solution, both the activities of undesalted and desalted ammonium sulfate precipitation fractions were significantly decreased (Figure 5B). This may result from the loss of antimicrobial agents during the precipitation process. Hence, we can conclude that 85% ammonium sulfate can be used to precipitate the antimicrobial substance from the ZYD4 supernatant.

The antimicrobial activity was significantly recovered and concentrated by 80% ethanol. At a 1-time dilution, about 64% of cell reduction was inhibited by 80% ethanol-precipitated supernatant. The inhibition of the precipitated fractions increased with the concentration of ethanol. However, after 24 hours, the antimicrobial activity of the dissolved pellet significantly decreased (Figure 6). Thus, we can conclude that the structure of the antimicrobial substances in the ZYD4 supernatant could possibly be damaged by an ethanol solution, causing this reduction of inhibitory activity. A high concentration of ethanol should be avoided in the following purification process. Considering the stability of antimicrobial compounds, the precipitation with ethanol is not a proper way to precipitate antimicrobial substances from ZYD4 supernatant in further studies.

Figure 5. Precipitation of Antimicrobial Agents from ZYD4 Supernatant with Ammonium Sulfate. Data represent the average of three experiments, and results are means ± SD.

The antimicrobial activity of 55% ammonium sulfate precipitation (desalting and without desalting) and 85% ammonium sulfate precipitation (desalting and without desalting) were tested by AlamarBlue assay. Ammonium sulfate was added to ZYD4 supernatant to 55% saturation. After centrifuge and remove the pellet, ammonium sulfate was continued to add to the supernatant to 85% saturation. All the pellet was dissolved in 50mM Tri-EDTA and desalting was performed against Tri-EDTA in 100-500Da cut-off membrane. The antimicrobial activity of 55% and 85% ammonium sulfate precipitation without desalting was both recovered. After desalting, the antimicrobial activity of 55% ammonium sulfate precipitation was significantly decreased ($p<0.05$), but not 85% ammonium sulfate precipitation.

Figure 5A.

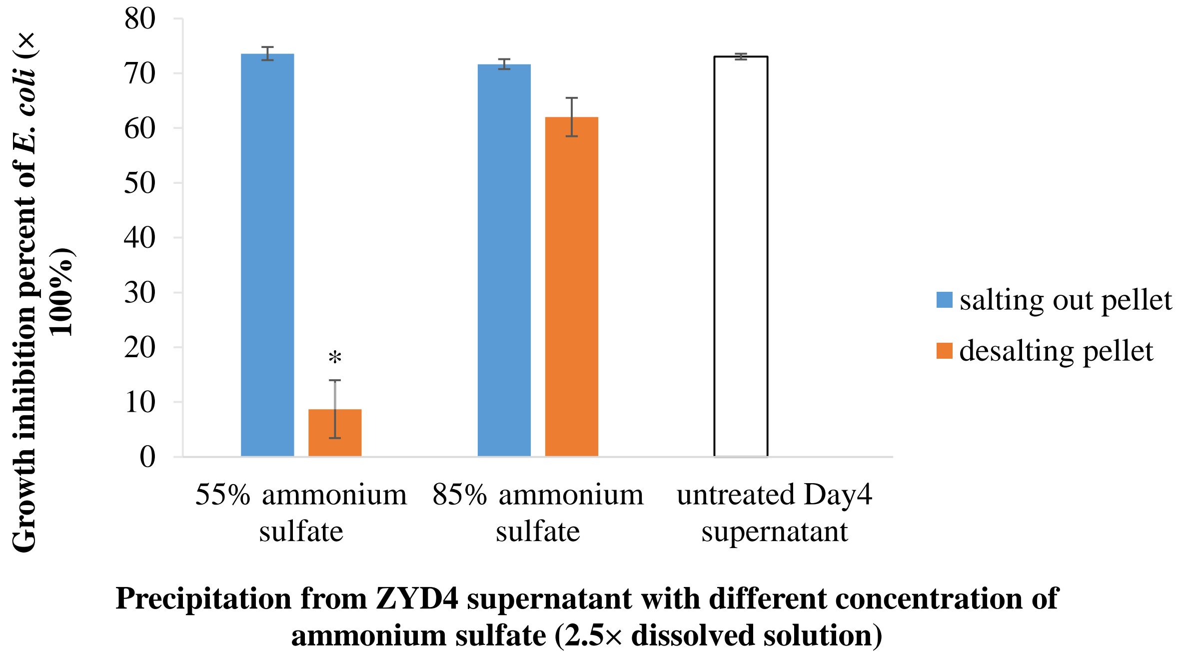


Figure 5B.

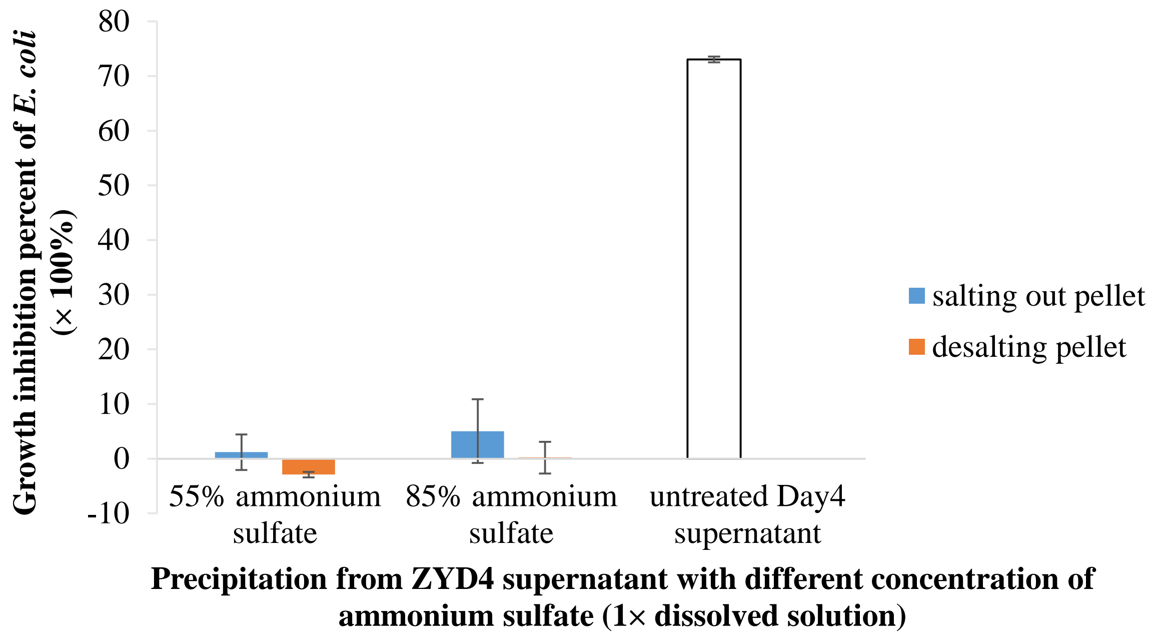
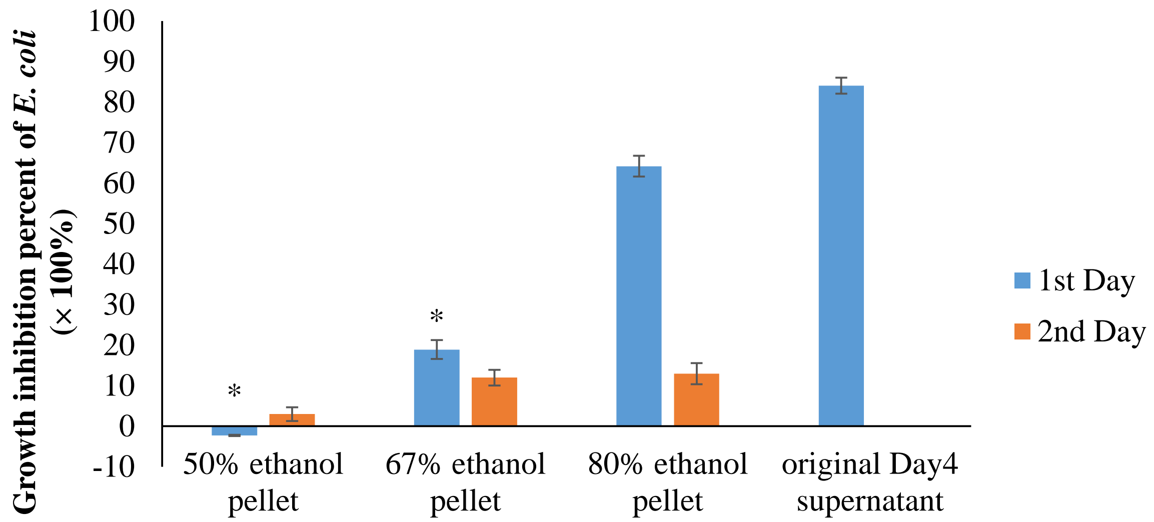


Figure 6. Precipitation of Antimicrobial Agents from ZYD4 Supernatant with Ethanol and Determination of Inhibitory Activity on the First and Second Day. Data represent the average of three experiments, and results are means ± SD.

