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Research Article

CHARACTERIZATION OF BIOPESTICIDE COMPOUNDS FROM *Bacillus subtilis* AAF2 UAAC 20701 FERMENTATION PRODUCTS

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ABSTRACT

The characterization of biopesticide compounds from extract of fermentation products using *Bacillus subtilis* AAF2 UAAC 20701 has been investigated. The fermentation process was carried out in a 1 liter volume bioreactor using a modified corn immersion media with 110 rpm agitation, 37^{0}C temperature, initial pH of 7, for 48 hours. Supernatant from fermented products that have been separated from bacterial cells, then extracted and fractionated using organic solvents: hexane, dichloromethane and ethyl acetate. Each fraction was dried and tested for its antimicrobial activity against the *Ralstonia solanacearum* fungal test, *Xanthomonas campestris*, *Fusarium oxysporum* and *Ssclerotium rolfsii*. The separation of active substance compounds was performed by preparative Thin Layer Chromatography (TLC) and subsequently characterized using Ultra violet-Visible (UV-Vis), Spectroscopy, Fourier Transform Infra Red (FTIR) Spectroscopy, Liquid Chromatography-Mass Spectra (LC-MS). The results showed that the highest antibiotic activity was obtained from ethyl acetate extract with 100% resistance to both fungi isolates, followed by dichloromethane extract with 76.0% resistance to *Fusarium oxysporum* and 63.3% to *Sclerotiumrolfsii*, and the lowest activity was obtained in hexane extract with resistance of 72.0% to *Fusarium oxysporum* and 38.3% to *Sclerotium rolfsii.* The ethyl acetate extract has two compounds, with Rf of 0.78 (AAF2[1]) and 0.59 (AAF2[2]) respectively. The AAF2[2] compounds had high antibiotic activity against *Fusarium oxysporum* (92.0%) and *Sclerotium rolfsii* (91.7%), compared with AAF2[1] to *Fusariumoxysporum* (70.0%) and *Sclerotiumrolfsii* (55.0%). Both AAF2[1] and AAF2[2] is a potential compound to be developed as biopesticide. The AAF2[2] compound is allegedly to be L- Homocysteine ($C_8H_{16}N_2O_4S_2$).

Keywords: characterization, biopesticide, fermentation, *Bacillus subtilis* AAF2 UAAC 20701

INTRODUCTION

Microbial secondary metabolites are important sources in the development of drugs and other chemical products. It begins since the discovery of penicillin as a potential antibiotic. Apart from the many antibacterial and antifungal agents available on the market, it is still necessary to search for new compounds. This is due to increased resistance to pathogenic microbes.

Bacillus subtilis was reported to have ability to inhibit soil phytopathogen because it has the ability to produce antibiotics, such as lipopeptide antibiotics[1,2]. Antibiotic compounds produced by *Bacillus subtilis* are surfactine[3,4], iturine[4,5], bacillomycine[6], fengicine[5,6], and subtilicine[4]. In our previous research, *Bacillus subtilis* AAF2 was reported to have the ability to inhibit the growth of phytopathogen. This ability is due totheproduction of secondary metabolite compounds with antibiotic activity. This strain has the best inoculum age at 8 hours and stationary phase is starting at 20 hours. The best fermentation conditions of *Bacillus subtilis* AAF2 was found on modified corn immersion media with glucose as carbon source, peptone nitrogen source, inoculum concentration 5.0%, nitrogen concentration 15.0%, 3.0% corn immersion concentration, pH 7, agitation 110 rpm, and the best fermentation time 48 hours.

To find out the compound produced by *Bacillus subtilis* AAF2, it is necessary to isolate and purify the compound before the characterization process. This stage is a critical stage, because in many cases, compounds are produced in small amounts of the entire fermentation fluid[7,8,9]. The purpose of this research is tocharacterize thebiopesticide compounds from the extract of *Bacillus subtilis* AAF2 fermentation. Stages performed for the compound characterization including: 1) separation of insoluble products by centrifugation; 2) separation of dissolved product by using liquid-liquid extraction mechanism (using solvent based on polarity); 3) test of antibiotic activity of each extracted fraction; 4) purification of compounds using preparative thin layer chromatography (TLC); and 5) identification of compounds through UV-vis, FT-IR, and LC-MS analyzes.

