Antagonistic Activity

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Antagonistic Activity of Extra Cellular Product and Component Bacteria of *Pseudomonas* sp. against *Aeromonas hydrophila* from Tilapia Aquaculture in East Borneo

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Abstract. This research aims to evaluate the antibacterial activity of 103 protein fractions obtained from the extra cellular product (ECP) and four different fractions from the cellular component (whole cell product, heat-killed whole cell product, intra-cellular product and crude extra cellular product) of *Pseudomonas* sp. against *Aeromonas hydrophila* pathogen on tilapia fish. Antibacterial activities were measured by the zone of inhibition in a sensitivity test against *A. hydrophila*. Intra-cellular product, whole cell product, and heat-killed whole cell product of *Pseudomonas* sp. exhibited inhibition zone of 10 mm and crude of fraction ECP showed inhibition zone of 11 mm. All protein fractions of ECP displayed potential activity against *A. hydrophila* in *Nila tilapia*. 73.8% protein fractions had resistant inhibition and 20.4% possessed intermediate inhibition. 5.8% fractions caused sensitive inhibition. Our results showed that protein fractions of ECP were found to be the most effective to inhibit *A. hydrophila* growth.

INTRODUCTION

A. hydrophila is a bacterial pathogen that has various hosts. Freshwater, brackish, and seawater reported to be infected by the bacteria, which caused hemorrhagic septicemia [1]. The bacteria are facultative or opportunistic which means they can live in water without a host for long time. They are found almost throughout the year in fish farms [2]. The incubation period is relatively short to let bacteria achieves the optimum growth after 18-24 h. The bacteria is highly pathogenic to the host and only needs less than 24 h to grow and develop virulence. The fish deaths caused by this bacterial strain is very high [3].

Bacterial infections of *A. hydrophila* are always accompanied by the infection of *Pseudomonas* sp. Both bacterial strains are always found in healthy and diseased tilapia with different levels of pathogenicity. According to Hardi (2012) [4] and Hardi and Pebrianto (2012) [3], fishes infected with *A. hydrophila* show changes in the external organs that appear to be faster than the infection of *Pseudomonas* sp. The existence of *Pseudomonas* sp. in fish is expected to be a bio control for *A. hydrophila*. However, research related to the antagonistic properties of extra cellular products of *Pseudomonas* sp. to obstruct the *A. hydrophila* cell is still limited.

The use of proteins produced by bacteria to suppress the growth of pathogens has been done with good results. Selection of bacterial products to kill bacterial pathogens is considered safer and more effective because it does not cause problems of resistance in farmed fish. Results of research conducted by Vijayan et al. (2006) [5], show that the supernatant of *Pseudomonas* sp. PS-102 is antagonistic against vibrio bacteria by 73%, and is safe for shrimp the size of the PL-9. Nour and El-Ghie (2011) [6] tested the in-vitro antibacterial activity of *P. fluorescens* against *A. hydrophila*. Applications of several *Pseudomonas* species as a fungicide to protect the food from toxic fungi such as

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Penicillium and Botrytis have also been reported. The protein patterns of the different strains of *Staphyloccus aureus* and *Lactobacillus sanfranciscensis* are resistant to specific antibiotics [7-9].

Research about controlling pathogenic bacteria in fish aquaculture is already being developed in aquaculture [6,10,11]. Several types of *Pseudomonas* sp. are antagonistic to some pathogenic bacteria in fish and shrimp farming [5,12,13]. Das et al. in 2006 [10] used the components of Pseudomonas to inhibit the growth of *A. hydrophila* bacteria, four fractions of cellular components (i.e. whole cell product, heat killed whole cell product, intra cellular product, and extra cellular product) of *Pseudomonas fluorescens*, *P. aeruginosa* and *P. putida* were equally effective in reducing the growth of *A. hydrophila* strains. Novelty from this research is in terms of the protein fractions from ECP Pseudomonas bacteria as an antagonistic component to *A. hydrophila*. From the previous research by Hardi et al. in 2014 [14], *Pseudomonas* sp. (EP-01) produces 103 protein fraction with a molecular weight of about 15:21-113.10 kDa that is putative as bio control to *A. hydrophila*.

MATERIALS AND METHODS

Isolation of Bacteria

A. hydrophila (EA-01) and *Pseudomonas* sp. (EP-01) were isolated from tilapia aquaculture at Loa Kulu, Kutai Kartanegara, East Kalimantan. Both bacterial strains were cultured in BHIB (Brain Heart Infusion Broth) and BHIA (Brain Heart Infusion Agar) media for 24 h at 30 °C.

Preparation of Different Cellular Components

Four antigenic components from *Pseudomonas* sp., i.e. heat killed whole cell product (HK), whole cell product (WCP), intra cellular product (ICP), and crude extra cellular product (ECP) were prepared by the method previously reported by Das et al. in 2006 [10].

