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Research Article

SELECTION OF MEDIUM FOR BIOPESTICIDES FERMENTATION PROCESS BY *Bacillus subtilis* AAF2 UAAC 20701

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ABSTRACT

Selection of the optimum medium type for biopesticide fermentation process by endophytic bacteria *Bacillus subtilis* AAF2 UAAC 20701 has been done. The medium type used were: Tryptic Soy Broth (TSB) medium, Luria Bertani (LB), Glucose Soybean Flour medium, and modified corn immersion. Erlenmeyer flask containing the medium to be used in the fermentation process. Then it was incubated at 27^{0}C with agitation of 120 rpm. Next step, inoculum was inoculated as much as 10% (v/v) into Erlenmeyer containing the fermentation medium and incubated at 27^{0}C with 120 rpm agitation. The parameters measured were pH fluctuation, bacterial cell count, growth inhibition zone of test bacteria and fungal growth inhibition measured every 24 hours for 72 hours. The results showed that the best fermentation condition for producing the highest biopesticide activity using *Bacillus subtilis* AAF2 UAAC 2070 from four type of testing medium was found in Corn Immersion Medium. The inhibition zone towards *Ralstonia solanacearum* and *Xanthomonas campestris* wth inhibition towards *Fusarium oxysporum* was 64.3% while towards *Schlerotium rolfsii* was 67.1 % with 48 hours of incubation time and pH changes during fermentation range from 7.0 to 7.8.

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

INTRODUCTION

US EPA (The United States Environmental Protection Agency) defines biopesticides as types of pesticides derived from natural materials such as animals, plants, bacteria and some minerals[1]. Based on the active composition, biopesticides are categorized as microbial pesticides, plant pesticides, and biochemical pesticides[2]. Djunaedy (2009) divides biopesticides into two groups based on the material sources: first, phytopesticides, is the result of extraction of certain parts of the plant either from leaves, fruit, seed or root[3]. These pesticides are commonly used to control pests (insecticidal) or disease (bactericidal or fungicidal). Second, biological pesticides, has formulations containing certain microbes either in the form of viruses, bacteria, or fungi that are antagonistic to other microbes (which caused the plant diseases) or produce certain compounds that are toxic to both insects and nematodes.

Endophytic bacteria were reported to have the ability to produce bioactive compounds. Such compounds may inhibit the growth of phytopathogen[4,5]. This ability is also demonstrated by the 14 strains used in the antibiotic test against *Ralstonia solanacearum*, *Xanthomonas campestris*, *Fusarium oxysporum*, dan *Sclerotium rolfsii*. The 14 strains were *Bacillus indicus* BJF1, *Bacillus indicus* TCF1, *Bacillus indicus* MCF2, *Bacillus pumilus* CAF4, *Bacillus* sp. CAF1, *Bacillus subtilis* AAF2, *Bacillus subtilis* MCF1, *Bacillus subtilis* CAF3, *Bacillus subtilis* MCF3, *Pseudomonas psychrotolerans* AAF1, *Pseudomonas oryzihabitans* AAF3, *Pantoea agglomerans* CAF2, *Pantoea stewartii* AAF4, and *Kocuria kristinae* CSF1.

Bacillus subtilis AAF2 UAAC 20701 is a potential strain to be developed as a biopesticide producer from our previous research[6]. The strain has antibiotic activity against all test microbes and has high antibiotic activity, so the authors are interested to do the biopesticide fermentation process using the strain. *Bacillus subtilis* has been reported as endophytes and has the ability to inhibit soil phytopathogen[7], as against *Xanthomonas campestris, Ralstonia solanacearum*[9], *Fusarium oxysporum*[10,11] and *Sclerotium rolfsii*[12]. Some research reported that *Bacillus subtilis* has potential in producing antibiotic, such as lipopeptide antibiotic[13].

Production of biopesticide compounds was conducted in this study using liquid fermentation method. Some topics that need to be studied in the fermentation process are inoculums rceselection, nitrogen source, inoculum concentration, medium pH, agitation, and some fermentation process needed addition of inducer, or inhibitor[14]. These things need to be optimized for optimal fermentation process. The purpose of this research is to find the optimum type of medium and the best biopesticide fermentation process condition of *Bacillus subtilis* AAF2UAAC 20701.