MATERIALS AND METHOD

Biopesticide-producing bacteria, which is *Bacillussubtilis* AAF2 UAAC 20701 and test bacterial *Ralstonia solanacearum*, *Xanthomonas campestris*, *Fusarium oxysporum*, *Sclerotium rolfsii* obtained from pure culture stored at UAAC Culture Central, Biotechnology Biotan Sumatra Laboratory, Andalas University, Padang, Indonesia. Materials and tools used in this

research were ethanol 70%, 90%, *nutrient agar* (NA), *potato dextrose agar* (PDA), NaCl physiologis 0,85%, hexane, dichloromethane, ethyl acetate, and DMSO. The tools used in this research is micropipette, Erlenmeyer flask, Petri dish, test tube, measuring pipette, separator flask, beaker glass, filter membrane, paper disc, vial glass, centrifuge, silica gel G60 F_{254}coated by aluminum plate (20 x 20 cm), UV-Vis, FT-IR, and LC-MS.

Sterilization

The materials and heat-resistant tools used in this study were sterilized using an autoclave at 121°C at a pressure of 15 lbs for 15 minutes. The non-heat-resistant tools were sterilized using 90% ethanol[10,11].

Extraction of Bioactive Compound

The extraction method was based on modified de Melo *et al.* (2009) procedure. The fermentation results were centrifuged at 4,000 *x g* for 45 minutes and the supernatant obtained was used for multistage extraction with hexane, dichloromethane and ethyl acetate (1: 1) which was repeated three times. Each organic phase is taken and dried with anhydrous sodium sulfate and evaporated[12]. The dried extraction results are then weighed.

Bioactivity Test of Extraction Product

Bioactivity test of extraction product for phytopathogen bacteria (*Ralstonia solanacearum* and *Xanthomonas campestris*) was performed with reference to disc diffusion method with extract concentration of 5% (v/v). The bioactivity test of extract on fungal phytopathogen (*Fusarium oxysporum* and *Sclerotium rolfsii*) was done using food poisoning technique method with extract concentration 0.1%(v/v).

TLC Analysis and Bioactivity Test Results

The best extraction results which showed the highest quantity has been chosen then followed by TLC analysis. The TLC analysis was performed using *Silica gel G60 F254 alumina backed plates* (1× 11cm) for separation. *Aliquots* (10 µL) of each extract obtained as described above were applied and the layer was developed with acetyl/methanol (4:1). UV active absorption points are visualized at 254 and 366 nm[13].

The TLC bioactivity test for phytopathogen bacteria (*Ralstonia solanacearum* and *Xanthomonas campestris*) was conducted with reference to the paper disc method. The extract concentration used was 25 ppm. The extract bioactivity test on fungal phytopathogen (*Fusarium oxysporum* and *Sclerotium rolfsii*) was done by using food poisoning technique method with extract concentration of 25 ppm (v/v).

UV-Vis Spectroscopy Analysis

UV-Vis analysis was performed to obtain λ_{max}value. This analysis was performed by derivatizing TLC results which positive inhibiting the growth of bacterial and fungal phytopathogen with diazomethane and dried. The tools used were UV-Visible spectrometers (Perkin-Elmer Lambda 19, 170 nm-3200 nm wavelength, dual monochrometer). Standard solutions were prepared with different solvents (dichloromethane, sterile distilled water, chloroform and ethyl acetate) at concentrations of 0.5 ppm, 10 ppm, 15 ppm, and 20 ppm. Absorbance versus wavelengths of the solution were measured using a UV-Visible spectrometer (performed three replications). Then the absorption curve was constructed[13].

FT-IR Spectroscopy Analysis

Infrared spectrum apparatus used in this analysis was Perkin-Elmer model Spectrum 400 FTIR Spectrometer, based on *Universal Attenuated Total Reflectance sensor* (UATR-FTIR). The scanning range used was 4000-650 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scanners. The crystals required in this technique contain diamonds in the upper layers and zinc selenida focus elements. The spectrum of each sample were performed with six replications. FTIR spectrum analysis was performed to determine the functional groups of fermented metabolite compounds that had been isolated.