The antimicrobial activity of 50%, 67% and 80% ethanol precipitation were all examined by AlamarBlue assay. Ethanol was added into ZYD4 supernatant to reach 50%, 67% or 80% volume concentration. After centrifuge, the pellet was dissolved in 50mM Tris-EDTA, and the antimicrobial activity was tested on the same day of precipitation and the second day. The antimicrobial activity of 80% ethanol precipitation was recovered, and 50% and 67% ethanol precipitation was significantly reduced ($p<0.05$) on the first day. All of 50%, 67% and 80% ethanol precipitation lost antimicrobial activity on the second day.


Growth inhibition percent of E. coli (× 100%)
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
50% ethanol pellet
67% ethanol pellet
80% ethanol pellet
original Day4 supernatant
1st Day
2nd Day
Precipitation from ZYD4 supernatant with different concentrations (v/v) of ethanol (1× dissolved pellet)

De-proteinization of Supernatant with Sevag Reagent

Since the antimicrobial substances can be precipitated by both ammonium sulfate and ethanol, the antimicrobial substances could possibly be a protein or polypeptide. Sevag reagent is a mild de-proteinization reagent which does not have an effect on other macromolecules, such as polysaccharides. The antimicrobial activity was significantly decreased (Figure 7) after two cycles of de-proteinization with Sevag reagent (Figure 8). Hence, we can conclude that the antimicrobial substances in the ZYD4 supernatant are highly likely to be small proteins or polypeptides. After the third treatment with Sevag reagent, the antimicrobial activity slight increased comparing to that after the second treatment with Sevag reagent (Figure 7). Since the components of Sevag reagent were chloroform and butanol, which have a solubility of 0.809 g/100 ml and 7.3 g/ml in water respectively, there could be a small portion of Sevag reagent dissolved in the supernatant layer. The organic reagent can be cytotoxic to the growth of bacteria. Therefore, we consider that the dissolved chloroform and butanol in supernatant layer caused a higher antimicrobial activity after the third treatment of Sevag reagent than the second treatment of Sevag reagent.

Extraction of Supernatant with Amberlite XAD-16 Resin

After washing the XAD-16 resins sequentially with methanol, acetone and ethyl acetate, each of the three types of eluent was concentrated to 5-time solution. Comparing to untreated supernatant, only the methanol eluent restored the antimicrobial activity (Figure 9). Since about 30% inhibitory activity was left in the supernatant after XAD-16 absorption, there could be two different antimicrobial substances in the ZYD4 supernatant. One compound was extracted by methanol, and the other was left in the supernatant. Also, since methanol has a larger relative

Figure 7. Antimicrobial Activity of ZYD4 Supernatant after Three Cycles of Treatment with Sevag Reagent. Data represent the average of three experiments, and results are means ± SD.

Sevag reagent was added to ZYD4 supernatant and shook for 20 min. The antimicrobial activity of ZYD4 supernatant after the treatment of Sevag reagent was tested by AlamarBlue assay. After three cycles of de-proteinization with Sevag reagent, the antimicrobial activity were all significantly decreased ($p<0.05$). The antimicrobial activity of supernatant after the third treatment was higher than the second treatment, with a decreasing amount of protein contained in the supernatant.

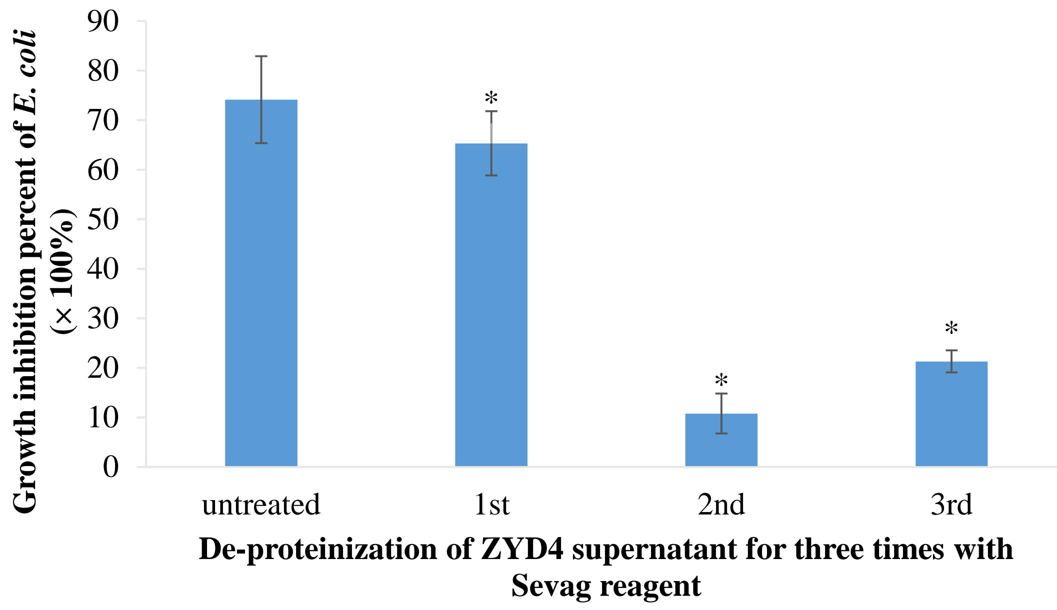

Growth inhibition percent of E. coli (× 100%)
90
80
70
60
50
40
30
20
10
0
*
*
*
untreated
1st
2nd
3rd
De-proteinization of ZYD4 supernatant for three times with Sevag reagent

Figure 8. Determination of Protein Concentration in ZYD4 Supernatant after Three Cycles of De-proteinization by Sevag Reagent. Data represent the average of three experiments, and results are means ± SD.

The protein concentration in ZYD4 supernatant was determined by Bradford assay. The protein contained in ZYD4 supernatant decreased from 0.82 mg/ml to 0.65 mg/ml (after the first Sevag treatment), 0.56 mg/ml (after the second Sevag treatment), and 0.54 mg/ml finally (after the third treatment with Sevag reagent).

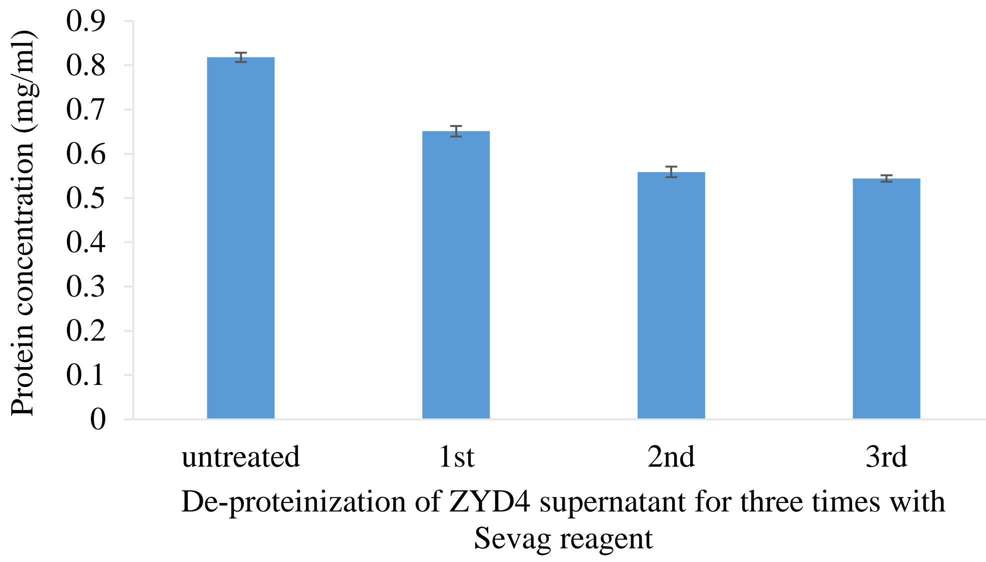
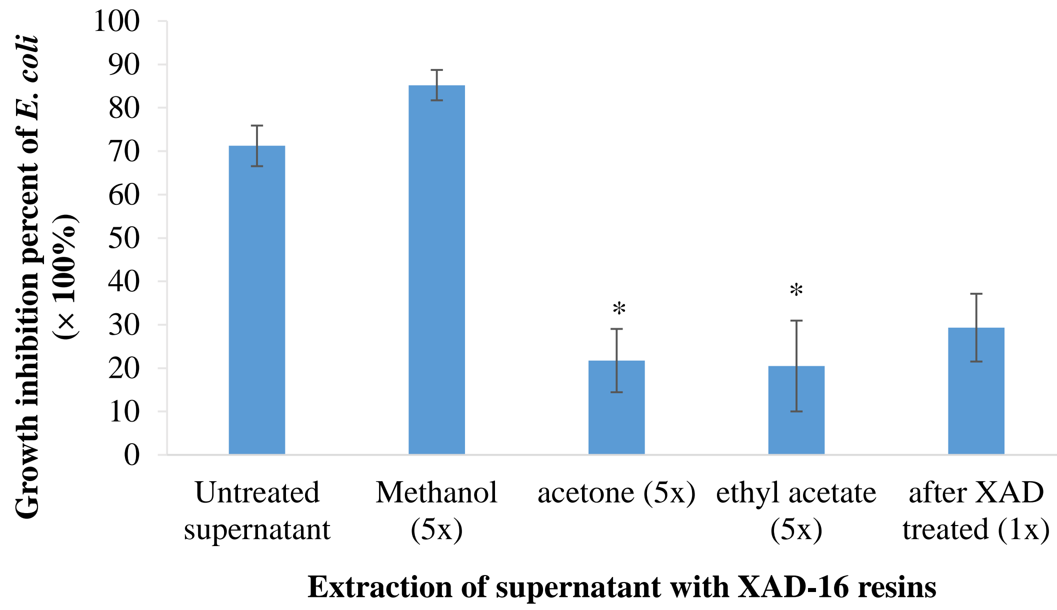