MATERIALS AND METHOD

Material used in this research were *Bacillus subtilis* AAF2UAAC 20701 isolate from UAAC Culture Centre, Laboratory of Biotechnology Biotan Sumatra, Universiy of Andalas, Padang, Indonesia, sterile aquadest, TSA medium, TSB medium, Luria-Bertani (LB) medium, NA medium, PDA medium, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$, $CaCO_3$, NH_4NO_3, $(NH_4)_2SO_4$, HCl, glucose, soybean flour, corn imersion liquid, and physiological saline (NaCl)0.85%. Tool used in this research were micropipette, Erlenmeyer flask, Petri dish, reaction tube, measuring pipette, Beaker glass, filter membrane, *shaker incubation*, and spectrophotometer.

Materials and 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% alcohol[15].

Inoculum Source Production

The preparation of the inoculum source was carried out by taking one Ose e and inoculated into an Erlenmeyer flask containing10 mL TSB medium. Then as much as 10% (v/v) inoculum was inoculated into Erlenmeyer flask containing a liquid TS medium with a final volume of 100 mL and incubated at 27^0C with agitation of 120 rpm for 24 hours[16].

Determination of Growth Curve of *Bacillus subtilis* isolate AAF2 U01

Preparation of *Bacillus subtilis* AAF2 UAAC 20701 isolate growth curve of used in this study was conducted by taking 10% (v/v) inoculum source with cell number 10^6 cells/mL and inoculated into liquid medium to be used in the fermentation process (TSB, LB, glucose of soybean flour, and medium developed by Djamaan *et al.* (2012)[17] then it was incubated at 27°C for 24 hours with agitation of 120 rpm. Sampling was done every 2 hours to calculate the total number of bacteria by the method of pour plate [18,19]. From the data obtained growth rate constant (k) and generation time (g) was calculated using the formula by Willey *et al.* (2008)[20]. Growth rate constant:

$$k = \frac{n}{t} = \frac{\log Nt - \log No}{0{,}301\ t}$$

where:
No=starting population amount, Nt=population amount at t-time

Generation time was calculated by formula:

$$g = \frac{1}{k}$$

Selection of Medium Fermentation Type

The selection of fermentation medium was done on four media, those were: TSB medium (17 g tripton; 3 g *soypeptone*; 2.5 g glukosa; 5 g NaCl; and 2,5 g K_2HPO_4 L^{-1} medium), LB medium g yeast extract, and 5 g NaCl L^{-1}medium), Glucose Soybean Flour medium (15 g of soybean flour; 0.002 g $FeSO_4$; 0,02 g $ZnSO_4$; 0,02 g $MnSO_4$; and 0,3 $MgSO_4$ L^{-1} medium), and modified corn immersion medium (Djamaan *et al.*, 2012)(30 ml of corn immersion liquid; 5 g $CaCO_3$; 1 g $FeSO_4$; 2 g $MgSO_4$; 0,1 g $ZnSO_4$; 0,02 g $MnSO_4$;0,9 g KH_2PO_4; and 5 g Na_2HPO_4 L^{-1} medium).

The fermentation process started by inoculating 10% (v/v) inoculum with cell concentration of 10^6 cells/mL into the Erlenmeyer flask containing the medium to be used in the fermentation process. Then it was incubated at 27^0C with agitation of 120 rpm corresponding to best inoculum age from growth curve. Next step, inoculum was inoculated as much as 10% (v/v) into Erlenmeyer containing the fermentation medium and incubated at 27^0C with 120 rpm agitation.

The parameters measured were pH fluctuation, bacterial cell count, growthinhibition zone of test bacteria and fungal growth inhibition measured every 24 hours for 72 hours[21]. Fluctuations in pH was measured by pH meters; the calculation of bacterial cell count was done by the method of pouring cups which refers to Cappucino & Sherman (1987) [18]and Harley & Prescott (2002)[19]; the width of the inhibition zone produced was measured by the disc diffusion method and the fungal growth inhibition sing the food poisoning technique[6].