LC-MS Analysis

LC-MS analysis was performed at LIPI Chemical Research Center, Bogor, Indonesia. This analysis was conducted to determine the concentration and molecular weight of the compound obtained. The purity of the compound can be seen from the peaks emerging from the result. This analysis was performed using Mariner Biospectrometry equipped with a binary pump. HPLC is connected with a Q-tof mass spectrometer and equipped with an ESI source. *Full-scan* mode from 100 to 1200 m/z wasdone at 140 °C. The HPLC columns used for the analysis were Phenomenex 5µ C8, 150 × 2 mm. The solvent used was 80% methanol with 0.3% formic acid. The solvent is delivered at a flow rate of 0.2 mL/min. The solvent proceeds with isocratic elution.

RESULTS AND DISCUSSION

Extraction process of *Bacillus subtilis* AAF2 UAAC 20701 fermentation productwas done gradually using an organic solvent, i.e. hexane, dichloromethane, and ethyl acetate. The ethyl acetate extract yielded a higher amount of crude extract with a weight of 280.5 mg/L, followed by dichloromethane extract with a weight of 232.0 mg/L, and hexane weighed 72.0 mg/L (Table 1). All extraction results were lost antibiotic activity against bacterial isolate test, whereas to the whole fungal extract still showed antifungal activity. The highest antifungal activity was obtained from ethyl acetate extract with 100% resistance to both testing fungal isolates (Figure 1), followed by dichloromethane extract with 76.0% resistance to *Fusarium oxysporum* and 63.3% towards *Sclerotiumrolfsii*, and the lowest activity obtained on hexane extract with 72.0% resistance towards *Fusarium oxysporum* and 38.3% to *Sclerotium rolfsii*.

The use of organic solvents is required to damaging the cells, so the compounds present in microbial cells can be isolated. Polarity differences in solvents are required to isolate secondary metabolite compounds[14]. Several solvents used were hexane and diethyl ether to isolate non-polar compounds, while chloroform, dichloromethane and ethyl acetate were used to isolate the semi-polar compounds [15].

Table 1: The amount of extract obtained per liter of fermentation for 48 hours

No.	Extract types	Extract Amount (mg/L)	Inhibition Zone (mm)		Resistance (%)	
			Ralstonia solanacearum	*Xanthomonas campestris*	*Fusarium oxysporum*	*Ssclerotium rolfsii*
1.	Hexane	72.0	0.0	0.0	42.0	38.3
2.	Dichloromethane	232.0	0.0	0.0	76.0	63.3
3.	Ethyl Acetate	281.0	0.0	0.0	100.0	100.0

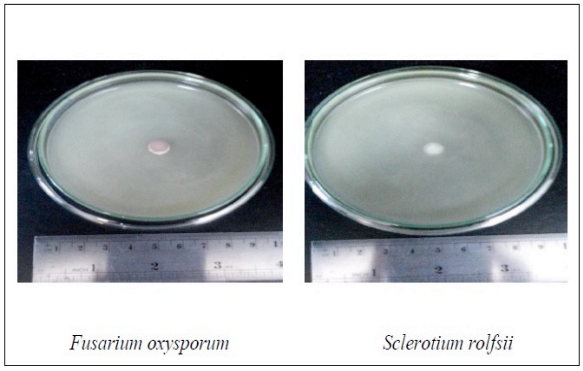

Figure 1: Antimicrobial activity profile of extract of ethyl acetate from fermentation *B. subtilis* AAF2 tested against test microbes *Fusarium oxysporum* and *Sclerotium rolfsii*

The rough extracts obtained in this study were six times higher than the crude extracts from plants. Hanson (2003)[16] reported that the secondary metabolites obtained from the crude extract of plants only range from 0.01% of the dry weight of the plant or only about 100 mg kg of the dry weight of the plant. This is showed that the fermentation process can be a solution in the production of bioactive compounds, because it is more effective and efficient, and purification of compounds from the fermentation process is easier to do than if using plant organs[17].