Protein concentration (mg/ml)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
untreated
1st
2nd
3rd
De-proteinization of ZYD4 supernatant for three times with Sevag reagent

Figure 9. Extraction of Supernatant with XAD-16 Resins and Antimicrobial Activity of Different Organic Eluents. Data represent the average of three experiments, and results are means ± SD.

XAD-16 resins were added to ZYD4 supernatant and shook overnight. The resins were eluted sequentially with methanol, acetone and ethyl acetate. The antimicrobial activity of methanol, acetone, ethyl acetate elution and supernatant treated after XAD-16 were determined by AlamarBlue assay. The antimicrobial activity in acetone and ethyl acetate elution were significantly decreased ($p<0.05$), and only activity in methanol elution was recovered. The antimicrobial activity left in the supernatant after the treatment of XAD-16 was about 30%.


Growth inhibition percent of E. coli (× 100%)
100
90
80
70
60
50
40
30
20
10
0
*
*
Untreated supernatant
Methanol (5x)
acetone (5x)
ethyl acetate (5x)
after XAD treated (1x)
Extraction of supernatant with XAD-16 resins

polarity than acetone and ethyl acetate, the antimicrobial compound which can be concentrated by the XAD-16 resin tends to be more hydrophilic.

Extraction of Supernatant with Ethyl Acetate and n-Butanol

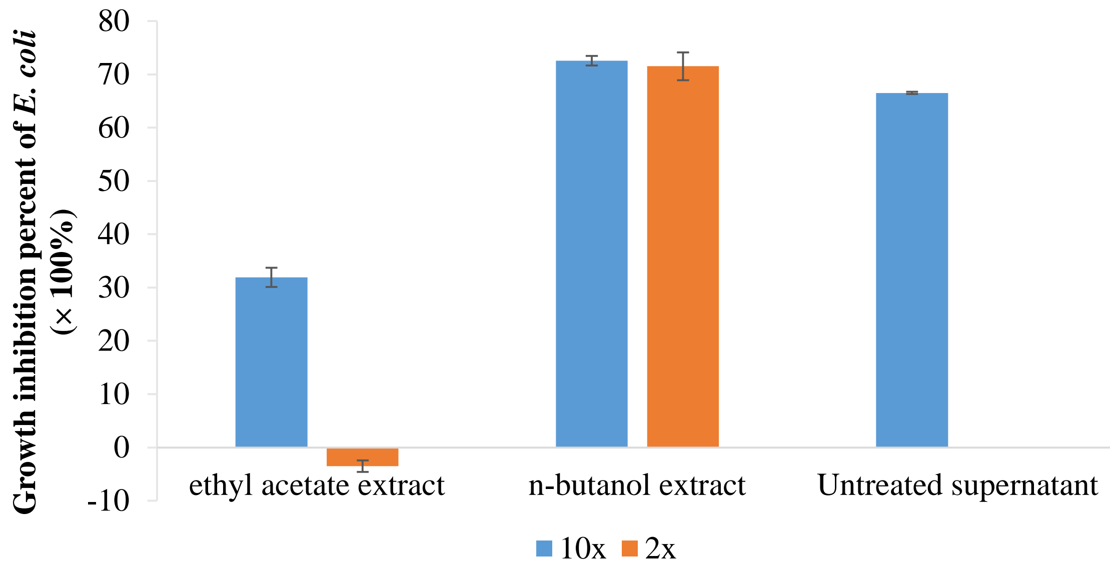
The antimicrobial activity was significantly recovered and concentrated by n-butanol but not ethyl acetate extraction (Figure 10). About 75% reduction of cell growth was observed by n-butanol extraction group, and ethyl acetate extracted at a 10-time concentrated supernatant caused only about 32% of reduction in cell growth. Even at 2-times concentration, n-butanol extract reduced the cell viability by 71%, while ethyl acetate extract showed no reduction on cell viability. Also, a white precipitation between the aqueous layer and the organic layer was found after both ethyl acetate and n-butanol extraction. Thus, n-butanol extraction was proved to be a convenient and effective way of concentrating the antimicrobial compounds found in the ZYD4 supernatant, which was used in the following purification process. Since the relative polarity of n-butanol (0.586) is greater than that of ethyl acetate (0.228), and antimicrobial agent was better extracted with n-butanol than with ethyl acetate, it suggests that the antimicrobial agent tends to be more hydrophilic.

Purification of Antimicrobial Reagents

On a Silica Gel plate, five substances were separated from the n-butanol extracted supernatant sample (spot 1-5), and two substances were separated from LB broth (spot 6 and 3’) (Figure 11A). There were unclear spots on the original line for the LB sample, which was not seen in the n-butanol sample, so we considered it to be bound too tightly to the silica gel, and could not be separated. Among the spots of LB broth, 3’ has a similar R_f value to spot 3 (from n-butanol supernatant), so we conclude that spot 3 and 3’ are the same substance. R_{f1} = 0.94, R_{f2} =

Figure 10. Antimicrobial Activity of Extraction of ZYD4 Supernatant with Ethyl Acetate and n-Butanol. Data represent the average of three experiments, and results are means ± SD.

The same volume of ethyl acetate was added to ZYD4 supernatant and extracted for three times. Then the same volume of n-butanol was added to aqueous layer and extracted for three times. Both organic solvent were evaporated to dry and dissolved in water. The antimicrobial activity of ethyl acetate and n-butanol extract were tested by AlamarBlue assay. The antimicrobial activity of ethyl acetate extract was about 30% at 10-time concentrated solution, but was lost at 2-time concentrated solution. For n-butanol extract, the antimicrobial activity were both recovered at 10-time and 2-time concentrated solution.



Extraction of ZYD4 supernatant with different organic solvent

Figure 11. n-Butanol extraction of ZYD4 Supernatant Applied on TLC.

On silica gel plate (Figure 11A), five substances were separated from 10x n-butanol extracted ZYD4 supernatant sample. R_{f1} = 0.94, R_{f2} = 0.62, R_{f3} = 0.46, R_{f4} = 0.41, R_{f5} = 0.35. Two substances were separated from LB broth sample. R_{f6} = 0.49, $R_{f3'}$ = 0.46. Spot 3 and 3' had similar R_f value. On alumina base plates (Figure 11B), five substance were separated from 10x n-butanol extracted ZYD4 supernatant. R_{f7} = 0.65, R_{f8} = 0.60, R_{f9} = 0.5, R_{f10} = 0.41, R_{f11} = 0.38. Two substances were separated from LB broth sample. $R_{f10'}$ = 0.41, R_{f12} = 0.74. Spot 10 and 10' had similar R_f value.