RESULTS AND DISCUSSION

The results of the *Bacillus subtilis* AAF2 UAAC 20701 curve used in this study are presented in Figure 1.

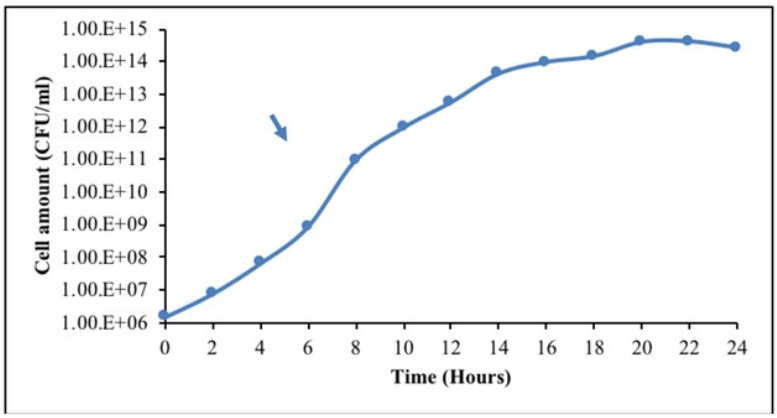

Figure 1: The curve of *Bacillus subtilis* AAF2 UAAC 20701 bacterial isolate used in this study

Figure 1 shows no lag phase in *Bacillus subtilis* AAF2UAAC 20701. This happened because the strain has been activated before and the amount of inoculum is adequately inserted. Astuti (2003) stated that active inoculum conditions can minimize the length of the lag phase in the fermentation process[22]. The length of the lag phase is, among others, influenced by the amount of inoculum and physiological conditions. In addition, the inoculum should be available in sufficient quantities, so it is important to provide an optimum amount of inoculum.

The best inoculums age for *Bacillus subtilis* AAF2 UAAC 20701 was at 8 hours. The best inoculum age was determined by ½ log phase. Determination of the age of the best inoculum is necessary, because the age of inoculum is one important factor in the production success of antibiotic compounds[23]. The stationary phase of *Bacillus subtilis* AAF2 UAAC 20701 was started at 20 hours, so it can be assumed that the production of antibiotic bioactive compounds was done in the same time. This is supported by research by de Carvalho *et al.* (2010) that stated bioactive compounds only found in the stationary phase[24]. Black (1999)[25]; Moat *et al.* (2002)[26]; and Madigan and Matinko (2006)[27] added that generally secondary metabolite products are produced at the stationary phase and the secondary metabolites have antibiotic activity.

The stationary phase in each strain occurs at different inoculums ages. de Carvalho *et al.* (2010)[24] reported that the stationary phase of *Bacillus subtilis* R14 was occurred from the age of inoculum 20 hours to 60 hours. The stationary phase was characterized by decreased growth when compared to the log phase. This condition occurred because the limitation of substrate concentration on the growth media of the three endophytic bacteria, in addition to the increase of toxic metabolism byproducts. This is supported by the opinion of Madigan and Martinko (2006) [27] which stated that bacterial growth was lower in the stationary phase. This can be the result of the limited substrate availability. Black (1999) [25] added that in the stationary phase there is an increase in metabolic byproduct concentration which is toxic to microorganisms, so the metabolism and growth of microorganism is lower.

Table 1: Cell amount, growth rate constant, and generation time of *Bacillus subtilis* AAF2 UAAC 20701 in growth curve production

No.	Time (Hour)	Cell amount (CFU/ml)	Growth rate constant (gen./hour)	Generation time (hour/gen.)
1.	0	.	1.76	0.57
2.	2	7.80×10^{6}		
3.	4	6.50×10^{7}		
4.	6	8.60×10^{8}		
5.	8	9.30×10^{10}		
6.	10	9.20×10^{11}		
7.	12	5.50×10^{12}		
8.	14	4.10×10^{13}		
9.	16	9.20×10^{13}		
10.	18	1.40×10^{14}		
11.	20	3.80×10^{14}		
12.	22	3.90×10^{14}		
13.	24	2.50×10^{14}		