Preparation of Fractionation Protein from Extra Cellular Product (ECP)

Before fractionation, *Pseudomonas* sp. was grown in a BHI medium and incubated for 24 h at a temperature of 30°C. Protein fractions from the ECP of *Pseudomonas* sp. were prepared using the method described by Laemmli in 1970 [15], Bradford in 1976 [16], and Rattanachuay et al. in 2010 [13].

Antagonistic Test

25 µm of each cellular component (HK, WCP, ICP, and crude ECP) and each protein fraction from the ECP of *Pseudomonas* sp. were impregnated on 7 mm diameter sterile discs and placed on a BHIA medium plates previously swabbed with *A. hydrophila* culture. The plates were incubated at 30 °C for 24 and 48 h. Inhibition zone was measured and recorded in mm.

RESULTS AND DISCUSSION

All the cellular components of *Pseudomonas* sp. bacteria possessed antibacterial activity against *A. hydrophila* pathogen at 24 h and decreased after 48 h. All cellular components from the bacteria displayed activity to suppress the pathogen bacteria with inhibition zone less than or 10 mm except for crude ECP at 11 mm, which was categorized as resistant or has weak antibacterial activity. However, Das et al.'s (2006) [10] investigation showed that heat whole cells and killed whole cell product from some strains of *Pseudomonas* sp. could suppress the growth of several strains of *A. hydrophila*. Commercial antibiotics used as the positive control of this study were Ciprofloaxcin/CIP, Norfloxacid/NOR, Nitroflorantion/F, Nalidixic Acid/NA. All antibiotics have a sensitive category to *Pseudomonas* sp. In addition, antibiotics Chloramphennicol/C, Oxytetracycline/OT, and Gentamicin/CN have showed resistant category (Table 1). Table 2 described the antibacterial activity of cellular component from *Pseudomonas* sp. against *A. hydrohila*.

Table 1 Sensitivities of *Pseudomonas* sp. to some commercial antibiotics

Antibiotic	Zone of clearance (mm)	Characteristic activity
Chloramphennicol/C	10	Resistant
Ciprofloaxcin/CIP	17	Sensitive
Norfloxacid/NOR	23	Sensitive
Nitroflorantion/F	18	Sensitive
Nalidixic Acid/NA	17	Sensitive
Oxytetracycline/OT	10	Resistant
Gentamicin/CN	10	Resistant

Table 2 Characteristic activity antibacterial of cellular component from *Pseudomonas* sp. against *A. hydrophila*

Cellular component	Zone of clearance(mm)	Characteristic activity
Heat killed whole cell product (HK),	6.5 – 10.5	Resistant
Whole cell product (WCP),	7 – 10.5	Resistant
Intra cellular product (ICP)	7.5 – 10.5	Resistant
Crude extra cellular product (ECP)	8 – 11	Resistant

Pseudomonas sp. (EP-01) was grown in a BHI broth medium and incubated for 24 h at 30 °C to produce 103 fractions protein. Test of ECP of *Pseudomonas* sp. antagonistic activity against *A. hydrophila* was done using paper discs with an incubation period of 24 h and 48 h. The results indicated that there were 10 fractions of ECP that retarded the *A. hydrophila* growth, with inhibition zone of more than 12 mm. i.e. fractions 10, 35, 37, 40, 57, 62, 71, 73, 84, and 101. Five fractions caused inhibition zone of more than 14 mm, while 4 fractions caused more than 15 mm. Only 2 fractions of ECP showed inhibition zone of more than 16 mm. Several numbers of ECP fractions from *Pseudomonas* sp. are likely as a candidate for *A.hydrophila* bio control. Inhibition zones of whole fractions was presented in Table 3.

Table 3 Antibacterial activity of protein fractions from ECP of *Pseudomonas* sp. against *A. hydrohila.*

Number of Protein Fraction ECP	Zone of clearance(mm)		Characteristic activity
	24 h	48 h	
1	10.0	8.0	Resistant
2	11.0	10.0	Resistant
3	12.0	12.0	Intermediate
4	11.0	10.0	Resistant
5	7.0	8.0	Resistant
6	10.0	10.0	Resistant
7	10.0	10.0	Resistant
8	10.0	10.0	Resistant
9	9.0	10.0	Resistant
10	14.0	11.0	Sensitive
14	9.0	10.0	Resistant
20	12.0	11.0	Intermediate
21	9.0	8.0	Resistant
22	12.0	10.0	Intermediate
23	9.0	9.0	Resistant
24	10.0	10.0	Resistant
25	11.0	11.0	Resistant
26	8.0	10.0	Resistant
27	9.0	9.0	Resistant
28	9.0	10.0	Resistant
29	9.0	10.0	Resistant
30	11.0	9.0	Resistant
31	12.0	11.0	Intermediate
33	10.0	11.0	Resistant
34	8.0	9.0	Resistant