Figure 11A. Silica Gel

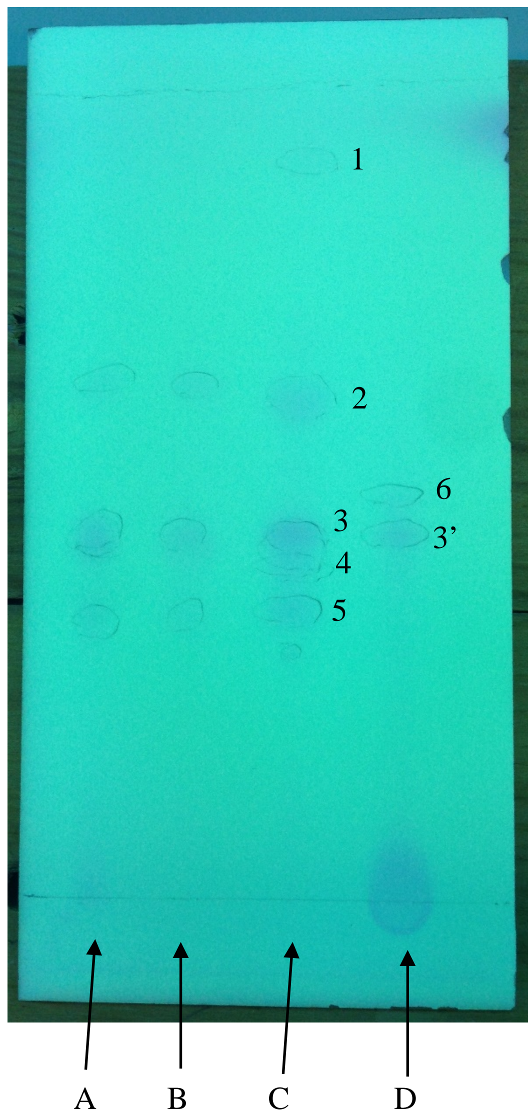
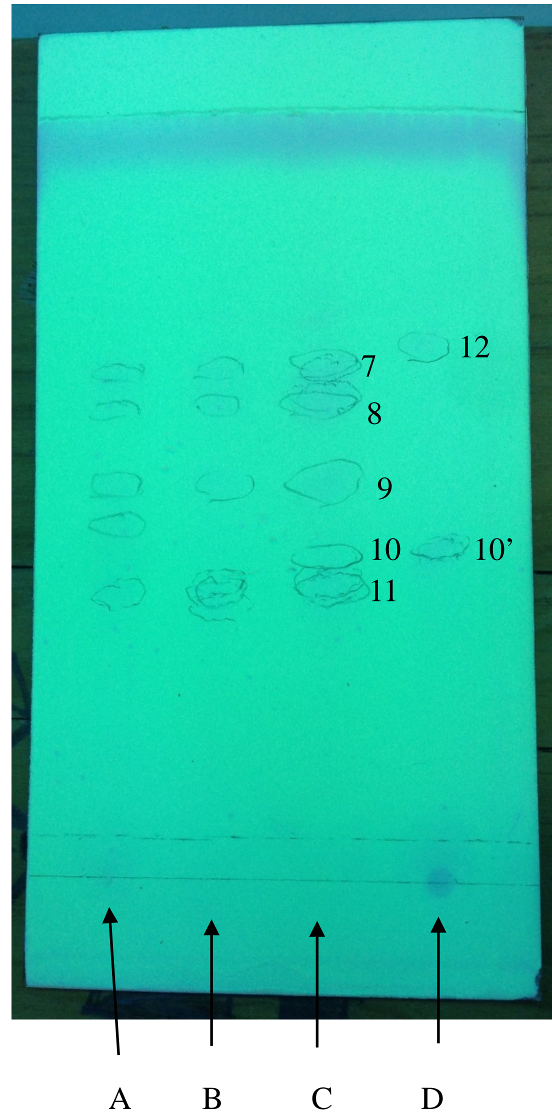


Figure 11B. Alumina Base



A: Untreated ZYD4 supernatant

B: 2x n-butanol extraction

C: 10x n-butanol

D: LB broth

0.62, R_{f3} = 0.46, R_{f4} = 0.41, R_{f5} = 0.35, R_{f6} = 0.49, $R_{f3'}$ = 0.46.

On an Alumina Base plate, five substances were separated from the n-butanol extracted sample (spot 7-11), and two substances were separated from LB broth (spot 10' and 12) (Figure 11B). Among the spots of LB broth, 10' has a similar R_f values to spot 10 (from n-butanol supernatant), so we conclude that spot 10 and 10' were the same substance. R_{f7} = 0.65, R_{f8} = 0.60, R_{f9} = 0.5, R_{f10} = 0.41, R_{f11} = 0.38, $R_{f10'}$ = 0.41, R_{f12} = 0.74.

Comparing the spots on the silica gel plate and the Alumina base plate, both n-butanol extraction samples shared a same spot with the LB broth sample (3 and 3', 10 and 10'). Thus, we can conclude that it is highly possible that spot 3 and spot 10 are same substance and are not the antimicrobial agent.

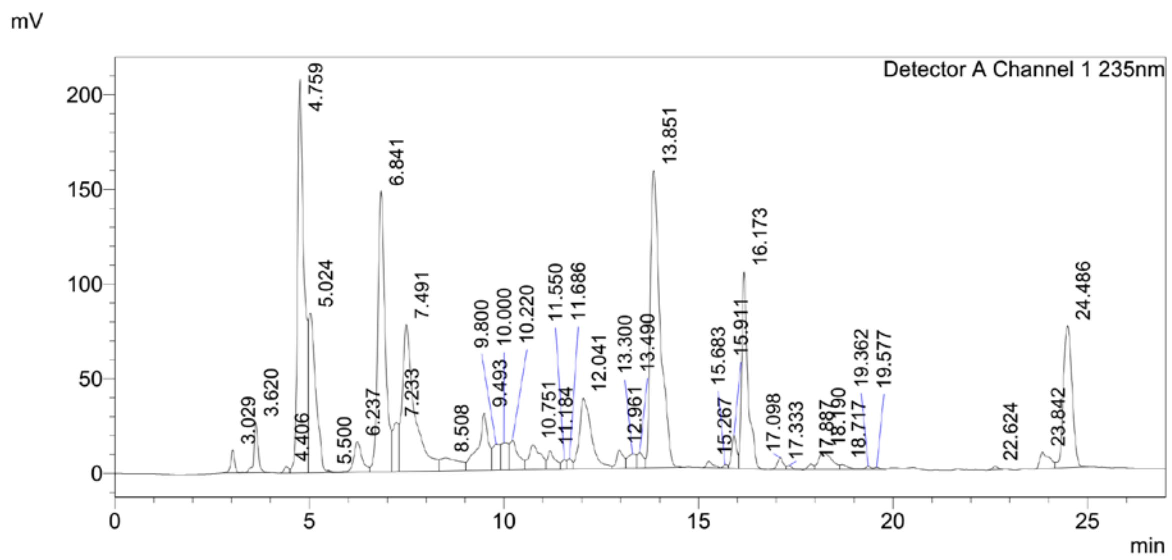
TLC can be used as an indicator assay in the further purification assay, which can help to determine whether the fraction contains the antimicrobial agents or not.

The HPLC chromatogram of 2-times concentrated n-butanol extraction of ZYD4 supernatant and 2-times n-butanol extraction of LB broth are shown in Figure 12A and Figure 12B. n-Butanol extraction of supernatant and the LB sample share the similar fractions around 11.8-12.1 min, 13.7-13.9 min, 16.1-16.3 min. The peaks around 4.5-5.5 min, 6.8-8 min, 9.4-10.5 min, 24.4-25 min were only found in n-butanol extraction of the ZYD4 supernatant sample. So we conclude that antimicrobial substances could be included in these fractions. Since tailing was observed when using water and acetonitrile as mobile phase, 0.05% TEA was added into the aqueous and organic phase to avoid tailing and improve peak shape. Chromatograms of the mobile phase containing 0.05% TEA are shown in Figure 13A (n-butanol extraction of ZYD4 supernatant) and Figure 13B (n-butanol extraction of LB broth). The tailing and efficiency of separation were not significantly improved. Considering TEA is a strong alkaline substance,

Figure 12. The HPLC Chromatogram of n-Butanol Extraction of ZYD4 Supernatant and n-Butanol Extraction of LB broth Run by Water and Acetonitrile as Mobile Phase.

Sample of n-Butanol extraction from ZYD4 supernatant and n-butanol extraction from LB broth were run by reverse phase HPLC system, and the wavelengths of UV detector were set at 235 nm and 255 nm. The mobile phase only contained acetonitrile and water. n-Butanol extraction from ZYD4 supernatant (Figure 12A) and n-butanol extraction from LB broth (Figure 12B) showed the same peaks around 11.8-12.1 min, 13.7-13.9 min, 16.1-16.3 min. The unique peaks that were only observed in n-butanol extraction from ZYD4 supernatant were around 4.5-5.5 min, 6.8-8 min, 9.4-10.5 min, 24.4-25 min.

Figure 12A.