Table 2: pH changing, cell amount, inhibition zone and growth inhibition on selection of fermentation medium by *Bacillus subtilis* AAF2 UAAC 20701

No.	Medium Type	Time (Hours)	pH	Cell amount (CFU/ml)	Inhibition zone (mm)		Inhibition growth (%)	
					Ralstonia solanacearum	*Xanthomonas campestris*	*Fusarium oxysporum*	*Sclerotium rolfsii*
1.	*Tryptic Soy Broth*	0	7.0	2.10×10^{6}	0.0	0.0	0.0	0.0
		24	6.8	3.80×10^{14}	3.0	2.0	60.0	58.8
		48	7.3	2.90×10^{14}	3.0	3.0	64.3	64.7
		72	8.2	4.50×10^{10}	2.0	2.0	47.1	42.4
2.	Luria-Bertani	0	7.0	1.30×10^{6}	0.0	0.0	0.0	0.0
		24	7.3	5.40×10^{14}	2.0	2.0	25.7	25.9
		48	8.0	3.20×10^{12}	2.0	2.0	28.6	29.4
		72	8.4	5.80×10^{10}	1.0	1.0	27.1	26.9
3.	Glucose - Soybean	0	7.0	1.60×10^{6}	0.0	0.0	0.0	0.0
		24	5.8	3.50×10^{14}	3.0	2.0	22.9	23.5
		48	6.5	1.10×10^{14}	3.0	2.0	27.1	29.4
		72	6.0	8.40×10^{12}	3.0	1.0	24.3	25.9
4.	Corn immersion	0	7.0	2.20×10^{6}	0.0	0.0	0.0	0.0
		24	8.0	7.50×10^{14}	3.0	3.0	57.1	56.5
		48	7.8	1.20×10^{14}	3.0	3.0	64.3	67.1
		72	8.4	3.80×10^{10}	0.0	2.0	57.1	49.4

The low growth in the stationary phase can also be caused by a decrease in the pH from the metabolism process by endophytic bacteria, thus it was interfering with bacterial cell metabolism. According to Black (1999)[25], Madigan and Matinko (2006)[27] pH greatly affects the enzymes activity. If the pH value is outside the minimum and maximum range, then the enzyme activity will be disrupted and resulted in disruption of activity inside the cell[14].

The growth rate constant (k) of *Bacillus subtilis* AAF2 UAAC 20701 in this study was 1.76 gen./hour and its generation time (g) was 0.57 hours/gen. (Table 1). The generation time showed that *Bacillus subtilis* AAF2 was doubled every 34.2 minutes. Willey *et al.* (2008) stated that the generation time of each bacterium depends on medium and growth conditions. This is evident from the reports which stated that *Bacillus subtilis* has a generation time

(g) 0.43 hours /gen., if it grown on a universal medium with a temperature of 40^0C.

Furthermore, in the selection process of fermentation medium to obtain the best results of *Bacillus subtilis* AAF2 UAAC 20701 has been done on four types of medium in this study. Ghribi *et al.* (2012) stated that the success of secondary metabolite fermentation was influenced by several factors, such as the type of fermentation medium, carbon source, nitrogen source, inoculum concentration, nitrogen concentration, corn immersion concentration, pH, and agitation. The results proved that these factors play an important role in the success of the fermentation process, especially antibiotic activity against test microbes.

Table 2 shows the best fermentation for *Bacillus subtilis* AAF2 UAAC 20701 was found on modified corn immersion medium with glucose as carbon source. This was demonstrated by the high antibiotic activity especially for the fungi (inhibition zonefor *Ralstonia solanacearum* and *Xanthomonas campestris* was 3 mm, and the growth inhibition towards *Fusarium oxysporum* was 64.3% and 67.1% for Sclerotium rolfsii) at 48 hours fermentation.