In this study, *Bacillus subtilis* AAF2 UAAC 20701 was produced more semi-polar compounds than non-polar compounds (Table 1). These results are similar to those obtained by de Melo *et al*. (2009)[13] and Bhoonobtong *et al*. (2012)[18], but the crude extract quantity obtained from this study was higher than the two researchers. Then, de Melo *et al*. (2009)[11]was obtained only 14 mg/L of hexane extract, 16 mg/L dichloromethane extract, and 16 mg/L ethyl acetate extract, while Bhoonobtong *et al*. (2012)[18] obtained 50 mg/L diethyl ether extract, 90 mg/L chloroform extract, and 100 mg/L ethyl acetate extract. These results are in accordance with Saifudin (2014)[15] which stated that only a few non-polar secondary metabolites and in general, secondary metabolites are semi-polar and soluble in organic solvents.

Extraction results may lose their antibiotic activity. This condition also found in research before where hexane, dichloromethane, and ethyl acetate extracts in the study lost antibiotic activity against some test microbes. According to Saifudin (2014)[15] the extraction process can reduce or eliminate the antibiotic activity. This may be due to: 1) the presence of a synergistic compound (the presence of one or several compounds causing amplification of effects), 2) additive effects of several compounds on a single molecular target, and 3) the complementary nature of the compound (some compounds have a target different molecule, but the same pathological properties).

In general, ethyl acetate extract is more effective toinhibits the growth of test microbes. De Melo *et al.* (2009)[13] reported dichloromethane and ethyl acetate extracts from *Bacillus pumilus* MAIIIM4A had antibiotic activity against strong bacteria and fungi, when compared with hexane extract. Battu & Reddy (2009)[19] reported that ethyl acetate extract from *Pseudomonas fluorescens* effectively inhibited the growth of test microbes 89% - 90%. Wang *et al*. (2012)[20] also reported that ethyl acetate extract from *Lactobacillus plantarum* IMAU 10014 has a stronger antifungal activity than extracts from other solvents (cyclohexane and dichloromethane).

It was also observed that ethyl acetate extract from *Bacillus* sp. has higher antimicrobial activity, while Sihem *et al*. (2011)[21] reported that hexane and dichloromethane extracts from *Bacillus* sp. have higher antimicrobial activity than ethyl acetate extract. These things are the basis of some researchers to use only ethyl acetate as a solvent in the fermentation extractionprocess, such as Kauria *et al.* (2012)[22], Niyaz (2012)[23], Handayani*et al*. (2015)[24], and Rivai *et al.* (2015)[17].

The purification process of *Bacillus subtilis* fermentation product was done through preparative method using thin layer chromatography[25]. Purification was performed only on extracts with highest bioactivity and provide the most amount of extract (ethyl acetate extract). The result of thin layer chromatography of ethyl acetate extract showed two separate stains, with Rf of 0.78 ($AAF2^1$) and 0.59 ($AAF2^2$) (Table 1 and Figure 1), respectively. The $AAF2^2$ compounds exhibit high antibiotic activity against *Fusarium oxysporum* (92.0%) and *Sclerotium rolfsii* (91.7%) compared to $AAF2^1$ compounds (Table 1).

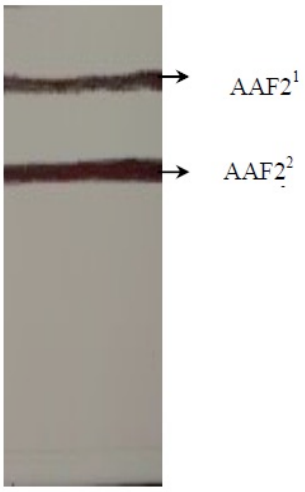

Figure 2: Chromatogram profile of preparative TLC of ethyl acetate extract on the separation process of both components in this research.

Table 2 shows a decreasing in the activity of the compound obtained compared with the crude extract of ethyl acetate (Table 1). This condition happened because the decrease in activity can be caused by the process of hydrolysis, auto oxidation, and compilation of compounds during the purification process[26]. Based on the criteria set by Saifudin (2014)[15], the $AAF2^2$ compound is a potent compound because it can inhibit the growth of test microbes (*Fusarium oxysporum* and *Sclerotium rolfsii*)> 75% at 25 ppm concentration.