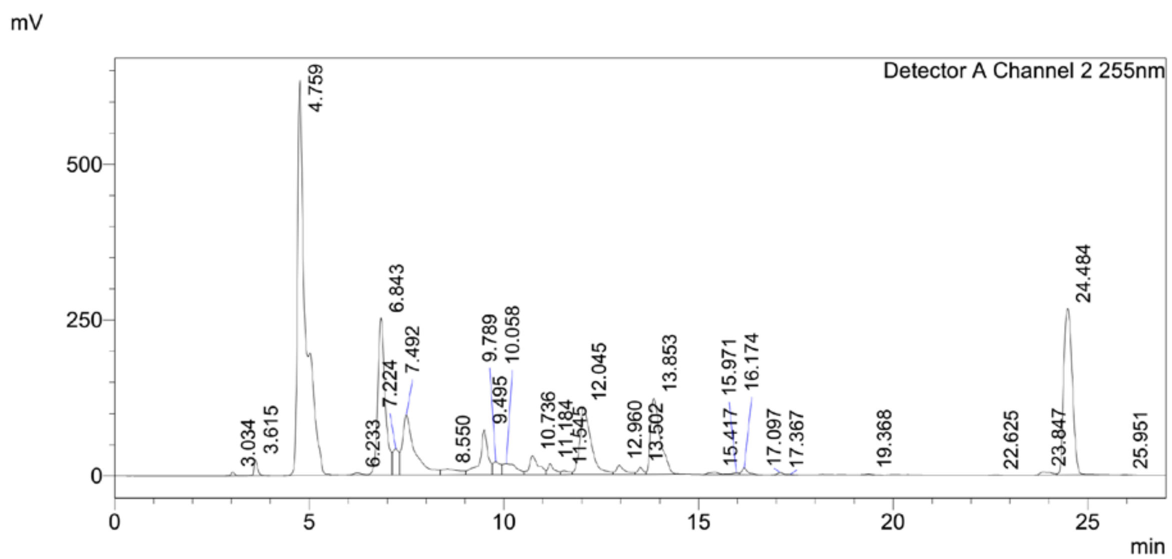
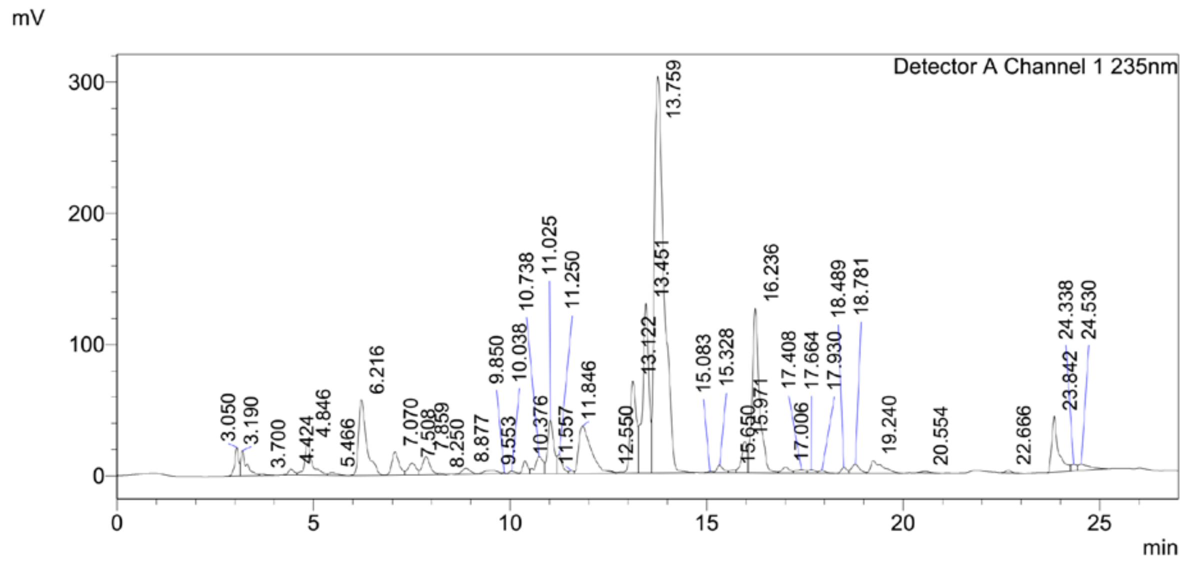


Figure 12B.



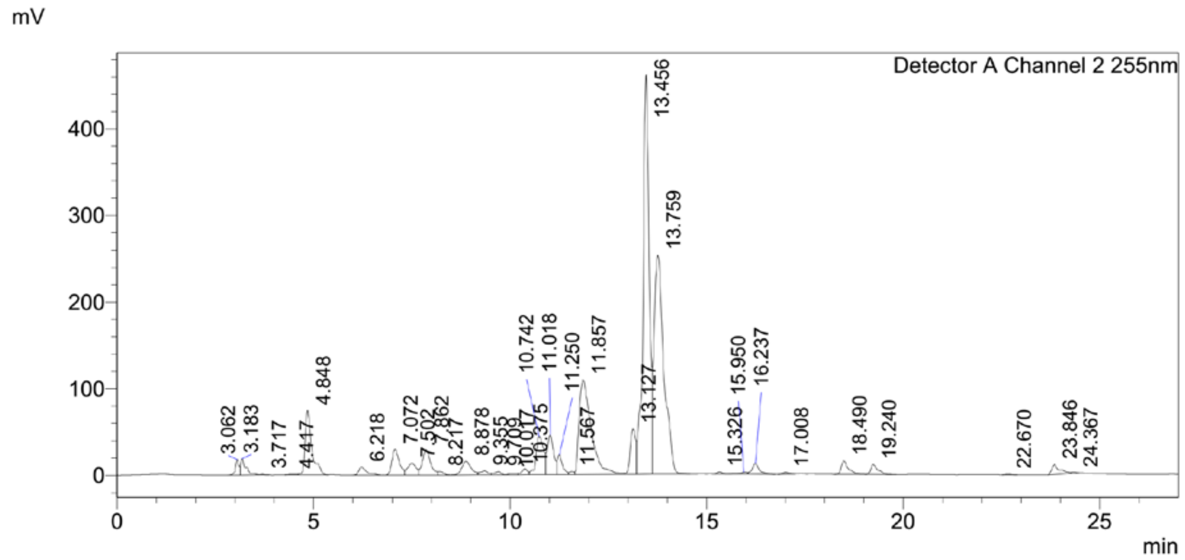
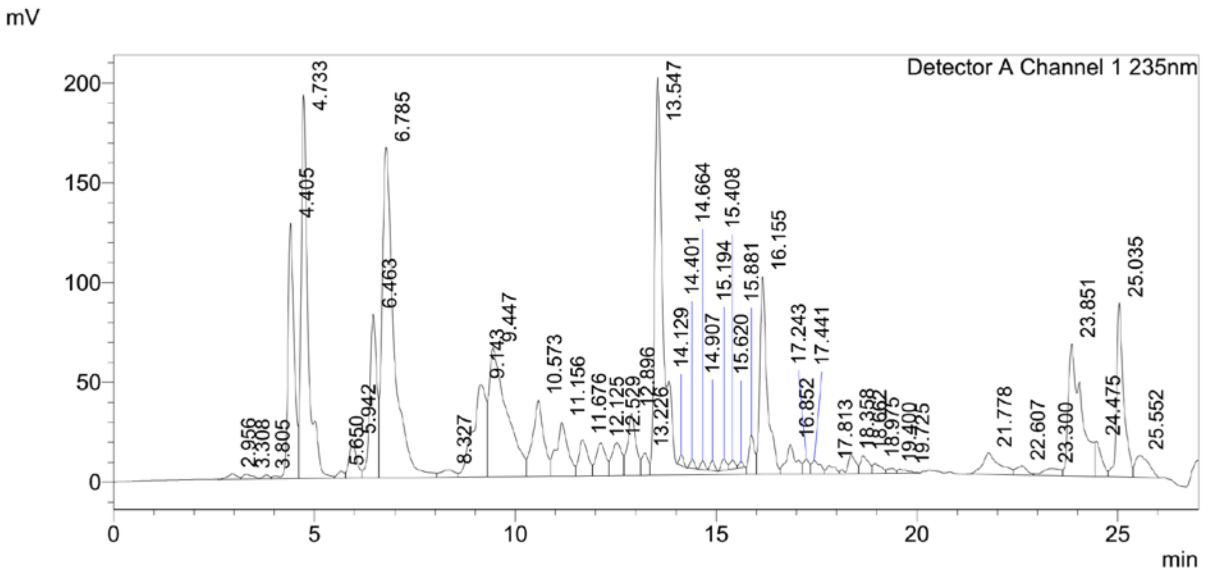


Figure 13. The HPLC Chromatogram of n-Butanol Extraction of ZYD4 Supernatant and n-Butanol Extraction of LB Broth Run by Water and Acetonitrile Containing 0.05% TEA (pH = 7) as Mobile Phase.