High levels of antibiotic activity suggest that the medium provides the nutrients needed in the production of antibiotic biopesticides. When viewed from the composition, this medium provides the nutrients needed in the production of biopesticides, such as carbon, N, P, Na, Mg, Fe, Zn, and Mn. Nutrition in the fermentation medium is known to play an important role in the metabolism of secondary metabolites[28] and plays an important role in the attack and intensity of secondary metabolites [29]. Cooper *et al.* (1981) reported that Mn in some *B. subtilis* strains can increase the production of secondary metabolites, and Fe is necessary for cell growth[30]. Duffy & Defago (1999) [31] and Beltran-Garcia *et al.* (2017) stated that mineral content, oxygen pressure, osmotic conditions, phosphates, carbon, and nitrogen sources was is abiotic factor that can affect the secondary metabolite production of bacteria in invitro[32].

High antibiotic activity on modified corn immersion medium showed that *Bacillus subtilis* AAF2 classified to synthetic medium-liking strain. According to Bashra *et al.* (2007) some *Bacillus subtilis* strain prefer synthetic medium to produces antibiotic compound to universal medium[33]. This is due to universal medium does not the necessary nutrient for antibiotic compounds production. Some bacterial strain will be showed decreasing of antibiotic activity when inoculated in complex medium[34] while *Bacillus subtilis* R14 is able to produce high antibiotic compounds in complex medium with limited oxygen [35]. Elibol & Mavituna (1998) stated that there is relationship between growth and antibiotic biosynthesis[36]. This condition showed that antibiotic production was induced by specific nutrition in fermentation medium[37].

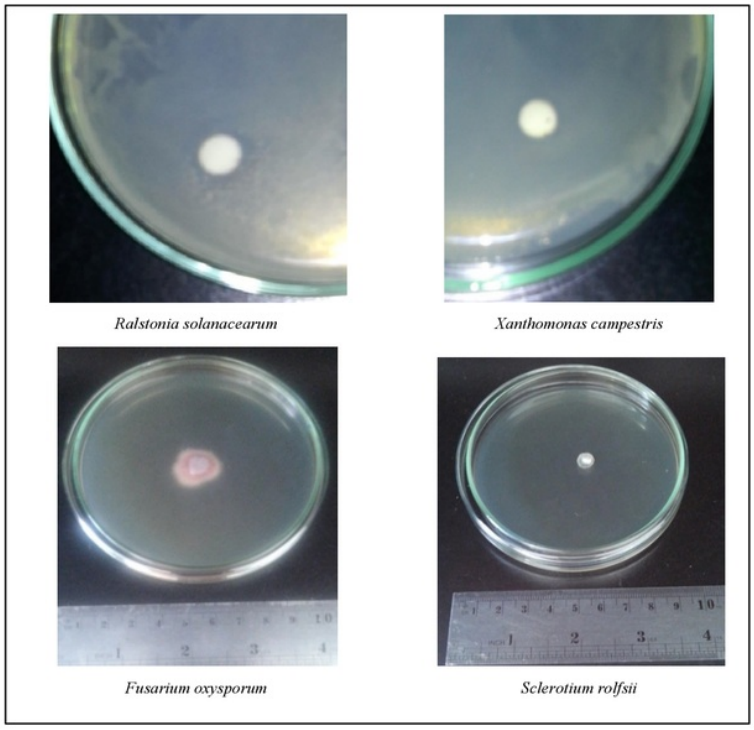

Figure 2: Profile of antibiotic activity from *Bacillus subtilis* AAF2 UAAC 20701 fermentation results towards several microbial test used in this research

Based on the data obtained in this research, the best fermentation time was 48 hours. Antibiotic activity found in 24 hour to 72 hour of inoculum age. Result from this research is corresponds with Demirkan & Usta (2013)[38], which stated that generally strains from *Bacillus* genera has antibiotic activity ranged from 24-72 hour of incubation time[39,40,41].

CONCLUSION

The best fermentation condition for producing the highest biopesticide activity using *Bacillus subtilis* AAF2 UAAC 2070 from four type of testing medium was found in Corn Immersion Medium. The inhibition zone towards *Ralstonia solanacearum* and *Xanthomonas campestris* was 3.0 mm and fungal growth inhibition towards *Fusarium oxysporum* was 64.3% while towards *Schlerotium rolfsii* was 67.1 % with 48 hours of incubation time and pH changes during fermentation range from 7.0 to 7.8.

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