Table 2: Retention factor (Rf) valueand resistance of the purified compounds

No.	Compounds	Rf Value	Resistance (%)	
			Fusarium oxysporum	*Sclerotium rolfsii*
1.	AAF2[1]	0.78	70.0	55.0
2.	AAF2[2]	0.59	92.0	91.7

The result of UV-Visible analysis showed that AAF2[2] compound has λ max of 263.40 nm (Figure 3). This showed that this compound has a chromophore and it is not visible in visible light. According to Saifudin (2014)[15], roughly if a compound can not be seen in visible light then it will have λ max> 400 nm. Compounds that are not visible in visible light, will have λ max between 240-380 nm, and the compounds seen in the wavelength range indicate the compound has a chromophore[27,28].

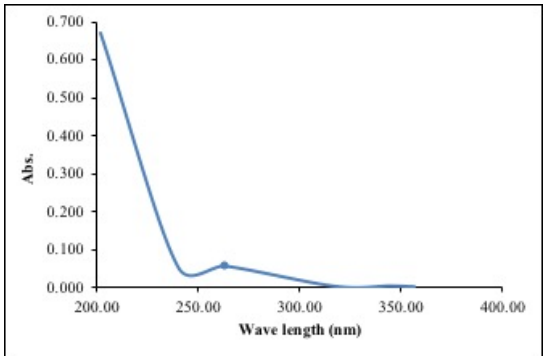

Figure 3: The ultraviolet spectrum of AAF2[2] which was separated from the *Bacillus subtilis* AAF2 UAAC 20701 fermentation product in this research

FT-IR analysis showed that AAF2[2] compound has wave number (cm^{-1}) of 3232, 2926, 2156, 2042, 1655, 1447, 1326, 1232, and 1070 (Figure 4). Based on criteria by Larkin (2011) the purified compound is allegedly to have a functional group-OH (3232 cm^{-1}), C-H aliphatic (2926 cm^{-1}), -N=C=O or -C=N (2156 – 2042 cm^{-1}), C=O (1655 cm^{-1}), CH_3 (1447 – 1326 cm^{-1}), and C-O-C (1070 cm^{-1}).

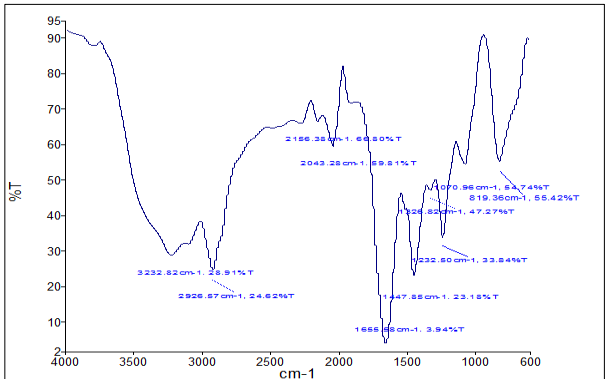

Figure 4: Infrared spectra of AAF2[2] compound of *Bacillus subtilis* AAF2 UAAC 20701 fermentation product in this research.

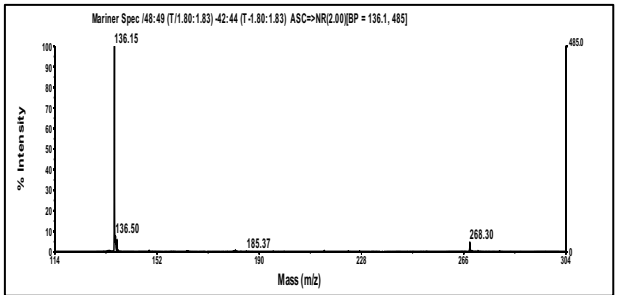

Figure 5: The Mass Spectra spectrum of AAF2 compounds separated from the *Bacillus subtilis* AAF2 UAAC 20701 fermentation product in this research

Annotation:

Index	Centroid Mass	Relative Intensity	Area
1	136.1457	100	2567.27
2	136.4955	7.66	146.15
3	137.1448	5.84	170.28
4	184.8553	0.07	124.80
5	185.3725	0.2	37.27
6	265.9377	0.08	52.49
7	268.3012	4.59	136.19