Sample of n-Butanol extraction from ZYD4 supernatant and n-butanol extraction from LB broth were run by reverse phase HPLC system, and the wavelengths of UV detector were set at 235 nm and 255 nm. The organic mobile phase was 0.05% TEA acetonitrile, and the aqueous mobile phase was 0.05% TEA water. n-Butanol extraction from ZYD4 supernatant (Figure 13A) and n-butanol extraction from LB broth (Figure 13B) showed the same peaks around 12.8-13 min, 13.5-14 min, 15.8-16.2 min. The unique peaks that were only observed in n-butanol extraction from ZYD4 supernatant were around 4.4-5.5 min, 5.9-7.1 min, 8.4-9.5 min, 24.4-25.6 min.

Figure 13A.



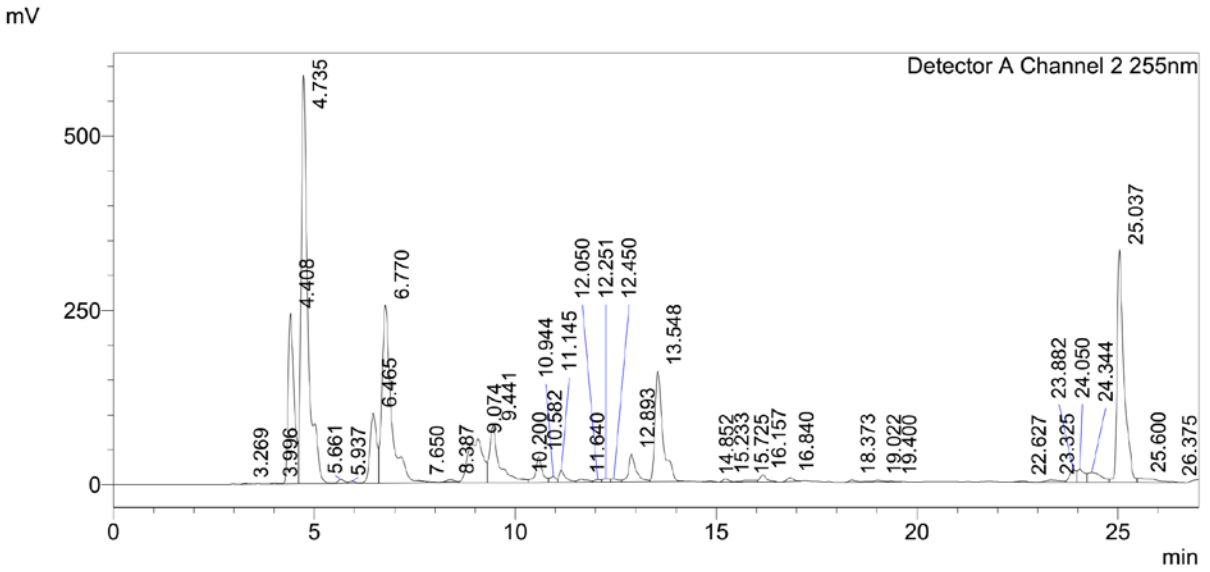
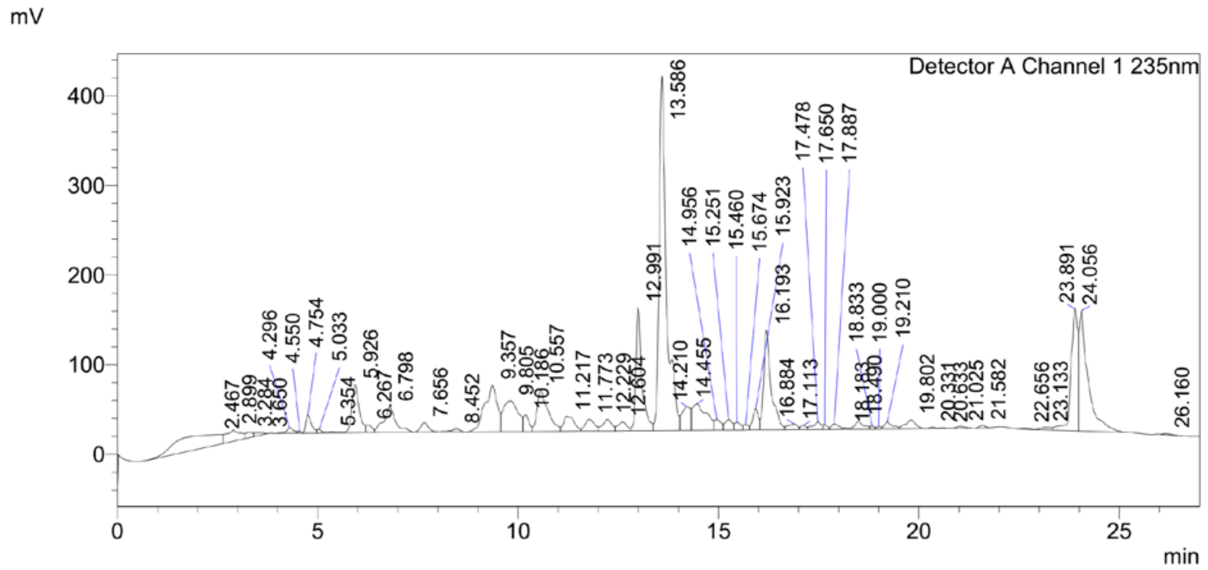
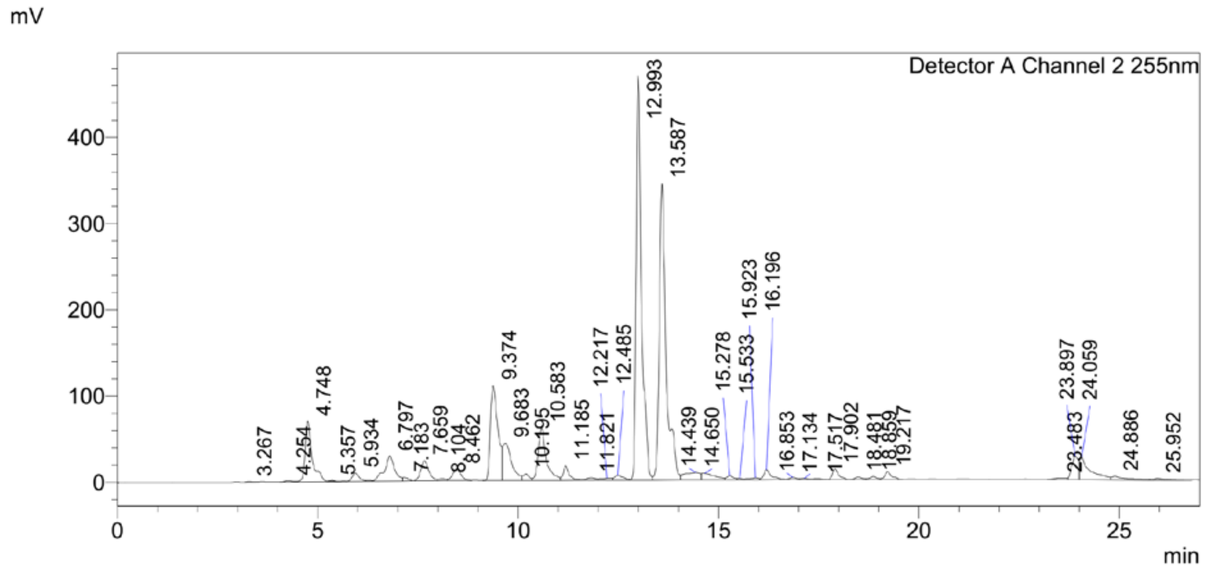


Figure 13B.





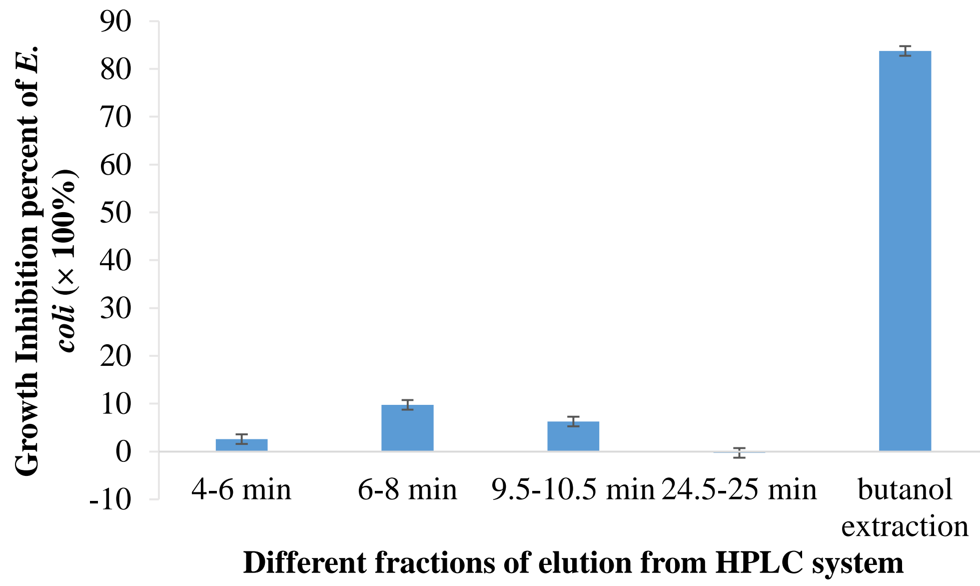
which may cause an increasing pH value and have effects on antimicrobial activity when concentrating eluent fractions, a 0.05% TEA mobile phase was not used in the following purification process.