35	10.0	15.0	Sensitive
36	12.0	12.0	Intermediate
37	16.0	13.0	Sensitive
38	10.0	11.0	Resistant
40	17.0	10.0	Sensitive
41	12.0	10.0	Intermediate
42	7.0	8.0	Resistant
43	10.0	10.0	Resistant
44	11.0	11.0	Resistant
45	10.0	9.0	Resistant
46	12.0	11.0	Intermediate
47	10.0	10.0	Resistant
48	7.0	10.0	Resistant
49	10.0	10.0	Resistant
50	9.0	9.0	Resistant
51	8.0	10.0	Resistant
52	9.0	10.0	Resistant
53	12.0	10.0	Intermediate
54	10.0	8.0	Resistant
55	8.0	7.0	Resistant
56	7.0	10.0	Resistant
57	14.0	13.0	Sensitive
58	9.0	11.0	Resistant
59	9.0	9.0	Resistant
60	12.0	11.0	Intermediate
61	8.0	9.0	Resistant
62	13.0	12.0	Intermediate
63	11.0	11.0	Resistant
64	10.0	10.0	Resistant
65	10.0	10.0	Resistant
66	11.0	11.0	Resistant
67	10.0	10.0	Resistant
68	10.0	9.0	Resistant
69	8.0	10.0	Resistant
70	10.0	11.0	Resistant
71	13.0	12.0	Intermediate
72	11.0	12.0	Intermediate
73	17.0	9.0	Sensitive
74	12.0	11.0	Intermediate
75	12.0	11.0	Intermediate
76	9.0	10.0	Resistant
77	9.0	10.0	Resistant
78	10.0	9.0	Resistant
79	10.0	11.0	Resistant
80	8.0	8.0	Resistant
81	10.0	11.0	Resistant
82	11.0	11.0	Resistant
83	12.0	10.0	Intermediate
84	16.0	9.0	Sensitive
85	8.0	10.0	Resistant
86	12.0	10.0	Intermediate
87	10.0	10.0	Resistant
88	10.0	10.0	Resistant
89	9.0	10.0	Resistant
90	8.0	10.0	Resistant

91	11.0	11.0	Resistant
92	7.0	10.0	Resistant
93	11.0	10.0	Resistant
94	9.0	8.0	Resistant
95	8.0	10.0	Resistant
96	11.0	10.0	Resistant
97	9.0	8.0	Resistant
98	10.0	10.0	Resistant
99	11.0	11.0	Resistant
100	11.0	12.0	Intermediate
101	12.0	13.0	Intermediate
102	8.0	9.0	Resistant
103	10.0	10.0	Resistant
104	11.0	10.0	Resistant
105	11.0	11.0	Resistant
106	10.0	10.0	Resistant
107	8.0	7.0	Resistant
108	11.0	11.0	Resistant
109	12.0	11.0	Intermediate
110	12.0	11.0	Intermediate
111	8.0	10.0	Resistant
112	12.0	11.0	Intermediate
113	9.0	9.0	Resistant

Inhibition zone from the protein fractions to *A. hydrophila* EA-01 strain was very varied. Seventy-six fractions of 103 or 73.8% fractions had resistant inhibition and 21 fractions or 20.4% possessed intermediate inhibition. Six fractions (5.8%) caused sensitive inhibition. This indicated that *Pseudomonas* sp. protein fractions of ECP might be developed as bio control to *A. hydrophila* infection in tilapia, especially fractions number 35, 37, 40, 57, 73 and 84. The highest inhibition zone by a number of ECP fractions ware caused by fractions 40 and 73 (17 mm).

The potential for ECP protein fractions of *Pseudomonas* sp. to suppress the growth of bacteria *A. hydrophila* was related to the content of antibiotics, bacteriocyn, siderophor [17], lysozyme, and other proteases [18]. Whole cells of *Pseudomonas* sp. (W3) can produce alkaline protease in extra cellular products that can suppress the growth of bacteria that causes disease like luminous vibrios in shrimps. This capability was due to *Pseudomonas* sp.'s ability to produce the proteolytic enzyme, lysozyme (N-acetylmuramidase), and lytic enzyme [13]. The supernatant of *Pseudomonas* sp. (I-2) contains antibacterial ingredients such asproteolytic, lipolytik, and amylolitic enzyme that suppresses the *Vibrio harveyi* growth [5].

The conclusion from this research is that the fraction protein ECP of *Pseudomonas* sp. is putative to be developed as a bio control against *A. hydrophila* in tilapia aquaculture.

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Antagonistic Activity

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