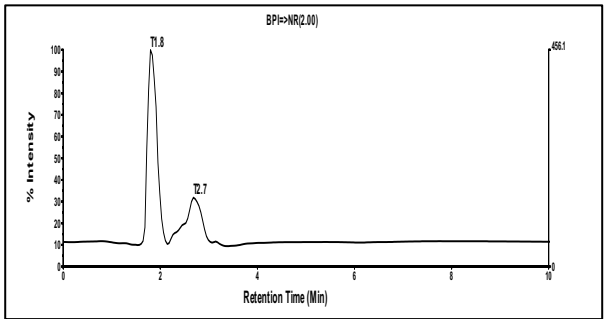

Figure 6: Chromatogram of AAF22 compound separated from *Bacillus subtilis* AAF2 UAAC 20701 fermentation product in this research

The AAF2 compounds were then analyzed using Liquid Chromatography-Mass Spectroctrometry (LC-MS). The LC-MS analysis was performed using Phenomenex 5µ C8 columns, with a 80% of methanol mobile phase and 0.3% formic acid. The detector used is a Q-t of mass spectrometer equipped with a ESI positive ion. The results of the analysis showed that the AAF2[2] compounds were separated from the fermentation fluid of *Bacillus subtilis* AAF2 UAAC 20701 in this study (Figure 5). The first peak has a retention time of 1.795 minutes, and the second peak has a retention time of 2.679 minutes. The highest peak of AAF2[2] compound was weighed as 268.301 (m/z).

Results above showed that the compound obtained in this research was different from general compound produced by *Bacillus subtilis* such as surfactine that has weight of 994 – 1.065 m/z[3,4,6] iturine which is weighed 1.028 – 1.109 m/z [4,5], bacillomycine that has weight 1081 m/z[6], fengisine weighed 1.421 – 1.566 m/z[3,4,5,6], and subtilisine which has weight 3.399 – 3.473 m/z[4].

Based on the searching through www.massbank.jp., the AAF2[2] compound with highest chromatogram peak was thought to be L-Homocysteine, with the chemical formula $C_8H_{16}N_2O_4S_2$. L-Homocysteine is a chemical compound consisting of two homocysteines connected by a disulfide bond[22,29]. Homocysteine is a derivative of methionine (www.sigmaaldrich.com). There have been no reports of antibiotic activity of these compounds. The L- Homocysteine structure is shown in Figure 7.

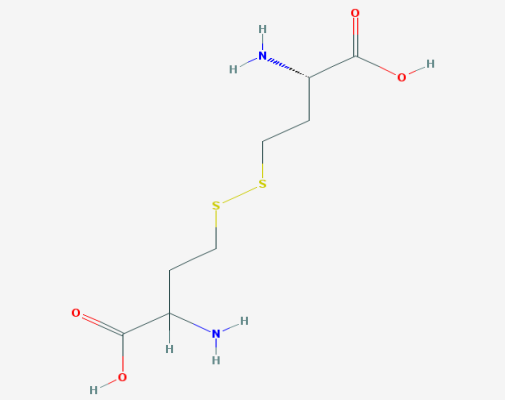

Figure 7: Chemical Structure of L- Homocysteine (pubchem.ncbi.nlm.nih.gov)

CONCLUSION

The highest antibiotic activity on fungi was obtained from ethyl acetate extract with 100% resistance to both isolates of fungi, followed by dichloromethane extract with 76.0% resistance to *Fusarium oxysporum* and 63.3% to *Sclerotium rolfsii*, and the lowest activity was obtained in extract hexane with a resistance of 72.0% to *Fusarium oxysporum* and 38.3% to *Sclerotium rolfsii*. Second, the ethyl acetate extract has two compounds, with Rf of 0.78 (AAF21) and 0.59 (AAF2^2), respectively. The AAF2^2 compounds had high antibiotic activity against *Fusarium oxysporum* (92.0%) and *Sclerotium rolfsii* (91.7%), compared to AAF2^1 compounds to *Fusarium oxysporum* (70.0%) and *Sclerotium rolfsii* (55.0%). Both AAF2^1 and AAF2^2 are a potential compound to be developed as biopesticide. Thirdly, the AAF2^2 compound separated from the *Bacillus subtilis* AAF2 UAAC 20701 fermentation product in this research allegedly to be L-Homocysteine ($C_8H_{16}N_2O_4S_2$).

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