The antimicrobial activity of different fractions are shown in Figure 14. Among the fractions only existing in the chromatogram of n-butanol extracted ZYD4 supernatant sample, only peaks around 6-8 min and 9.5-10.5 min are observed to contain inhibitory activity against indicator strain, indicating that the antimicrobial agents could be contained in these peaks.

Each fraction separated by the C18 column in the HPLC system was injected into the mass spectrophotometer. The possible molecular weight of antimicrobial agents: 1) peak a (retention time = 7.133 min), 1263 m/z (base peak); 2) peak b (retention time = 7.483 min), 1499 m/z (base peak); 3) peak c (retention time = 10.200 min), 357 m/z (base peak) (Figure 15). The mass peaks in the mass chromatograph that are different from the base value with smaller abundance could be the fragments broken by the ESI interface.

Figure 14. Antimicrobial Activity of Different Fractions of Elution of n-Butanol Extracted ZYD4 Supernatant Compared to n-Butanol Extracted LB Broth from HPLC. Data represent the average of three experiments, and results are means ± SD.

The eluted fractions were dried and dissolved in water. The antimicrobial activity of the peaks existing only in n-butanol extracted ZYD4 supernatant were determined by AlamarBlue assay. Peaks around only peaks around 6-8 min and 9.5-10.5 min were recovered with partial antimicrobial activity.



Growth Inhibition percent of E. coli (× 100%)
90
80
70
60
50
40
30
20
10
0
-10
4-6 min
6-8 min
9.5-10.5 min
24.5-25 min
butanol extraction
Different fractions of elution from HPLC system

Figure 15. Mass Spectrum Graph of Possible Antimicrobial Agents.

The mass spectrum was connected to HPLC system, and the separated fractions from n-butanol extracted ZYD4 supernatant were directly injected into the mass spectrum. The possible molecular weight of antimicrobial agents. Peak a with the retention time of 7.133 min showed a m/z of 1263 as base peak value (Figure 15A). Peak b with the retention time of 7.483 min showed a m/z of 1499 as base peak value (Figure 15B). Peak c with the retention time of 10.200 min showed a m/z of 357 as base peak value (Figure 15C).

Figure 15A.

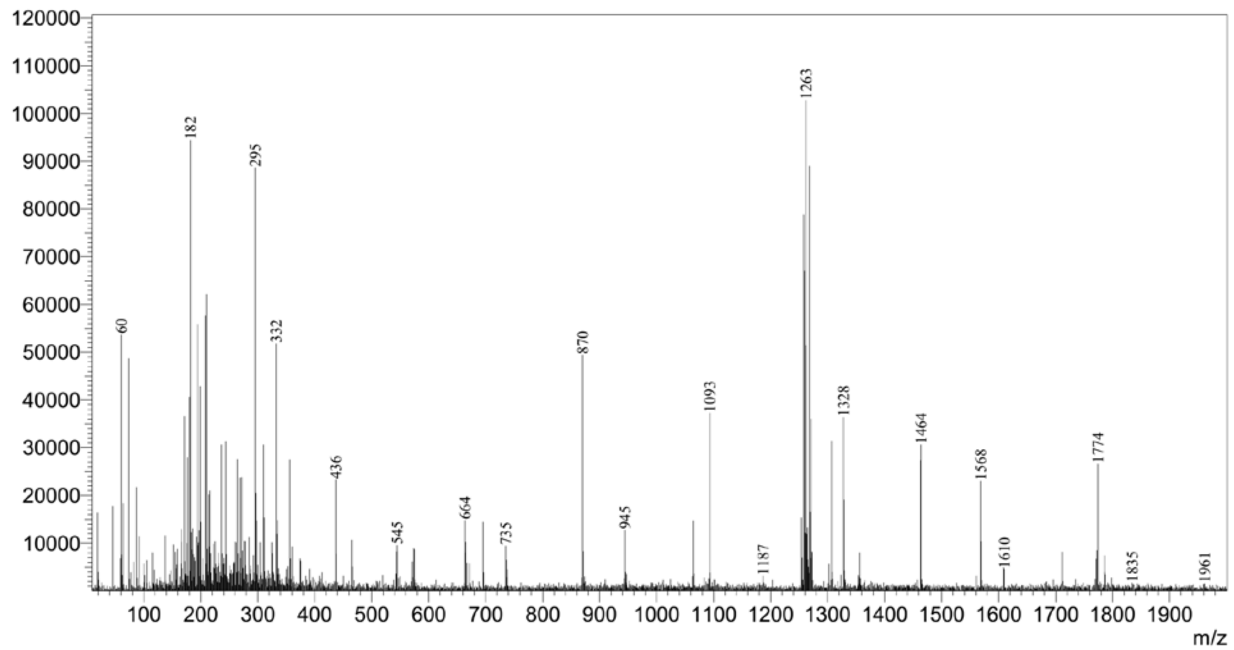


Figure 15B

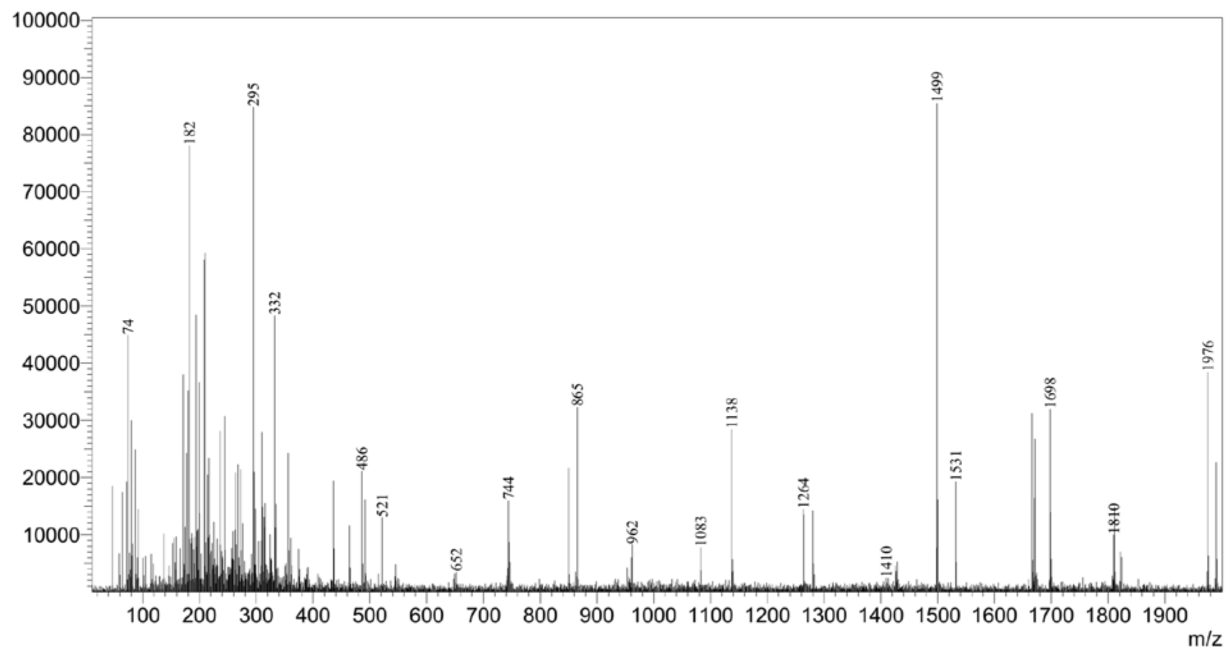
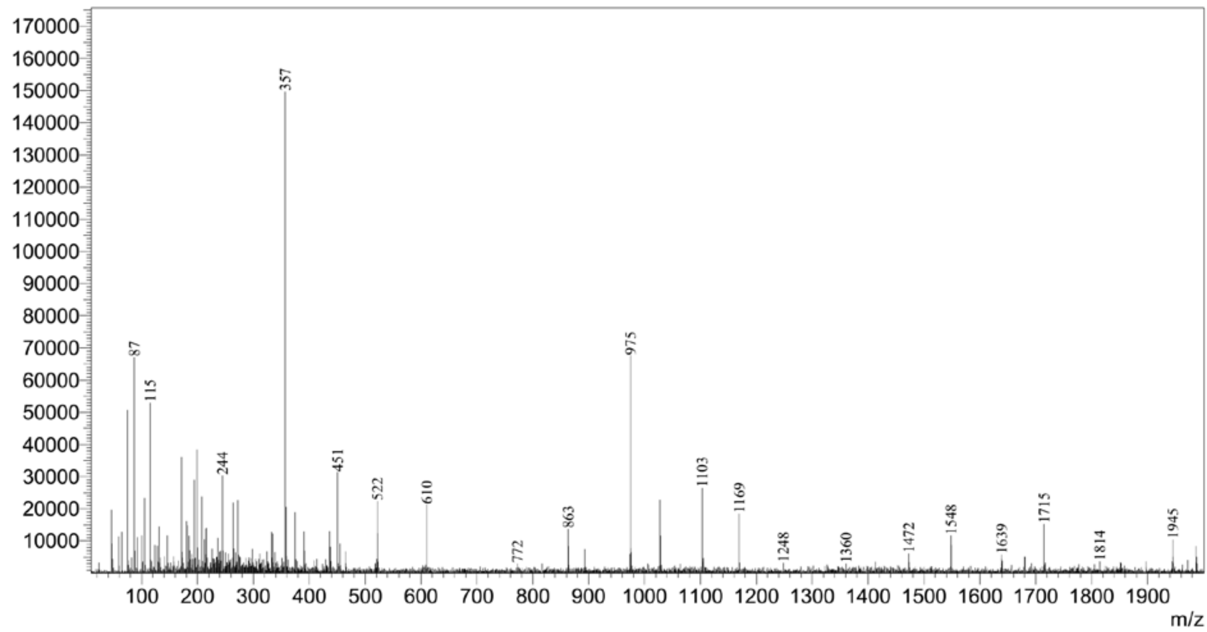


Figure 15C



IV. DISCUSSION

Antibiotics are the front line of defense against bacterial infections. However, multiple antibiotic resistance of clinical significant pathogenic microbes has been developing and spreading rapidly[14]. The increasing number of bacterial species that develop antibiotic resistance poses a serious threat to human health. Because of the great diversity of bacteria, screening of natural habitats in different parts of the world is anticipated to result in isolation of new microorganisms producing potential antimicrobial agents. Here, a potential isolate producing antimicrobial agents was identified as *Brevibacillus laterosporus* by its genotypic characteristics and named ZYD4. In previous studies, the antimicrobial agents produced by ZYD4 have proven to strongly inhibit the growth of 11 species of microorganismic pathogens of clinical importance, including *Staphylococcus aureus* (MRSA), *Staphylococcus saprophyticus, Enterococcus faecalis, Enterococcus faecium, Corynebacterium diphtheriae, Escherichia coli, Pseudomonas aeruginosa* and *Acinetobacter baumannii.* The broad spectrum of antimicrobial activity of the agents produced by ZYD4 suggest a high potential for application in future clinical research.

In refining the growth media for the optimal production of antimicrobial agent produced by ZYD4, the antimicrobial agents produced by bacteria grown in LB broth showed the strongest inhibitory activity. Among the three media (LB, TSN and NB), LB broth contains the richest nutrients. Interestingly, our results seem to contradict the common conception that bacteria can produce more antimicrobial substances to cope with environmental stress.

Accordingly, the antimicrobial agents can be precipitated by ammonium sulfate and high concentrations of ethanol, indicating that the agents could be proteins or peptides. The reduction

of inhibitory activity against indicator bacteria strains when removing the protein from the supernatant produced by ZYD4 further strengthens this conclusion. Amberlite XAD 16 is one member of the Amberlite XAD resin series (macroporous resin), which can be used to absorb peptides and proteins according to their different physical properties. Macroporous resins have adsorption and desorption capacities for different substances according to their polarity, hydrophilic or hydrophilic residues. Other XAD resins such as XAD-7 and XAD-4 have been reported to successfully isolate soluble proteins from an aqueous solution[15][16]. Diaion HP-20 resin also belongs to the family of macroporous resins, which have been used for crude extraction of a novel bacteriocin named sonorensin from cell cultures of *Bacillus sonorensis* MT93[17]. Usually serially-diluted methanol or ethanol are used to elute target proteins or peptides from macroporous resins. However, according to the results of ethanol precipitation of antimicrobial substances from the ZYD4 supernatant, the results imply that ethanol may have an effect on the inhibitory activity of target substance; the recovery of elution from XAD-16 should therefore be done quickly to avoid the damage to antimicrobial substance.

Small proteins and peptides produced by bacteria, which can inhibit the growth of similar or non-closely related bacteria strains, are called bacteriocins[18]. Bacteriocins do not harm the bacteria themselves due to specific immunity proteins. More than 99% of bacteria can produce at least one bacteriocin, most of which have not been identified[19]. To maintain a population and reduce the numbers of competitors in the same environment in order to obtain more nutrients and living space, the ability to secrete bacteriocin is considered a successful strategy for bacteria. Unlike most antibiotics, which are secondary metabolites, bacteriocins are ribosomally synthesized and sensitive to proteases while generally harmless to the human body and surrounding environment. In our project, the antimicrobial agents are shown to be heat-stable,

alkaline-stable but acid-unstable. The antimicrobial agents are also resistant to proteinase K. Since different proteinases can recognize and cleave their own specific type of peptide bond, it is possible that proteinase K does not destroy the active structure of the antimicrobial agents. Alternatively, the antimicrobial agents could be small molecules that do not contain a peptide bond. In the preliminary studies, the inhibitory activity of ZYD4 supernatant worked better on Gram-positive bacteria than Gram-negative bacteria (data not shown in this thesis). Coincidentally, bacteriocins produced by Gram-positive strains are mostly inhibitory to Gram-positive strains and are less effective against Gram-negative strains[20].

Currently, the mechanism of action of antimicrobial peptides remains unclear. Early studies revealed that antimicrobial peptides can interact with the target cell membrane through the formation of ion channels and transmembrane pores, which cause membrane leaking and eventually result in the lysis of the microbial cells[21][22].

In the purification process, it is essential to use various chromatographic techniques depending on the different physical and chemical properties of bacteriocin[23][24] to obtain bacteriocin for further research. For example, gel filtration, including SP-Sepharose and Sephadex G-25, and affinity chromatography have been reported[25][26] to be applied after the ammonium sulfate precipitation. Gel filtration is a separation based on molecular size and is also called molecular exclusion. In our project, the molecular size was determined by using dialysis membrane tube. The range of molecular size of the antimicrobial agents was 100 to 12,000 Da. Comparing to dialysis membrane, gel filtration is more convenient to narrow down the range of molecular size, by running the column with an indicator substance which has a known molecular weight. Gel filtration can also be used for desalting in the substitution of the dialysis membrane[27].

Alternatively, organic solvents can be applied to complete extraction from cell culture supernatant, such as mineral ether, ethyl acetate, and 1-butanol[28][29]. In our project, by using 1-butanol, the antimicrobial agents were successfully collected and concentrated. After extraction with 1-butanol, a small amount of precipitation existed between the 1-butanol phase and aqueous phase, which did not disappear even after 24h, indicating no emulsification. Considering that the mixture of n-butanol and chloroform can de-proteinize the supernatant from ZYD4, it is possible that some proteins contained in the supernatant were precipitated, but not including the target substances. Compared to the other methods applied in this project, extraction with n-butanol does not have any obvious reducing effect on antimicrobial agents, and may not need too many sequential steps to complete crude purification, like desalting with dialysis tubing, so n-butanol extraction is an easier and more convenient method to obtain antimicrobial agents from the ZYD4 supernatant.

A mass spectrum was finally used to identify the exact molecular weight of the antimicrobial agents. LC-MS is a widely used technique that has high sensitivity, making it useful in many applications. It combines the separation, general detection and potential identification of chemicals of particular masses in the presence of other substances. LC-MS is especially useful in separating natural products from natural extracts, or bioanalysis in the study of metabonomics[30]. In purification of antimicrobial substance from bacteria culture, the application of LC-MS/MS has been reported to analyze the sequence of peptide and lipopeptide[31][32]. LC-MS/MS technique is more sensitive and specific than the LC-MS system, which highly improves the accuracy and speed in bioanalysis work at large molecular scales[33][34]. For example, it can be used for analysis of amino acids sequences of proteins or peptides [35].

In summary, this is a novel antimicrobial peptide from the soil microorganism, *Brevibacillus laterosporus*, and it is active against a wide range of clinically-related pathogens. Moreover, the antimicrobial compound has displayed thermostability, extreme high pH stability and enzyme stability; thus, it could be a promising candidate for use against bacterial infection in the near future. Further research can focus on the influence of growth conditions on the antimicrobial agents. Carbon source limitation, nitrogen source or phosphorous limitation can all be applied to investigate how this bacterium adapts to the environment.

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